European Journal of Medicinal Plants
14(1): 1-16, 2016, Article no.EJMP.25300
ISSN: 2231-0894, NLM ID: 101583475

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Hepatoprotective Effects of *Rubus idaeus L.* Leaves against CCl_4-Induced Liver Injury via Antioxidant, Anti-inflammatory and Antiapoptotic Mechanisms

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/EJMP/2016/25300
Editor(s):
(1) Marcello Iriti, Professor of Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Fernando Jose Cebola Lidon, New University of Lisbon, Portugal.
(2) Anonymous, The University of Hong Kong, Hong Kong, China.
(3) Dinithi Peitis, University of Sri Jayewardenepura, Sri Lanka.
Complete Peer review History: http://sciencedomain.org/review-history/14014

Original Research Article

Received 26th February 2016
Accepted 26th March 2016
Published 5th April 2016

ABSTRACT

Aims: To investigate the hepatoprotective effects of red raspberry (*Rubus idaeus L.*) leaves against oxidative stress, inflammation and apoptosis induced by carbon tetrachloride (CCl_4) in rats.
Study Design and Methodology: Forty rats were divided into 5 groups, 1) normal control; 2) Raspberry (Rsp) control (100 mg/kg); 3) CCl_4 control; 4) and 5) CCl_4 groups pre-treated with 50 and 100 mg/kg of Rsp leaves, respectively, once daily for 10 days. Liver injury was induced on the 11th day in groups 3, 4 and 5 by intraperitoneal injection of CCl_4 (1.0 ml/kg) in 50% olive oil (1:1). Serum and liver were separated and prepared for biochemical and histological assay. Antioxidant, inflammatory and apoptotic markers were evaluated.
Results: CCl_4-induced liver damage was manifested by increased activities of serum marker enzymes, elevated levels of lipid peroxidation and by decreased hepatic antioxidants including reduced glutathione, glutathione peroxidase, superoxide dismutase and catalase. CCl_4 also resulted in elevated levels of hepatic inflammatory mediators including tumor necrosis factor-α (TNF- α), interleukin -6 (IL-6), IL-1β and nitric oxide (NO) as well as upregulation of apoptotic markers including caspase-3, FasL and Bax. Hepatic levels of Bcl2, an anti-apoptotic factor, was

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decreased by CCl_4. Pretreatment with Rsp leaves significantly ameliorated the elevation in serum liver enzymes and prevented lipid peroxidation and the depletion of the antioxidants. In addition, Rsp leaves exhibited anti-inflammatory effects via attenuation of increased hepatic levels of TNF-α, Il-6, IL-1β and NO. Additionally, CCl_4-Induced apoptosis in the liver cell was attenuated by Rsp pretreatment. With regard to histological examination, Rsp leaves significantly alleviated degeneration and necrosis of hepatocytes, accompanied by decreased inflammatory cell infiltration. **Conclusion:** These results suggest that Rsp leaves have protective effects against CCl_4-induced acute liver injury, and this protection is likely due to antioxidant, anti-inflammatory and anti-apoptotic mechanisms.

Keywords: Liver injury; red raspberry; tumor necrosis factor-α; interleukin-6; caspase-3; nitric oxide; oxidative stress; fas ligand.

1. INTRODUCTION

Liver injury is one of the major worldwide health problems that frequently results from non-adequate nutrition, drug abuse, viral infection, toxins or hepatic ischemic-reperfusion injury [1]. Acute liver injury is usually referred as the rapid development of hepatocellular dysfunction with a poor prognosis. It has been demonstrated that oxygen-derived free radicals, lipid peroxidation and inflammation play a critical role in the pathogenesis of various liver diseases [2,3].

Carbon tetrachloride (CCl_4) intoxication is a frequently used model of liver injury in different animal models via induction of oxidative stress and inflammatory mediators [4,5]. The toxicity of CCl_4 results from its reductive dehalogenation by cytochrome P450 into the highly reactive free radical trichloromethyl radical ($CCl3^{\cdot}$). In the presence of excess oxygen, $CCl3^{\cdot}$ can transform into trichloromethylperoxy radical ($CCl3OO^{\cdot}$), another highly reactive species. This molecule can also attack polyunsaturated fatty acids and cause lipid peroxidation, which contributes to severe cellular damage [6]. In addition, free radicals probably activate Kupffer cells and mediate the hepatic inflammation process through producing tumor necrosis factor-α (TNF-α) and other pro-inflammatory cytokines [7]. Serious hepatocyte apoptosis is also a major cause of CCl_4-induced liver damage. CCl_4 destroys not only plasma membrane but also phospholipid bilayer in mitochondria [8], which triggers caspase-3-dependent apoptosis [9]. Caspase-3 is one of the major death proteases, catalyzing the specific cleavage of key cellular proteins [10].

Traditional herbal medicine is rich source of bioactive components that have desirable health benefits on the prevention of human diseases. In this context, many studies have been reported that herbal extracts or natural products have markedly protective effects against oxidative stress and inflammation induced by CCl_4 [11-15].

The red raspberry (*Rubus idaeus L*., family *Rosaceae*) is a species widely known for its edible fruits. Although they are most commonly known as food products, they are also a popular anti-inflammatory and antimicrobial remedy used in traditional medicine for treating disorders such as stomatitis, sore throats, tonsillitis, coughs, and fevers and as a facilitator of child birth [16]. Impact of berries for improving human health and promoting quality life have been documented [17]. A polyphenolic-enriched red raspberry extract has been reported to show anti-inflammatory effects *in vitro* [18] and *in vivo* [19]. Although the most common part in folk medicine is the fruit, the leaves of *R. idaeus* have also been used. Tea made from the leaves of red raspberry is recommended in traditional medicine as a remedy for upper airway inflammation and has been used for centuries as a folk medicine to treat wounds, diarrhoea, colic pain and as a uterine relaxant [16]. Experimental data indicates that polyphenolic extracts obtained from the leaves of *Rubus* species possess anticancer, antioxidant, antimicrobial, and relaxant properties [16,20,21].

The antioxidant and free radical scavenging activities of red raspberry leaves were documented [22]. Previous reports indicated that the red raspberry leaves are a rich source of flavonoids, ellagic acid, proanthocyanidins and other polyphenols, which made up almost 30% of the dry leaf extracts [23,24], and possess antioxidant capacity [25]. The total amount of phenolic compounds in the leaves varied from 4.8 to 12.0 mg of gallic acid equivalents in 1 g of plant extract. Quercetin and rutin were identified in the extracts [22]. The biological activity of red raspberry leaf polyphenols was confirmed and

the daily intake of this valuable natural antioxidant, exhibited beneficial health effects such as cytotoxic and cytoprotective activity on human laryngeal carcinoma and colon adenocarcinoma [21]. Furthermore, the anti-inflammatory effects of the polyphenolic content of raspberry have been reported [19].

Although red raspberry leaves have been reported to possess many benefits and medicinal properties, however, its protective effects against CCl_4-induced liver injury have not been clarified. The liver injury could be prevented by blocking or retarding the process of oxidative stress and inflammation [2,3]. Being a rich source of antioxidant and anti-inflammatory substances, we hypothesized that these properties of red raspberry leaves could provide a rational assumption for the protective effectiveness of this plant against liver toxicity. In the present study, the capability of red raspberry leaves to protect against CCl_4-induced hepatotoxicity was firstly investigated.

2. MATERIALS AND METHODS

2.1 Animals

This study was conducted on adult male albino Wistar rats (180 – 220 g). The animals were provided by the Experimental Animal Center of King Saud University, College of Pharmacy, Riyadh, KSA. All rats were housed in a temperature-controlled room (23–25℃) and kept on a 12-h light/dark cycle. They had free access to standard rat chow and tap water. The research was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Animal Care Committee of King Saud University.

2.2 Chemicals and Kits

CCl_4, thiobarbituric acid (TBA), Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid), trichloroacetic acid (TCA), sulfanilamide and N-(1-naphthyl) ethylenediamine were purchased from Sigma-Aldrich chemical Co. (St Louis, MO, USA). Commercial kits used for determining albumin and liver enzymes were purchased from Randox Laboratories Ltd. (CRUMLIN, CO. Antrim, UK). ELISA kits for assay of TNF-α, interleukin-6 (IL-6), and IL-1β and FasL were obtained from R&D Co. (Quantikine, R&D systems, Minneapolis, MN, USA). ELISA kits for assay of caspase-3, Bax and Bcl2 were purchased from Cloud-Clone Corp Co. (Houston, USA). Kits used for determining superoxide dismutase (SOD) and glutathione peroxidase (GPx) were obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA). All other chemicals used were of high analytical grade.

2.3 Preparation of Red Raspberry Leaves Suspension

Red raspberry (Rsp) dried leaves powder filled in vegetarian capsules (480 mg in each) was manufactured by Nature's Way Products Co. (Utah, USA) to be taken as a dietary supplement with food or water. The dried leaves powder was suspended 1% gum Arabic solution in normal saline and administered orally (by gavage) at the doses of 50 and 100 mg/kg body 10 days before CCl_4 injection. The dose selection was based on a preliminary study performed in our lab using 10, 25, 50, 100 and 200 mg/kg dried leaves with the best liver function (ALT and AST) improvement obtained with the doses 50 and 100 mg/kg.

2.4 Experimental Design

Forty animals were divided into 5 groups (eight rats each), as follows: group 1 (normal control group); group 2 (Rsp control group treated with 100 mg/kg of Rsp leaves once daily for 10 days); group 3 (CCl_4 control); groups 4 and 5 (CCl_4 groups pre-treated with 50 and 100 mg/kg, respectively, of Rsp leaves once daily for 10 days. During the ten days period of treatment, rats in group 1 (normal control) and group 3 (model group) were administered with the drug vehicle (1% gum Arabic solution) to avoid the effect of this solvent on the results. On the 11th day, rats in groups 3, 4 and 5 were intraperitoneally (i.p.) injected with CCl_4 at a dose of 1.0 ml/kg in 50% olive oil (1:1) to induce acute liver damage.

2.5 Preparation of Serum and Tissue Homogenate

24 h after CCl_4 injection, the rats were sacrificed by cervical decapitation. Blood from the trunk was collected, allowed to coagulate and centrifuged at 3000 rpm for 15 minutes at 4℃. The serum was divided into aliquots to be used for the determination of albumin levels and liver enzymes activities. The livers were removed and cleaned from excess blood with ice-cold normal saline. A part of each liver was fixed with 4% formalin in phosphate buffered saline (PBS; pH 7.4) for at least 24 hrs and prepared for H&E

staining. One gram of each liver was sampled and homogenized (20% w/v) in cold PBS (NaCl 8 g/L, KCl 0.2 g/L, Na2HPO4 144 g/L and KH2PO4 0.24 g/L, pH 7.4) by using Ultra-Turax (IKA-USA) homogenizer. The resulting tissue homogenates were centrifuged at 3000 rpm for 10 minutes at 4℃. The collected supernatant was divided into aliquots and then kept at −80℃ till being used for the assay of oxidative stress, inflammatory and apoptotic markers.

2.6 Evaluation of Liver Function

Serum activities of liver enzymes; alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma glutamyl transferase (GGT), in addition to serum levels of albumin, were measured with routine laboratory methods using commercially available kits from Randox Co.

2.7 Assessment of Oxidative Stress Markers

2.7.1 Determination of lipid peroxidation

Lipid peroxidation was determined by estimating the level of thiobarbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA), according to the method of Ohkawa et al. [26]. MDA is an end product in the sequence of lipid peroxidation reactions and so was taken as an index for this process. Briefly, the reaction mixture (0.5 ml homogenate + 2.5 ml 20% TCA +1.0 ml 0.6% TBA) was heated for 30 minutes in a boiling water bath. The mixture was then cooled and centrifuged for 10 minutes at 4℃. The absorbance of the developed pink-colored product was measured at 535 nm against a reagent blank.

2.7.2 Assay of reduced Glutathione (GSH), a non-enzymatic antioxidant

Total GSH was determined according to the method described by Moron et al. [27] based on the reduction of 5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent) by sulfhydryl groups to form 2-nitro-5-mercaptobenzoic acid, which has an intense yellow color. Briefly, 0.5 ml of hepatic homogenate was mixed with 0.5 ml of 25% TCA and then centrifuged at 4℃ at 3000rpm for 10 minutes. 0.5 ml of supernatant was then added to 4.5 ml of Ellman's reagent and the produced yellow color was measured at 412 nm within 5 minutes.

2.7.3 Assay of CAT, SOD and GPx as enzymatic antioxidants

CAT activity was estimated as the decomposition rate of hydrogen peroxide according to the method of Aebi [28]. In brief, 50 µl of liver homogenate was diluted with 5 ml of phosphate buffer, and then 2 ml of the diluted homogenate was mixed with 1 ml hydrogen peroxide. The absorbance was read after15 and 30 seconds at 240 nm. Activities of SOD and GPx in the liver tissue were determined using assay kits according to the manufacturer's protocol.

2.8 Assessment of Inflammatory Mediators

2.8.1 ELIZA assay of TNF-α, IL-6 and IL-1β

The hepatic levels of TNF-α, IL-6 and IL-1β (as proinflammatory cytokines) were determined by enzyme-linked immunosorbent assay using rat immunoassay kits (R&D Systems) according to the manufacturer's instructions.

2.8.2 Assay of nitric oxide (NO)

Biologically produced NO is rapidly oxidized to the stable metabolites; nitrite and nitrate. Thus, nitrite concentrations can reflect NO production. Hepatic levels of nitrite were measured colorimetrically according to the method of Green et al. [29] in which nitrite is converted into a deep purple azo chromophore. Briefly, 100 µl of liver homogenate was added to 100 µl of Griess reagent (1:1 mixture of 1% sulfanilamide in 2.5% orthophosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine in distilled water). After 10 min. of color development at room temperature, the absorbance was measured at 540 nm.

2.9 Assessment of Apoptotic Markers: Assay of FasL, caspase-3, Bcl2 and Bax

The hepatic levels of FasL (a marker of extrinsic apoptotic pathway) and the levels of caspase-3, Bcl2 and Bax (markers of intrinsic apoptotic pathway) were determined by ELISA assay using rat immunoassay kits according to the manufacturer's instructions.

2.10 Histopathological Analysis

Five µm thick sections of liver samples were prepared for staining with H& E for routine histopathological examination. Examination of

the slides was performed under a light microscope, and digital images were captured using a Nikon microscope (Y-THS, Japan).

2.11 Statistical Analysis

The results are expressed as the mean ± SEM. Statistical comparisons between the groups were performed using one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post hoc test. Statistical analysis was conducted using Prism GraphPad software version 4 (San Diego, California, USA). P values less than .05 were considered statistically significant. *, ** and *** indicate P < .05, P < .01 and P < .001, respectively.

3. RESULTS

3.1 Effect of Rsp Leaves on Liver Function in CCl_4-induced Liver Injury

The effects of Rsp leaves pretreatment on the CCl_4-induced abnormalities in liver function are shown in Table 1. The administration of CCl_4 caused severe hepatotoxicity, as indicated by the significant elevation in serum activities of ALT, AST, ALP, LDH and GGT (P < .001) in addition to a decrease in albumin levels (P < .01). However, pretreatment with Rsp leaves at the dose 100 mg/kg prevented these elevations in serum enzymes and the decrease in albumin levels (P <.001) where the enzymes activities and albumin levels were similar to those observed in normal control group. Low dose of Rsp leaves (50 mg/kg) also, although with a lesser extent, significantly attenuated the alterations of serum ALT, ALP, GGT and LDH (P < .01), AST and albumin (P < .001) compared with the model group. Pretreatment with 50 mg/kg showed significantly higher serum ALT, AST, ALP and GGT (P <.001, P < .01) compared with normal control rats. In addition, rats pretreated with the high dose 100 mg/kg revealed significant improvement in ALT, AST, ALP and GGT compared to those pretreated with 50 mg/kg (P < .05). These results indicated the hepatoprotective effect of Rsp leaves and this effect is more prominent with 100 mg/kg.

3.2 Effect of Rsp Leaves on Liver Architecture in CCl_4-induced Liver Injury

Hematoxylin and eosin (H&E) stained sections are shown in Fig. 1. Normal liver lobular architecture, cell structure and vasculature were observed in normal control and Rsp control groups (Panels A and B). While the liver tissue of the rats treated with CCl_4 alone showed apparent morphological changes including cell degeneration, loss of hepatocyte architecture, sinusoid distortion and massive cellular inflammatory infiltration (Panels C and D). However, pretreatment with both 50 and 100 mg/kg Rsp leaves significantly attenuated both the cell degeneration and inflammatory infiltration (panels E and F) particularly with the dose 100mg/kg which markedly improve cellular infiltration, cellular degeneration and hepatic sinusoid congestion (Panel F).

3.3 Effect of Rsp Leaves on Lipid Peroxidation Extent in CCl_4-induced Liver Injury

Hepatic levels of MDA were determined to evaluate the extent of lipid peroxidation and, in turn, the degree of oxidative injury. As shown in Fig. 2, significant increase of MDA content was observed in the liver of rats exposed to CCl_4 (P < .001), while the pretreatment with Rsp leaves (50, 100 mg/kg) significantly suppressed the MDA levels in liver tissues compared with the model (P < .001). In addition, the hepatic MDA levels in rats pretreated with 50 and 100 mg/kg were similar to those observed in normal control group, confirming that the Rsp leaves effectively protect membrane lipids from oxidation by CCl_4-induced free radicals.

3.4 Effect of Rsp Leaves on Antioxidant Markers in CCl_4-induced Liver Injury

Fig. 3 revealed that the hepatic levels of GSH and the activities of GPx, SOD and CAT showed significant decreases in the rats intoxicated with CCl_4 compared with normal control group (P < .001). However, rats pretreated with 100 mg/kg Rsp leaves significantly attenuated this antioxidant depletion compared to CCl_4-injured rats (P < .001), and showed a significant improvement towards the control values. Therefore, the Rsp leaves have markedly antioxidant activities and suppressed CCl_4-induced oxidative liver injury. Rsp leaves at the dose of 50 mg/kg could significantly attenuate the levels of GSH (P < .01) and the activities of GPx (P < .001), SOD (P < .05) and CAT (P < .05) compared to model group, but the levels were still significantly higher than those observed in normal control rats (Fig. 3). The antioxidant effect was more prominent with the dose 100 mg/kg which showed significant improvement in GSH and SOD (P < .05) and in GPx and CAT (P < .01) compared to the dose 50 mg/kg.

Table 1. Effect of 50 and 100 mg/kg of Rsp leaves on liver function markers in CCl_4-induced liver injury

	Control	Rsp 100	CCl_4	CCl_4+Rsp 50	CCl_4 + Rsp 100
ALT (U/ml)	66.13±5.56	58±5.06	201.2^{a***}±23.65	125.6^{a**b**}± 6.4	71.68$^{b***\ c*}$±3.8
AST (U/ml)	121.8±8.72	102±3.65	326 ^{a***}± 32.6	217.3^{a**b***}±10.4	138.4 $^{b***\ c*}$±8.2
GGT (U/ml)	20±0.93	20.67±0.71	36.3 ^{a***}±1.5	27.5^{a**b***}±1.33	22.5$^{b***\ c*}$±1.25
LDH(U/ml)	928.1±46.5	771.8±84.4	1528^{a***}±168.9	1090 ^{b**}±43	977 ^{b***}±22.6
Albumin (g/dl)	4.5±0.13	4.77±0.18	3.5 ^{a**}± 0.08	4.2 ^{b***}± 0.14	4.4 ^{b***}±0.17

*Values are expressed as Mean ± SEM. **a**: significantly different from normal control group; **b**: significantly different from CCl_4-intoxicated group; **c**: significantly different from Rsp 50-treated group. *** P < .001, ** P < .01, * P < .05*

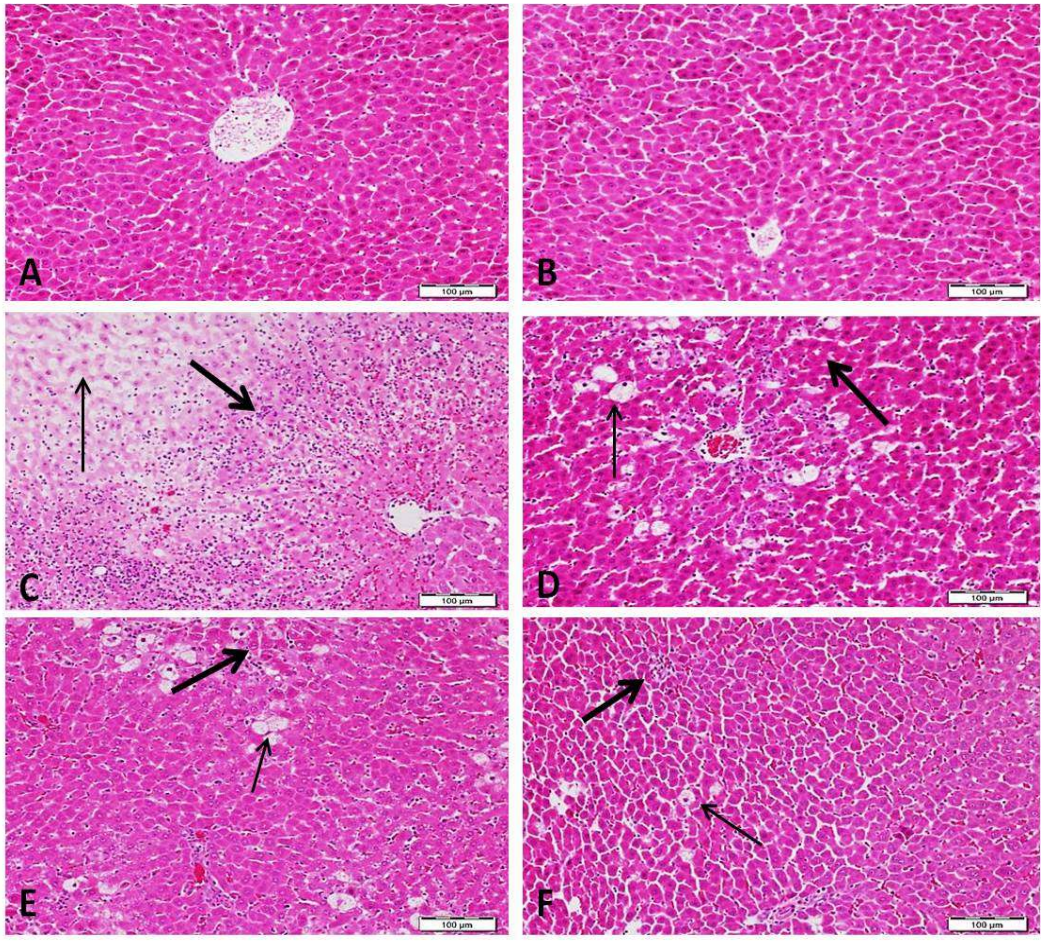

Fig. 1. Light photomicrographs of liver sections stained with H&E, scale bar=100 µm

(A) and (B) Liver sections from normal control and Rsp leaves control, respectively showed normal hepatocytes and sinusoids, while (C and D) represent liver sections of rats received CCl_4 only, showing hepatic toxicity in the form of hepatocytes degenerations, widening of the blood sinusoids and hemorrhage (thin arrow, panel C), cellular degeneration (thin arrow, panel D) and severe inflammatory cellular infiltration (thick arrows). (E) section of liver from rat received CCl_4 and pretreated with 50 mg/kg Rsp leaves showing decreased cellular degeneration where only local small foci of cellular degeneration were observed (thin arrow) also the cellular infiltration decreased than that observed with CCl_4 only (thick arrow). (F) liver section from rat pretreated with 100mg/kg Rsp leaves showing marked regeneration of cellular degeneration with very small patches of cellular infiltration (thick arrow) and very few scattered degenerated solitary degenerated hepatocytes (thin arrow)

3.5 Effect of Rsp Leaves on Proinflammatory Markers in CCl_4-induced Liver Injury

As shown in Fig. 4, the 24-hour exposure to CCl_4 alone led to a remarkable elevation in the proinflammatory markers, TNF-α, IL-6 and IL-1β compared to normal control rats ($P < .001$). Pretreatment with the high dose of Rsp leaves (100 mg/kg) significantly alleviated CCl_4-induced inflammatory response compared with the model group ($P < .001$). No significant differences in the hepatic levels of TNF-α and IL-1β were observed in rats pretreated with the dose 100 mg/kg compared to normal control group reflecting the powerful anti-inflammatory effects of Rsp leaves at that dose. Similarly, but to lesser extent, pretreatment with 50 mg/kg significantly suppressed the levels of TNF-α, IL-6 ($P < .01$) and IL-1β ($P < .05$) compared to CCl_4-injured liver, however the levels were still significantly higher compared to normal control (Fig. 4). The anti-inflammatory effects of the dose 100 mg/kg were significantly pronounced compared to the dose 50 mg/kg ($P < .05$).

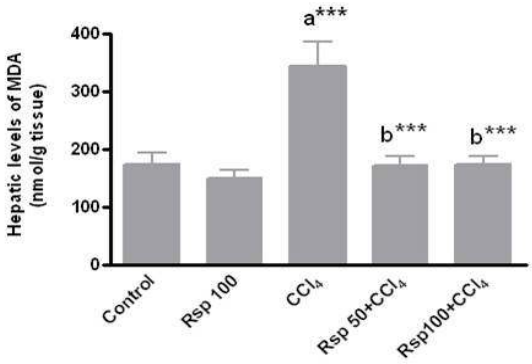

Fig. 2. Effect of 50 and 100 mg/kg of Rsp leaves on hepatic levels of MDA; a marker of lipid peroxidation in CCl_4-induced liver injury

*Values are expressed as Mean ± SEM. a: significantly different from normal control group; b: significantly different from CCl_4-intoxicated group. *** P <.001*

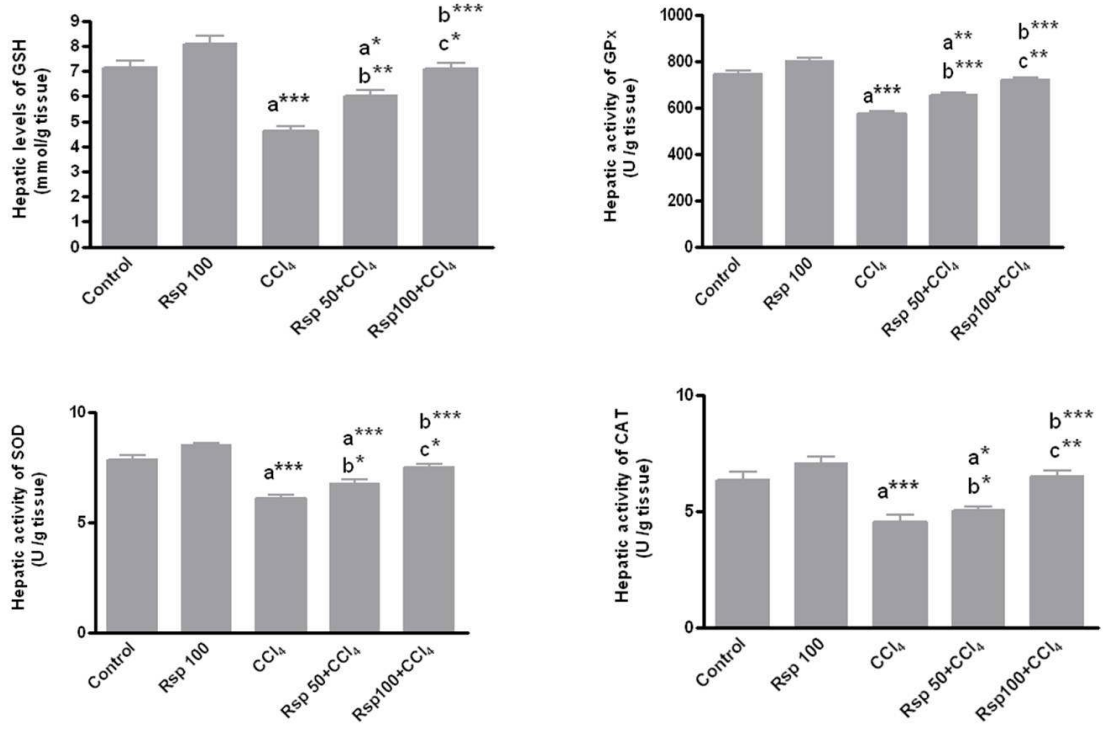

Fig. 3. Effect of 50 and 100 mg/kg of Rsp leaves on hepatic levels of reduced glutathione (GSH) and activities of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) enzymes in CCl_4-induced liver injury

*Values are expressed as mean ± SEM. **a**: significantly different from normal control group; **b**: significantly different from CCl4-intoxicated group; **c**: significantly different from Rsp 50-treated group. *** P < .001, ** P < .01, * P < .05*

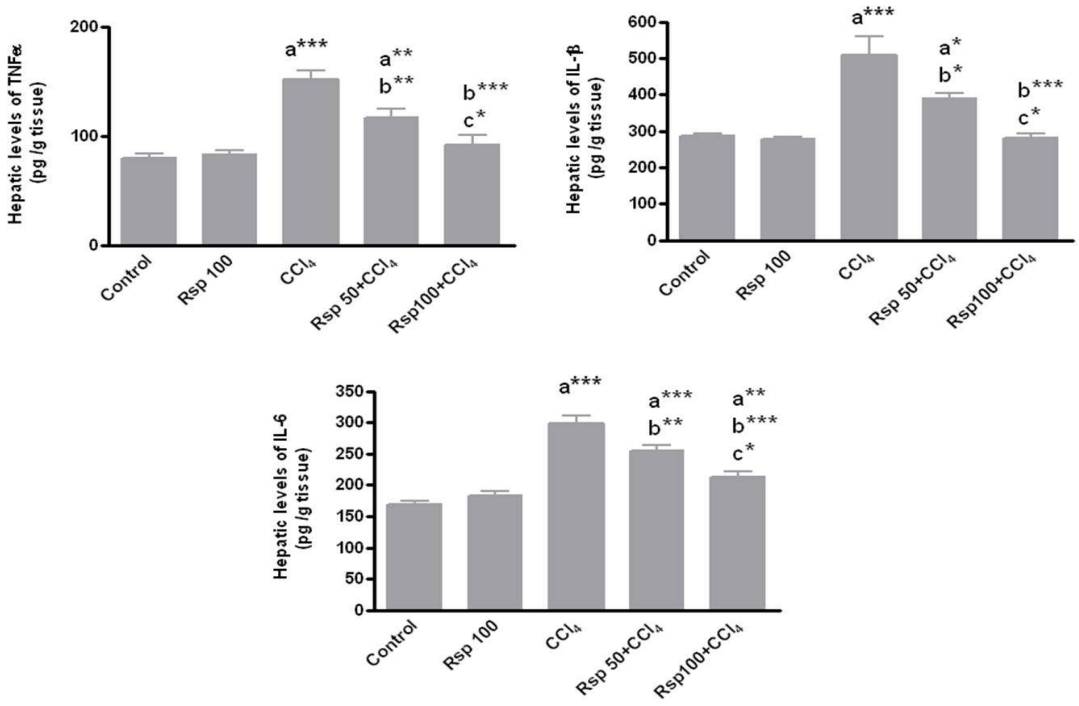

Fig. 4. Effect of 50 and 100 mg/kg of Rsp leaves on hepatic levels of proinflammatory cytokines in CCl_4-induced liver injury

*Values are expressed as mean ± SEM. a: significantly different from normal control group; b: significantly different from CCl_4-intoxicated group; c: significantly different from Rsp 50-treated group. *** P < .001, ** P < .01, * P < .05*

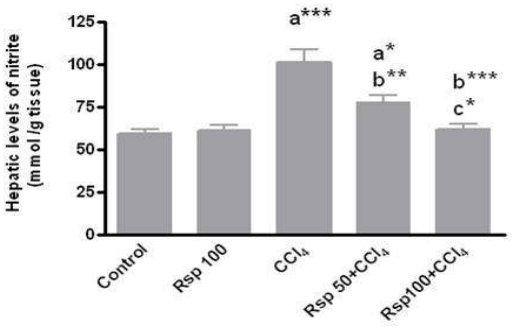

Fig. 5. Effect of 50 and 100 mg/kg of Rsp leaves on hepatic levels of nitrite (an index of nitric oxide) in CCl_4-induced liver injury

*Values are expressed as mean ± SEM. **a**: significantly different from normal control group; **b**: significantly different from CCl_4-intoxicated group; **c**: significantly different from Rsp 50-treated group. *** P < .001, ** P < .01, * P < .05*

3.6 Effect of Rsp Leaves on Nitrite Levels in CCl_4-induced Liver Injury

The hepatic nitrite levels were measured as index of NO production in liver. Fig. 5 showed that CCl_4 induced NO production in liver as indicated by the remarkable increase in nitrite levels compared to normal control group (P < .001). This elevation was attenuated by pretreatment with both 100 mg/kg (P < .001) and 50 mg/kg (P < .01) of Rsp leaves compared to model group. In addition, the hepatic levels of nitrite were similar to those in normal control group particularly with the dose 100 mg/kg. The dose 100 mg/kg significantly improved the nitrite levels compared to the dose 50 mg/kg (P < .05).

3.7 Effect of Rsp Leaves on Apoptotic Markers in CCl_4-induced Liver Injury

Fig. 6 showed the effect of CCl_4 and Rsp leaves on apoptotic markers in liver tissue. FasL levels were determined as a marker of extrinsic pathway of apoptosis. Hepatic levels of caspase-3 (the effector apoptotic factor), Bax (a proapoptotic factor) and Bcl2 (an anti-apoptotic factor) were evaluated as markers of intrinsic pathway of apoptosis. Results showed that CCl_4 resulted in significant increases in the hepatic levels of FasL, caspase-3 and Bax (P < .001),

while Bcl2 levels were significantly reduced ($P < .001$). Pretreatment with Rsp leaves (100 mg/kg) significantly attenuated the change in all these apoptotic factors ($P < .001$) compared to CCl_4-injured rats. No significant differences in the hepatic levels of FasL, caspae-3, Bax and Bcl2 were observed in rats pretreated with the dose 100 mg/kg compared to the normal control group confirming the potential antiapoptotic activity of Rsp. Rsp leaves (50 mg/kg) also prevented the increase in FasL ($P < .01$), caspase-3 ($P < .001$) and Bax ($P < .001$) and attenuated the reduction in Bcl2 ($P < .05$). The dose 50 mg/kg successfully preserved the levels of Bax at those of normal control rats, while the levels of FasL, caspase-3 and Bcl2 were still higher compared to normal control (Fig. 6). The improvement in the levels of caspase-3 was significantly better at the dose 100 mg/kg compared to the dose 50 mg/kg, otherwise, no significant differences were observed in case of FasL, Bax and Bcl2.

4. DISCUSSION

In the last decade, red raspberry leaves gain most acceptance as they contain numerous bioactive compounds such as phenolics, tannins, flavonoids, ellagic acids and proanthocyanins [22,23]. Although many biological activities of red raspberry leaves have been studied, however, to date, the protective effects of raspberry leaves in liver diseases have not been elucidated. Being a rich source of antioxidants and exhibiting potential aspects as anti-inflammatory and free radical scavenger, we considered that raspberry leaves are useful in the prevention of liver injuries induced by oxidative stress.

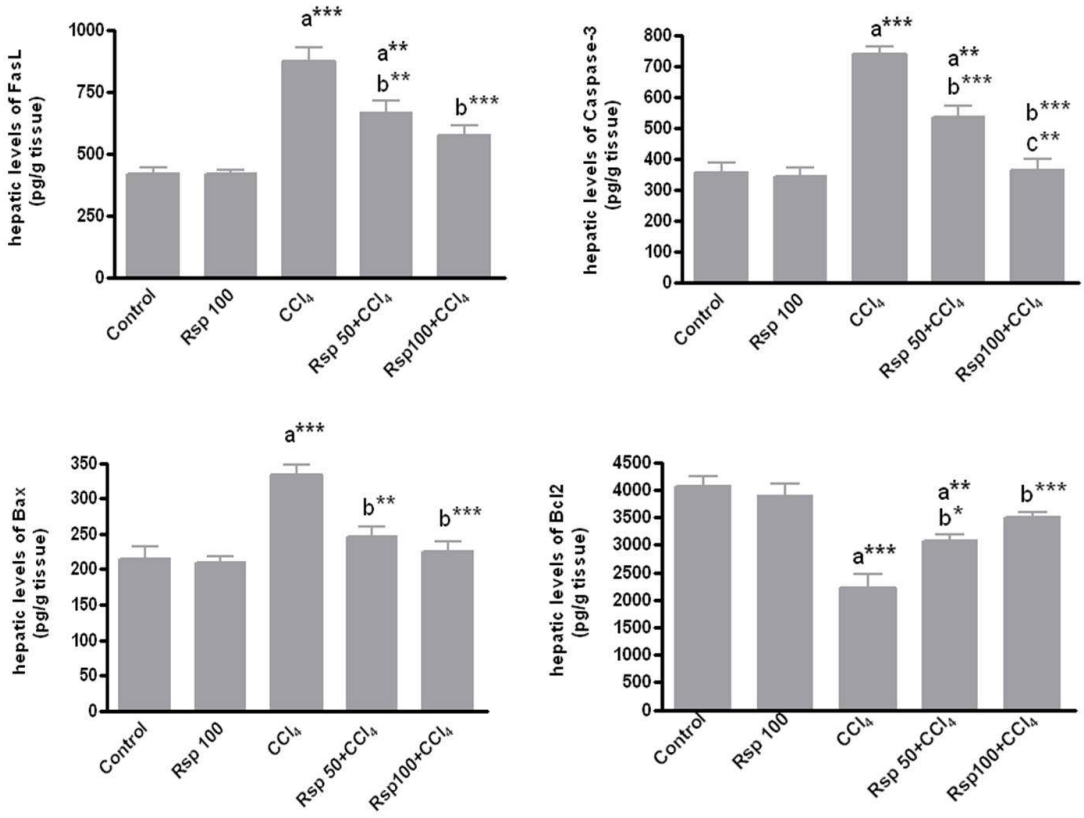

Fig. 6. Effect of 50 and 100 mg/kg of Rsp leaves on hepatic levels of FasL, caspase-3, Bax and Bcl2 in CCl_4-induced liver injury

*Values are expressed as mean ± SEM. **a**: significantly different from normal control group; **b**: significantly different from CCl_4-intoxicated group; **c**: significantly different from Rsp 50-treated group. *** P < .001, ** P < .01, * P < .05*

Carbon tetrachloride (CCl_4)-induced liver injury is the best characterized system for xenobiotic-induced hepatotoxicity via oxidative stress and inflammation in experimental animals [4,5]. Therefore, this model of hepatic injury has been extensively used to evaluate the potential of drugs and dietary antioxidants against the oxidative damage and inflammatory response. The principle of injury is that CCl_4 is biotransformed into a highly reactive trichloromethyl (-CCl3•) and trichloromethylperoxy (OOCCl3•) free radicals by cytochrome P450 (CYP)-2E1 inside the ungranulated endoplasmic reticulum which is found excessively in the liver. These free radicals promote the accumulation of lipid peroxidation products that cause liver injury, resulting in cell necrosis.

The present study showed that serum ALT, AST, LDH, GGT and ALP activities increased rapidly and dramatically in CCl_4-intoxicated rats (Table 1), indicating severe and acute hepatocellular damage. The obtained results are in accordance with those of the previous reports [13,30,31]. Elevations of serum liver enzymes could be attributed to $CCl3^{\bullet}$ and $OOCCl3^{\bullet}$ radicals which are highly unstable and immediately activate oxidation of polyunsaturated fatty acids of cell membrane leading to damage and increased permeability of the hepatocellular membranes and finally the leakage of liver enzymes [32]. ALT and AST are cytosolic enzymes of the hepatocyte, and their dramatically elevated serum levels reflect the loss of integrity and increased permeability of cell membrane, therefore, their serum levels are the most commonly used biochemical markers of liver injuries [33]. LDH is a well-known marker of cell damage and its increase in serum is indicative to the hepatocellular necrosis that leads to leakage of the enzyme into the bloodstream. ALP and GGT are hepatocyte cell membrane enzymes, an increase of them has been related to damage of the liver cell membrane, therefore they are sensitive markers for liver injury [34]. Impaired liver function was further emphasized by lowered serum albumin levels indicating impaired synthetic capacity of the damaged liver. Histological observations of liver samples strongly supported the release of liver enzymes by damaged hepatocytes (Fig. 1).

The present results indicated that pretreatment with 50 and 100 mg/kg Rsp leaves markedly attenuated the CCl_4- induced elevation in serum liver enzymes. The reduction in liver enzymes toward the normal control values, particularly at the dose 100 mg/kg, is a sign of the stabilization of plasma membranes reflecting the effectiveness of Rsp in protection against CCl_4 hepatotoxicity. Furthermore, Rsp leaves could significantly improve serum albumin, which demonstrated that Rsp could promote liver functional recovery. This biochemical improvement was coincided with the attenuation of histological alterations so that treated livers showed only minor hepatocellular necrosis and inflammatory cell infiltration and marked regeneration of hepatic architecture, particularly with the dose 100 mg/kg. These results suggest that the Rsp leaves could have potential clinical applications for treating liver disorders.

It has been reported that lipid peroxidation of hepatocyte membrane is central to the toxicity of CCl_4, and is mediated by the free radicals derivatives of CCl_4 [5]. Lipid peroxidation leads to destruction of the plasma membrane and intracellular organells which in turn results in massive destruction of hepatocytes and tissue necrosis as revealed in the present study by H&E staining (Fig. 1). MDA is a natural lipid peroxidation end product and its increase in liver tissue is the hallmark of oxidative stress. In the current study, the elevation of MDA content in the liver of CCl_4-intoxicated rats (Fig. 2) indicates lipid oxidation and oxidative stress that lead to tissue injury. GSH plays an excellent role in protecting cells from CCl_4-induced hepatotoxicity as it combines with the toxic metabolites of CCl_4, which contributes to detoxification of CCl_4. SOD, CAT and GPx, comprise the major antioxidant system in mammalian cells and the inhibition of their activities cause accumulation of reactive oxygen species (ROS) which accentuate a cascade of free radical formation [7]. It is crucial to maintain the balance between ROS and antioxidant enzymes, which serves as a major mechanism in preventing damage elicited by oxidative stress [7]. However, the imbalance can produce toxic effects within the body and damage all the single aspects of a cell, including its protein, lipids and DNA. In consistent with the current results, CCl_4-derived free radicals lead to impaired antioxidant defense system either enzymatic -such as SOD, CAT and GPx - or non enzymatic such as GSH (Fig. 3). The decrease of GSH by CCl_4 is probably related to a reduced synthesis by the diseased liver or may be associated with its rapid consuming for scavenging ROS and free radicals in injured liver. In addition, $CCl3^{\bullet}$ free radicals can react with sulfhydryl group in GSH. The activities of

SOD, CAT and GPx are reduced by the produced lipid peroxides or ROS. In addition, the liver is the main organ in the metabolism and homeostasis of selenium in the body and because GPx is a selenoprotein which is predominantly synthesized and secreted by the liver [35] so, a decrease of GPx activity was observed in CCl_4-intoxicatd rats compared with normal liver.

In the present study, pre-treatment with 50 or 100 mg/kg Rsp leaves exhibited potential protective effects against CCl_4-mediated oxidative stress as indicated by the alleviation of MDA elevation and attenuated hepatic GSH and antioxidants depletion 24 h after CCl_4 injection (Figs. 2 and 3). These results suggest that the antioxidant properties may be one mechanism by which the Rsp leaves protects against CCl_4-mediated liver damage. The antioxidant effect was more prominent with the dose 100 mg/kg reflecting that the effect may be dose-dependent. This protective action of Rsp leaves could be attributed to free radical scavenging function due to high levels of bioactive antioxidant substances including polyphenolics, tannins, flavonoids, ellagic acids and proanthocyanins [22,23,25]. Dudzinska et al. [25] demonstrated that antioxidant capacities of the Rsp extracts remained relatively high and corresponded well to the determined total polyphenol contents. In parallel with our results, Durgo et al. [21] found that Rsp leaf extract increased total GSH level in HEp2 cells. This effect was reinforced after 24 hours of recovery, indicating that induction was caused by products formed during cellular metabolism of compounds present in the extract. In conclusion, Rsp leaves might prevent hepatic damage through scavenger activity, inhibitory action on lipid peroxidation, maintaining the normal oxidant/antioxidant balance of the liver and preservation of membrane integrity.

The second phase of CCl_4-induced hepatotoxicity involves the activation of Kupffer cells (macrophages in the liver) [5,7] which results in the production of a vast array of proinflammatory mediators in the liver [7,36] including IL-1, IL-6, IL-8, TNF-α and NO. These inflammatory mediators are believed to aggravate CCl_4-induced hepatic injury and induce toxicity either by direct cellular damage or by chemoattracting neutrophils and lymphocytes [37]. TNF-α is a major endogenous mediator of hepatotoxicity in several experimental liver injuries through its direct cytotoxicity [38], NO production [39], and the triggering of an inflammatory cascade. Gabele et al. [40] demonstrated through bile duct ligation model that TNF-α actively potentiates hepatotoxicity and fibrogenesis. In addition, TNF-α acts as mediator of liver apoptosis which is also linked to cytotoxicity induced by CCl_4 [41]. IL-1β plays a key role in inflammatory conditions, usually leading to tissue destruction. Furthermore, IL-1β has been previously shown to antagonize hepatocyte proliferation [42,43]. In agreement with these data, our results revealed elevated levels of TNF-α, IL-6 and IL-1β in CCl_4-injured livers compared to normal control. The histological examination from the current study revealed massive inflammatory cell infiltration confirming the inflammatory response involved in the process of acute chemical liver injury. To investigate the anti-inflammatory mechanism of Rsp leaves, we evaluated the effects of their administration on the hepatic levels of the above mentioned key cytokines tightly related with inflammation. Pretreatment with both 50 and 100 mg/kg Rsp leaves significantly attenuated the increase in TNF-α, IL-6 and IL-1β production indicating the hepatoprotective effect through alleviation of the inflammatory response. The anti-inflammatory effects of Rsp leaves could be attributed to their polyphenolic compounds such as anthocyanins and ellagitannins which have been reported to show anti-inflammatory effects in both *in vitro* and *in vivo* models [18,19,44].

It has been also reported that overproduction of NO is associated with CCl_4-induced inflammatory hepatic injury [45-47]. NO levels increase rapidly within minutes to hours in response to inflammatory stimuli [48]. NO is derived from two sources in liver; hepatocytes and Kupffer cells. These cells contain inducible NO synthase (iNOS), the activity of which is markedly increased in inflammation. It has been demonstrated that TNF-α stimulates iNOS in liver parenchymal and non-parenchymal cells to generate NO which cause nitrosative stress [49]. Thus, NO is a crucial factor during acute inflammation and has been used as a marker of inflammation in many studies [50,51]. Furthermore, and importantly, excessive NO production could exaggerate the oxidative hepatic damage. That is because excess NO reacts with superoxide anion to produce the highly reactive peroxynitrite radical [52] which further cause nitrosative stress and subsequent nitration of protein tyrosine residues, which plays an important role in the pathogenesis of hepatic necrosis [6]. Therefore, the prevention of NO production may have an important role in

ameliorating hepatocellular injury. In the present study pretreatment with both doses of Rsp leaves significantly ameliorated the elevation in NO production as evidenced by lowered levels of nitrite; the metabolic product of NO. This modulatory effect on NO is probably related to reduced levels of TNF-α by the Rsp leaves with subsequent suppression of iNOS stimulation. Collectively, we can conclude that liver inflammation caused by CCl_4 intoxication was significantly ameliorated by the Rsp leaves pretreatment. According to our results, the anti-inflammatory properties of Rsp leaf extract are considered to be achieved through inhibiting the production of inflammatory cytokines and NO.

Hepatocyte cell death occur in both acute and chronic liver diseases. Indeed, excessive cell death has been identified as a central mechanism of liver damage. Apoptosis, a highly organized and genetically controlled process, is the most investigated and best defined form of programmed cell death. CCl_4 administration causes the incidence of apoptosis in the liver by activating caspase-3 [9], which is one of the major death proteases, catalyzing the specific cleavage of key cellular proteins [10]. In consistent with this fact, the present study demonstrated a significantly higher levels of hepatic caspase-3 compared to normal control indicating the hepatocyte death. However, pretreatment with Rsp leaves significantly ameliorated this increase at both doses, providing another mechanism of its hepatoprotection via antiapoptotic effect. The mechanism by which Rsp leaves ameliorate casapse-3 activation was further investigated in the present study.

Apoptosis is initiated by either membrane receptors (extrinsic pathway) or intracellular stimuli (intrinsic pathway). However, both pathways result in the activation of effector caspase 3, which execute the final apoptotic changes. Extrinsic pathway of apoptosis is initiated by stimulation of death receptor (DR) which is a member of tumor necrosis factor receptors superfamily, leading to formation of death inducing signaling complex, which in turn recruit and activate caspase 8 and 10 leading to activation of effector caspase, caspase-3 [53]. Fas receptor is one of DRs that are considered to play a role on liver apoptosis. Fas promotes apoptosis after being activated through binding to Fas ligand (FasL), a transmembrane protein with a homotrimeric structure. It has been suggested that that apoptosis via the Fas/FasL signalling pathway play an important role in the development of liver failure [54].

In intrinsic pathway, apoptotic stimuli such as CCl_4 produce changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore, loss of mitochondrial transmembrane potential and release of pro-apoptotic proteins such as cyctochrome c. cytochrome c binds and activates procaspase-9, which in turn cleaves and activates the executioner caspase-3 thereby triggering the apoptotic pathway [55]. The regulation of pro-apoptotic events occurs through members of the Bcl-2 protein family that may be pro-apoptotic such as Bcl-10, Bax, Bad and Blk or anti-apoptotic proteins including Bcl-2, Bcl-x, Bcl-xL and BAG [56]. The intrinsic pathway involve interaction between pro-apoptotic and death factors such as cytochrome c and apoptosis inducing factor released from mitochondria , while the other members of Bcl-2 such as Bcl-2 serve as anti-apoptotic factors and inhibit recruitment of pro-apoptotic members to the mitochondria.

In the current study, elevated levels of caspase-3 (Fig. 6B) indicated enhanced apoptosis in the livers of CCl_4 treated rats. Moreover, CCl_4 increased the levels Fas L (Fig. 6A) and Bax (pro-apoptotic protein, Fig. 6C), and decreased the levels of Bcl-2 (anti-apoptotic protein, Fig. 6D) which are important regulators of apoptosis in mitochondrial pathway [56]. These results reflect the activation of both extrinsic and intrinsic apoptotic pathways by CCl_4. Both doses of Ras leaves significantly ameliorated these alteration in apoptotic markers indicating that the heaptoprotective effect of Rsp leaves is mediated partly by suppressing apoptosis pathway, including up-regulating Bcl2 and markedly decreasing the levels of caspase-3, FasL and Bax in the liver tissue. In consistent with our results, previous reports suggested that a dietary supplement with red raspberry effectively protects against chemically induced hepatic lesions in rats via inhibition of induced apoptosis [57]. This anti-apoptotic effect of Rsp may be attributed to its high contents of flavonoids and polyphenols which are known to reduce apoptosis [58]. TNF-α is able to induce apoptosis via caspase activation pathways [5,41,59,60], therefore, we can conclude that Rsp leaves protect from CCl_4-induced apoptosis by inhibiting TNF-α-mediated activation of caspase. Except for caspase-3, the dose 100 mg/kg showed no significant difference in FasL, Bax and Bcl2

compared to 50 mg/kg. However, collectively, the results showed that the dose 100 mg/kg was more effective than 50 mg/kg particularly as antioxidant and anti-inflammatory.

5. CONCLUSION

In conclusion, the present study demonstrates, for the first time, that Rsp leaves significantly prevented CCl_4-induced hepatotoxicity, *in vivo*, possibly by scavenging free radicals, improving the endogenous antioxidant system, blocking the inflammation response and inhibiting apoptosis in liver tissue. Our findings suggest that consumption of raspberry leaf products should therefore be encouraged not only in healthy people, but especially in those with increased risk of toxic liver damage.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The research was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Animal Care Committee of King Saud University.

ACKNOWLEDGEMENTS

I want to express my sincere thanks to the technicians in the department of Histology, College of Medicine, King Saud University for their assistance in performing the histological examination.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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