



## Edible Plants-Induced Recovery from Hepatic Disorders through Matrix-Metalloproteinase-9 Evaluation

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### ABSTRACT

Liver fibrosis is a pathologic phenomenon characterized by destruction of the liver parenchyma and imbalance between fibrogenesis and fibrolysis forming scars or fibrous tissues. Without effective treatment at an early stage, reversible hepatic fibrosis progresses to irreversible cirrhosis. The objective of the present study was aimed to early detection of liver fibrosis using matrix metalloproteinase-9 (MMP-9) as a diagnostic marker and to evaluate the curative and prophylactic roles of the two natural edible plants Blackberry and Rosemary (*Rubus ursinus* and *Rosmarinus Officinalis*) in mitigating the oxidative stress and inflammatory risk factors which have the principle role in liver fibrosis. This study demonstrated that matrix metalloproteinase-9 (MMP-9) may be an excellent diagnostic marker for early detection of liver disorders induced by CCL<sub>4</sub>. Additionally, supplementation of either rosemary or blackberry extracts to CCL<sub>4</sub> treated rats, has been found to possess a beneficial protective effects against liver fibrosis through their antioxidant, anti-inflammatory, anti-apoptotic, and anti-fibrotic activities. Also, the histopathological pictures of liver sections of rats intoxicated with CCL<sub>4</sub> and treated with either rosemary or blackberry appeared more or less like normal with an improvement in the liver architecture compared to CCL<sub>4</sub> treated group.

**Keywords:** Liver fibrosis, Carbon tetrachloride, Blackberry, Rosemary, Matrix metalloproteinase-9(MMP 9), Apoptosis.

### INTRODUCTION

The liver is the key organ regulating homeostasis in the body, it is involved with almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy provision and reproduction.<sup>1</sup> Unique among the body's organs, the liver has the ability to regenerate or form new cells as needed. Up to 80% of the liver's cells can be surgically removed or damaged by disease before the liver ceases to function. However, the liver's ability to repair itself and recover from injury varies among individuals. This variability may strongly influence the rate of liver disease severity and progression.<sup>2</sup>

The development of liver fibrosis is a multi cellular process involving paracrine signaling between resident liver cells and nonresident inflammatory cells resulting in complex cell, matrix, cytokine and growth factor interactions.<sup>3,4</sup> Physiological fibrogenesis is initiated by parenchymal cell destruction owing to multiple harmful agents and mechanisms, followed by inflammation, which in turn activates 'resting' ECM-producing cells to produce and secrete extracellular matrix (ECM) molecules, cytokines, chemokines, matrix metalloproteinases (MMPs) and their specific inhibitors [tissue inhibitors of metalloproteinases (TIMPs)].<sup>5,6</sup> In addition during the process of fibrogenesis, apoptosis and its apoptotic markers such as (Caspase-3 and caspase-7) are activated with disease progression, so measuring the serum level of both caspases reflect the progression of liver disease.<sup>7,8</sup> Liver fibrosis representing the final common pathway of all chronic hepatic injury, it is a pathologic phenomenon

characterized by progressive and excessive accumulation of fibrillar extracellular matrix (ECM) proteins that occurs during chronic liver tissue damage.<sup>9,10</sup> The accumulation of ECM protein deranges the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes defines liver cirrhosis. Fibrosis is currently considered an abnormal wound-healing response to chronic liver injury.<sup>11</sup> Fibrosis is a common response to chronic liver diseases because of infective mostly hepatitis C virus (HCV) and hepatitis B virus (HBV), toxic drug- induced metabolic and auto-immune causes.<sup>12</sup> There are another stimuli of liver fibrosis including, Alcohol, Non-alcoholic steatohepatitis (NASH), Hepatotoxins.

There are many hepatotoxins that are present in our life and used as models for studying liver fibrosis, among them carbon tetrachloride (CCl<sub>4</sub>), nitrosamines and aflatoxins.<sup>13</sup> Carbon tetrachloride was used in our study for induction of liver disorders.

Carbon tetrachloride (CCl<sub>4</sub>) is a one of the chlorinated hydrocarbons, is widely used in various industries as a solvent. It is also used in medicine as a vermifuge in the treatment of hookworm disease. Exposure to CCl<sub>4</sub> by inhalation, ingestion or absorption through the skin resulted in many cases of poisoning.<sup>13</sup> In addition, CCl<sub>4</sub> is highly toxic chemical agent, the most famous drug used to induce liver damage experimentally for studying free radical-induced liver injury and screening hepatoprotective drugs.<sup>14</sup> Prolonged administration of CCl<sub>4</sub> causes fibrosis, cirrhosis and hepatic carcinoma.<sup>15</sup> The hepatotoxic activity of CCL<sub>4</sub> is initiated after its activation



by cytochrome P450-dependant monooxygenases to form the trichloromethyl radical (CCl<sub>3</sub>\*).<sup>16</sup> This radical can bind to cellular molecules (nucleic acid, protein, lipid), impairing crucial cellular processes such as lipid metabolism, with the potential outcome of fatty degeneration (steatosis). Adduct formation between (CCl<sub>3</sub>\*) and DNA is thought to function as initiator of hepatic cancer. This radical can also react with oxygen to form the trichloromethyl-peroxy radical (CCl<sub>3</sub>OO\*), a highly reactive species.<sup>17</sup> (CCl<sub>3</sub>OO\*) initiates the chain reaction of lipid peroxidation, which attacks and destroys polyunsaturated fatty acids, in particular those associated with phospholipids. This affects the permeability's of mitochondria, endoplasmic reticulum, and plasma membranes, resulting in the loss of cellular calcium sequestration and homeostasis, which can contribute heavily to subsequent cell damage.<sup>18</sup>

In view of the adverse effects associated with the synthetic drugs and as plants are safer, cheaper and much effective, herbal medicines are increasingly popular and becoming more mainstream in clinical practice and show a significant value in treating and preventing diseases.<sup>19,20a</sup> The new attractions of drug discovery using natural products remain an important issue in the current herbal medicine research. Many recent studies have suggested that dietary intake of phytochemicals could be a useful strategy for the chemoprevention against hepatic disorders. These substances have been shown to exert their effects via their antioxidant-related properties. The plant based compounds have many advantages; they are easily available, can be synthesized in large quantities, and can be administered without any deleterious side effects.<sup>21,22</sup> Blackberry (*Rubus ursinus*), belonging to the botanical family *Rosaceae* is a good source of natural antioxidants, which is one of the major reasons for increasing popularity in the human diet.<sup>23</sup> It has a high oxygen radical absorbance activity against peroxy radical (ROO\*), superoxide radical, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical(OH\*), and singlet oxygen (O<sub>2</sub>).<sup>24</sup> Blackberry has been shown to have various bioactivities including hepatoprotection against hepatotoxins<sup>25</sup>, protection against endothelial dysfunction and vascular failure in vitro<sup>26</sup>, attenuating the injury by toxins-induced endotoxic shock in rats<sup>27</sup> and exhibiting cytotoxic effects on human oral, prostate<sup>28</sup>, lung cancer cells.<sup>29</sup>

The bioactive phytochemicals in Blackberry falls into several structural and chemical classes including phenolic acids (ellagic acid, hydroxycinnamic and hydroxybenzoic acids), flavonoids (anthocyanins, flavanols, flavonols), condensed tannins (proanthocyanidins), hydrolyzable tannins (ellagitannins and gallotannins), stilbenoids, lignans, triterpenes, sterols, vitamins (vitamin A, B complex, C and E), some minerals (Na, K, Ca, Se, Zn and P).<sup>23,28</sup>

Rosemary (*Rosmarinus Officinalis*), belonging to the botanical family Lamiaceae, is a woody plant and is a common medicinal and aromatic plant grown in many parts of the world. It is known as a common herb and

household plant broadly used around all the world for different medicinal purposes. It is indigenous to Southern Europe, particularly on the dry rocky hills of the Mediterranean region.<sup>30-32</sup> Rosemary is used as a naturally occurring antioxidant, culinary herb, flavoring agent, a beverage drink, as well as in cosmetics; in traditional Chinese medicine it is used as a tonic and stimulant, analgesic, anti-rheumatic, carminative, diuretic, expectorant, anti-epileptic, anti-spasmodic in renal colic, in case of dysmenorrhoea, for relief of respiratory disorders, improve the human fertility, and the stimulation of hair growth.<sup>30,33</sup>

Rosemary extract was found to contain several bioactive antioxidant phytochemicals, including phenolic diterpenes (carnosol, carnosic acid, methyl carnosate, rosmanol and epirosmanol), phenolic acids such as (rosmarinic acid, caffeic acids and ursolic acid), carotenoids, tannins, flavonoids and  $\alpha$ -tocopherol.<sup>33,34</sup> These high active constituents contribute to the biological activity of rosemary and could provide effective synergy. On the other hand results of many experiments showed that rosemary essential oil had antimicrobial, cognition-improving and certain glucose level lowering properties which makes it useful as a natural animal feed additive. The essential oil of rosemary contains several compounds at a rather different concentration. It is characterized by two or three major components at fairly high concentrations (1,8-cineole,  $\alpha$ -pinene, camphor and p-cymene) compared to other components present in trace amounts. They can act in synergic manner or regulate one another.<sup>31</sup>

Therefore, the present work was undertaken to explore the antifibrotic potentials of both blackberry and rosemary water extracts in carbon tetrachloride induced fibrosis in Wistar rats.

## MATERIALS AND METHODS

### Materials

All chemicals used in the study were of high analytical grade, products of Sigma Aldrich Chemical CO., St Louis MO, (USA). Carbon tetrachloride was obtained from EL-Naser Chemical CO. Abu Zaabal, Egypt.

### Plants

The two plants of Rosemary and Blackberry were obtained from Egyptian markets.

### Animals

60 adult male albino rats (120-150 g.) were obtained from the animal house of the National Research Centre, Dokki, Giza, Egypt. Rats were kept for two weeks to acclimatize to the environmental conditions; they were kept under constant environmental and nutritional conditions in individual metabolic cages and allowed free access to standard diet and water all over the period of the experiment. The standard diet was supplied by animal house of the National Research Centre, Dokki, Giza, Egypt. It composed of 72.2% carbohydrates, 3.4% fats, 19.8%



proteins, 3.65 cellulose, 0.5% vitamins, minerals and 0.5% salts.

### Experimental Design

The 60 rats were divided into 6 groups as follows: The first group (Normal control group), comprised of 10 animals that received normal diet and served as untreated normal control healthy group. The second group (carbon tetrachloride group), comprised of 10 animals and continued to receive normal diet and injected with CCL<sub>4</sub> subcutaneously in a dose of (0.15ml /100 g body weight) for 3 days/ week.<sup>35</sup> The third group (curative rosemary group), comprised of 10 animals and continued to receive normal diet and injected with CCL<sub>4</sub> subcutaneously as in the previously mentioned dose, then administered Rosemary in a dose of (1gm/Kg body weight) for 5 weeks.<sup>36</sup> The fourth group (curative blackberry group), comprised of 10 animals and continued to receive normal diet and injected with CCL<sub>4</sub> subcutaneously as in the previously mentioned dose, then administered Blackberry in a dose of (1.6 gm/Kg body weight) for 5 weeks.<sup>25</sup> The fifth group (prophylactic rosemary group), comprised of 10 animals and continued to receive normal diet and administered Rosemary in the previously mentioned dose, then injected subcutaneously with the previously mentioned dose of CCL<sub>4</sub>, the sixth group (prophylactic blackberry group), comprised of 10 animals and continued to receive normal diet and administered Blackberry in the previously mentioned dose, then injected subcutaneously with the previously mentioned dose of CCL<sub>4</sub>.

### Preparation of samples

After 6 weeks the animals were fasted overnight (12-14 hours); the blood samples were collected from each animal in all groups into sterilized tubes for serum separation. Blood samples were centrifugated at 3000 rpm for 10 minutes at 4°C. Serum was separated and stored in epindorff at - 20°C to be used for biochemical analysis. After blood collection, rats of each group were sacrificed under ether anesthesia; the livers were removed rapidly, weighed and small sections were cut with cold knife and put in 10% formalin solution and left for histopathological examinations. The other sections of livers tissue were immediately frozen at - 20 °C for other biochemical analyses.

### Preparation of liver homogenate for enzymatic analyses

0.2 g of tissue was homogenated in 2 ml bidistilled water and centrifuged at 3000 r.p.m for 10 min then the clear supernatant was used in the test.

### Preparation of liver homogenate for metabolite analyses

0.2 g of tissue was homogenated in 2 ml of 10% TCA and centrifuged at 3000 r.p.m for 10 min then the clear supernatant was used in the TBARs test.

### Blood analyses

Matrix metalloproteinase-9, caspase-3 and caspase-7, Interleukin-2 and paraoxonase-1 activities in serum were

measured by a sandwich Enzyme Linked Immune-Sorbent Assay (ELISA kit). Alanine aminotransferase was determined in blood serum using kit purchased from Stanbio (USA) according to Bergmeyer *et al.*,<sup>37</sup> Aspartate transaminase was determined in blood serum using kit purchased from Stanbio (USA) according to.<sup>38</sup> Alkaline phosphatase was determined in serum using the method described by Demetriou *et al.*,<sup>39</sup>

### Tissue analyses

Apoptosis was detected by qualitative analysis of DNA fragmentation by agarose gel electrophoresis according to the method of Bortner *et al.*,<sup>40</sup> The hepatic hydroxyproline content was determined using the method of Jamall *et al.*,<sup>41</sup> Catalase activity was assayed in tissue liver homogenate according to Lubinsky and Bewley.<sup>42</sup> Glutathione reductase was assayed in tissue liver homogenate according to Erden and Bor.<sup>43</sup> Glutathione was estimated according to the method of Moron *et al.*,<sup>44</sup> Superoxide dismutase (EC 1.15.1.1) was assayed in liver homogenate according to.<sup>45</sup>

### Histopathological examination

Immediately after removal from the animals, hepatic tissues were fixed in 10% buffered formaldehyde, processed for histopathological examination according to conventional method and stained with hematoxyline and eosin (H&E) for morphological evaluation<sup>46</sup> and with Masson's trichrome (MT) for assessment of fibrosis.<sup>47</sup> The histopathological slides were observed with a photomicroscope.

### Statistical analysis

Data were analyzed with SPSS (Statistical Package for the Social Sciences) version 9 software package (USA). Results were expressed as mean ± S.D. One-way analysis of variances (ANOVA), followed by a non-parametric post hoc test (LSD) were used to determine the differences among groups. A *p* value of 0.05 or less was considered statistically significant.

## RESULTS

Table 1 demonstrates that serum MMP-9, caspase-3,7, IL-2 levels were highly significantly elevated in CCL<sub>4</sub>- treated group (G2) compared to control group (G1) (P≤0.0001). Therapeutic and prophylactic treatment with either Rosemary or Blackberry were significantly decreased the level of these measured parameters in sera of animals. On the other hand, results in table (1) shows a significant depletion in the activity of PON-1 in serum of CCL<sub>4</sub> intoxicated rats compared to that of normal control one. However, the prophylactic treatment with either of the water extracts of the used plants significantly (P≤ 0.0001) increase the decreased activity of PON-1 in sera of rats. Table (2) demonstrates that both hepatic hydroxyproline (HP) and malonedialdehyde (MDA) (markers of fibrosis) were significantly increased in liver of CCL<sub>4</sub> intoxicated rats compared to that of normal control group (P≤ 0.0001). Prophylactic and curative treatment of animals

with the tow studied plants were significantly decreased the elevated levels of HP and MDA in liver tissues. Also, data listed in table (2) a great depletion in glutathione content and GR, CAT and SD enzymatic activities of CCL<sub>4</sub> treated rats compared to that of normal control group ( $P \leq 0.0001$ ). Prophylactic and therapeutic treatment of animals with the current plants showed a significant protection against CCL<sub>4</sub> treatment.

Data in table (3) illustrates a significant increase in liver function enzymes including ALT, AST and ALP in sera of CCL<sub>4</sub> treated rats compared to normal control healthy rats. Both prophylactic and therapeutic treatment of rosemary and blackberry water extracts significantly reduced the elevated liver enzymes.

**Table 1:** Effect of rosemary and blackberry on some selected serum parameters of different studied groups

Groups	-ve control	CCL <sub>4</sub>	Curative rosemary	Curative blackberry	Prophylactic rosemary	Prophylactic blackberry
MMP-9(ng/ml)	1.03 ± 0.27	16.47 ± 0.82 <sup>a</sup>	12.04 ± 0.85 <sup>a*</sup>	11.55 ± 1.32 <sup>a*</sup>	14.79 ± 0.76 <sup>a**</sup>	14.08 ± 0.31 <sup>a**</sup>
Caspase-3 (ng/ml)	5.18 ± 1.04	15.78 ± 1.32 <sup>a</sup>	7.85 ± 0.32 <sup>b*</sup>	7.83 ± 0.25 <sup>b*</sup>	7.35 ± 0.33 <sup>c*</sup>	7.02 ± 1.09 <sup>c*</sup>
Caspase-7(ng/ml)	8.70 ± 0.81	21.06 ± 2.25 <sup>a</sup>	12.35 ± 0.79 <sup>b*</sup>	11.26 ± 1.79 <sup>c*</sup>	12.52 ± 0.68 <sup>c*</sup>	11.50 ± 1.18 <sup>c*</sup>
IL-2(ng/ml)	8.55 ± 1.67	39.01 ± 1.74 <sup>a</sup>	24.62 ± 2.10 <sup>a*</sup>	19.85 ± 1.11 <sup>a*</sup>	31.43 ± 3.47 <sup>a**</sup>	25.20 ± 2.61 <sup>a*</sup>
PON-1(ng/ml)	126.60 ± 6.02	54.01 ± 4.64 <sup>a</sup>	78.17 ± 5.99 <sup>a*</sup>	87.09 ± 4.94 <sup>a*</sup>	68.01 ± 2.15 <sup>a**</sup>	77.17 ± 1.94 <sup>a*</sup>

Data is presented as mean±SD of 6 rats;  $aP \leq 0.0001$ ,  $bP \leq 0.001$ ,  $cP \leq 0.01$ , n, not significance compared with control group;  $*P \leq 0.0001$ ,  $**P \leq 0.001$ , compared with CCL<sub>4</sub> group.

**Table 2:** Effect of rosemary and blackberry on some selected parameters in liver tissues of different studied groups

Groups	-ve Control	CCL <sub>4</sub>	Curative rosemary	Curative blackberry	Prophylactic rosemary	Prophylactic blackberry
H.p (μ mol / g tissue)	89.52 ± 1.61	184.27 ± 24.7 <sup>a</sup>	120.58 ± 2.90 <sup>c*</sup>	102.58 ± 2.30 <sup>n*</sup>	128.19 ± 8.08 <sup>b*</sup>	114.45 ± 4.44 <sup>c*</sup>
MDA (n mol / g tissue)	19.65 ± 2.58	29.69 ± 1.47 <sup>a</sup>	21.78 ± 2.79 <sup>b**</sup>	20.51 ± 1.69 <sup>b*</sup>	26.49 ± 1.96 <sup>b***</sup>	25.21 ± 3.16 <sup>b***</sup>
GSH (μ mol / g tissue)	1.43 ± 0.43	0.38 ± 0.12 <sup>a</sup>	1.29 ± 0.21 <sup>n**</sup>	1.61 ± 0.20 <sup>n*</sup>	1.09 ± 0.13 <sup>a***</sup>	1.35 ± 0.31 <sup>n***</sup>
G.R (n mol/min /g protein)	14.62 ± 3.89	7.38 ± 0.71 <sup>a</sup>	14.08 ± 1.03 <sup>n*</sup>	15.31 ± 0.85 <sup>n*</sup>	12.16 ± 0.47 <sup>n**</sup>	13.39 ± 0.62 <sup>n*</sup>
CAT (n mol/ min/g protein)	9.43 ± 0.44	5.52 ± 0.29 <sup>a</sup>	8.75 ± 0.53 <sup>n*</sup>	9.77 ± 0.29 <sup>n*</sup>	8.75 ± 1.30 <sup>n*</sup>	9.59 ± 0.58 <sup>n*</sup>
SOD (μ mol / min/g protein)	22.66 ± 3.23	9.86 ± 1.22 <sup>a</sup>	16.53 ± 3.03 <sup>b*</sup>	18.66 ± 3.60 <sup>b*</sup>	10.33 ± 0.64 <sup>a**</sup>	10.40 ± 0.80 <sup>a**</sup>

Data is presented as mean ± SD of 6 rats;  $aP \leq 0.0001$ ,  $bP \leq 0.001$ ,  $cP \leq 0.05$ , n, not significance compared with control group;  $*P \leq 0.0001$ ,  $**P \leq 0.001$ ,  $***P \leq 0.01$  compared with CCL<sub>4</sub> group.

**Table 3:** effect of rosemary and blackberry on liver function enzymes in serum of different studied groups

Groups	-ve Control	CCL <sub>4</sub>	Curative rosemary	Curative blackberry	Prophylactic rosemary	Prophylactic blackberry
ALT (IU/L)	12.64 ± 0.38	20.83 ± 0.22 <sup>a</sup>	16.11 ± 1.38 <sup>b*</sup>	13.55 ± 0.56 <sup>n*</sup>	15.16 ± 1.17 <sup>c*</sup>	14.56 ± 1.53 <sup>c*</sup>
AST (IU/ml)	14.58 ± 1.32	22.56 ± 2.62 <sup>a</sup>	20.77 ± 1.56 <sup>a**</sup>	18.98 ± 1.09 <sup>b*</sup>	22.01 ± 0.47 <sup>a**</sup>	18.84 ± 0.85 <sup>b*</sup>
ALP (IU/ml)	19.99 ± 3.63	34.23 ± 6.18 <sup>a</sup>	26.05 ± 2.77 <sup>b*</sup>	23.02 ± 2.29 <sup>n*</sup>	26.96 ± 1.38 <sup>b**</sup>	25.14 ± 1.89 <sup>n*</sup>

Data is presented as mean±SD of 6 rats;  $aP \leq 0.0001$ ,  $bP \leq 0.001$ ,  $cP \leq 0.01$ , n, not significance compared with control group;  $*P \leq 0.0001$ ,  $**P \leq 0.001$ , compared with CCL<sub>4</sub> group.

Figure (1) illustrates that apoptosis was increased in CCL<sub>4</sub> treated rats compared to normal control healthy rats. This data represented by a marked increase in the apoptotic bands in CCL<sub>4</sub> treated rats compared to normal control healthy rats. This is due to DNA cleavage and multiple fragmentation of DNA strand as a result of hepatotoxic activity of CCL<sub>4</sub>. Both curative and

prophylactic groups of rats treated with blackberry or rosemary show a highly significant reduction in apoptotic bands compared to normal healthy control rats.

### Histopathological results

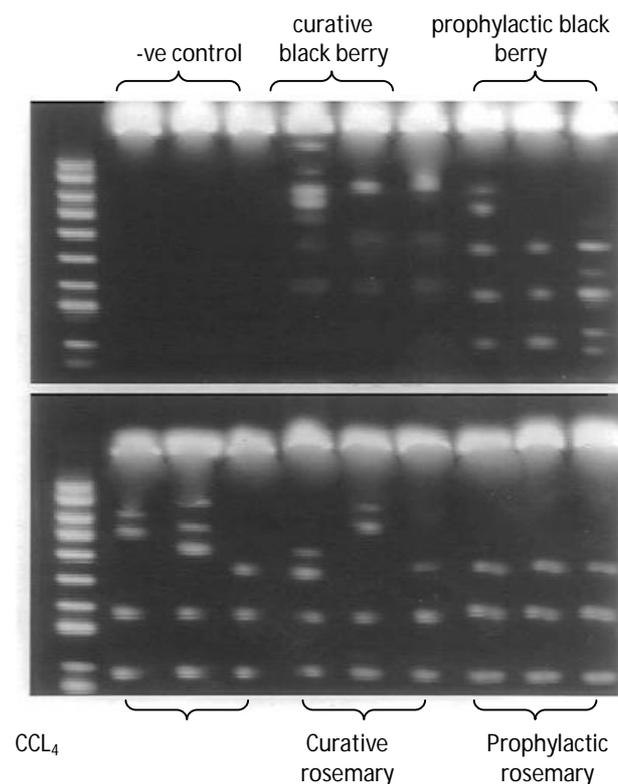
Histopathological examination of liver sections in the present study showed that the control group showed

normal architecture with the central vein (CV) of the hepatic lobule surrounded by the normal hepatocytes characterized by polyhedral shape, arranged in well-organized hepatic cords with acidophilic cytoplasm full of collagen granules and vesicular nuclei, and separated by narrow blood sinusoids (figure 2 and 8). While examined liver sections from group administered CCL<sub>4</sub> showed perivascular fibrosis (figure 3) indicated by the greenish blue color and also an obvious cytoplasmic edema was seen. Moreover, some liver sections showed areas of necrosis with faint cytoplasm, vacuoles of different shapes and sizes, many nuclei lost their vesicular appearance with hyperchromatic stain, and general the liver architecture was lost with obvious cellular damage (figure 9).

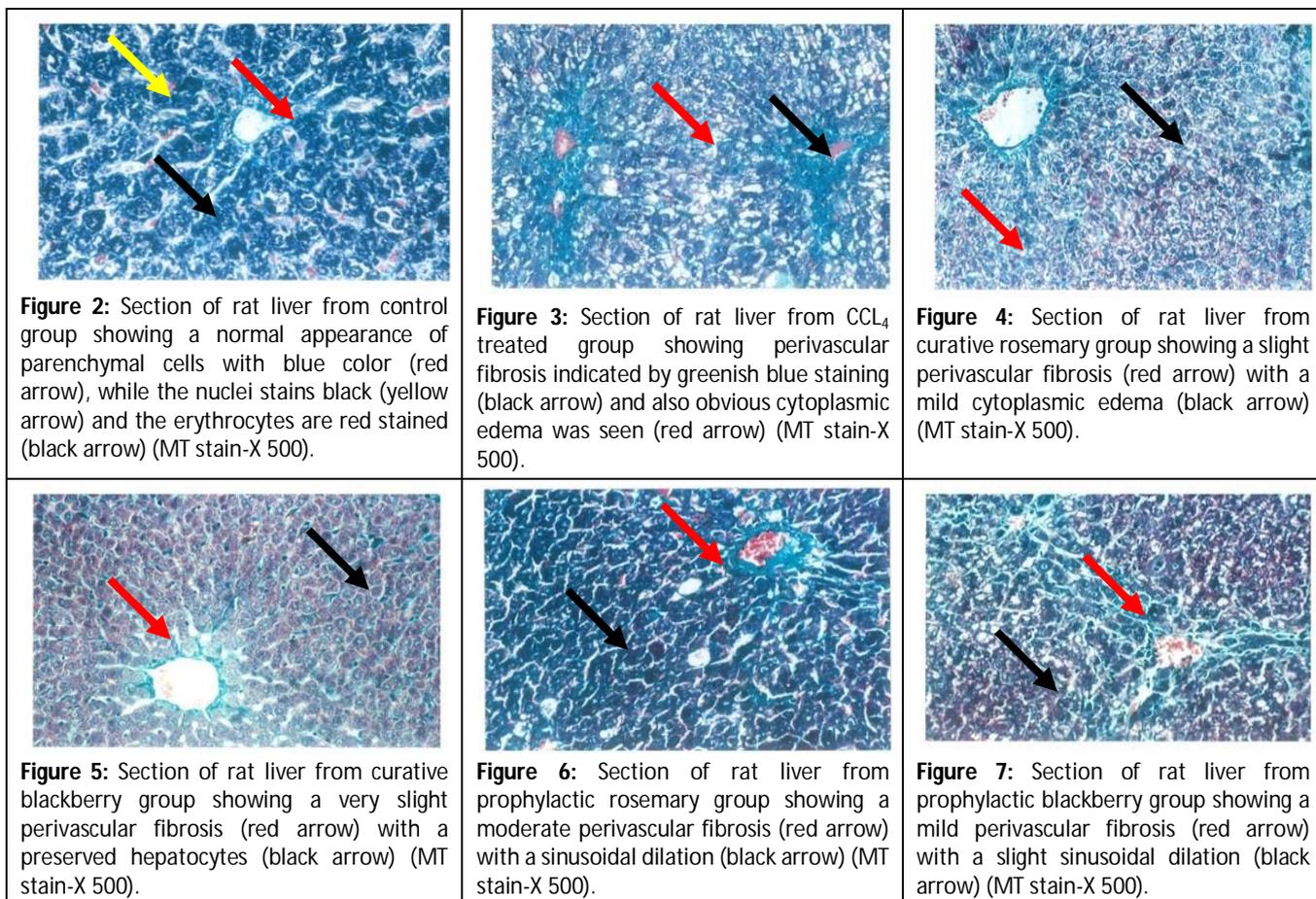
However, examination of liver sections of rats in both curative and prophylactic groups treated with rosemary showed mild appearance of perivascular fibrotic areas and mild cytoplasmic edema (figure 4 and 6). Additionally, some liver sections showed an improvement in the hepatic architecture compared to CCL<sub>4</sub> examined pictures indicated by a mild cytoplasmic swelling, appearance of many tinny spaces around central vein and the boundary of hepatocytes are intact (figure 10 and 12).

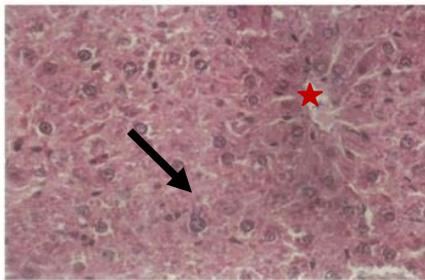
Microscopically, liver sections of rats in both curative and prophylactic groups treated with blackberry showed slight perivascular fibrosis (figure 5 and 7). Meanwhile, some liver sections showed a significant improvement in the hepatic architecture compared to CCL<sub>4</sub> examined liver

sections indicated by preserved hepatocytes with a very slight sinusoidal dilatation between intact cord of hepatocytes (figure 11 and 13)

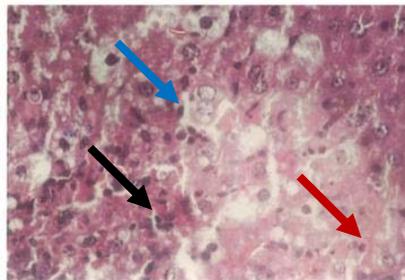


**Figure 1:** Agarose gel electrophoresis of apoptotic bands in different studied groups.

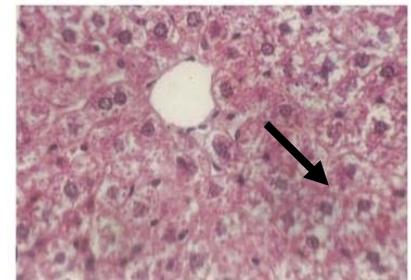




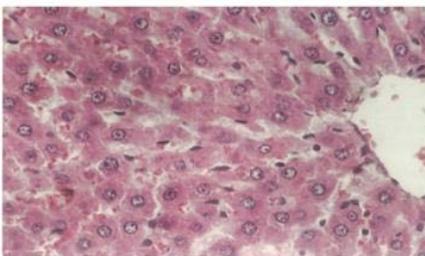
**Figure 8:** Section of rat liver from control group showing normal hepatic architecture with the central vein ★ of the hepatic lobule surrounded by normal hepatocytes, which composed of acidophilic cytoplasm full of collagen granules and vesicular nuclei (black arrow) (H&E stain-X 500).



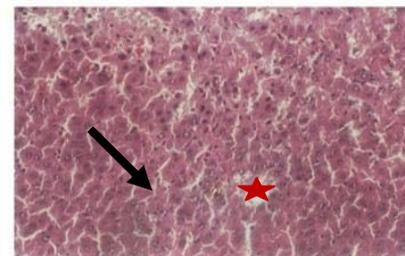
**Figure 9:** Section of rat liver from CCL<sub>4</sub> treated group showing areas of necrosis with faint cytoplasm (red arrow), vacuoles of different shapes and sizes (blue arrow), many nuclei lost their vesicular appearance and become darkly stained (black arrow). Generally the liver architecture is lost with obvious cellular damage (H&E stain-X 500).



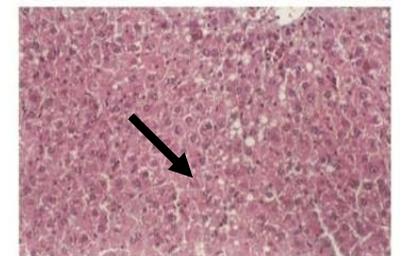
**Figure 10:** Section of rat liver from curative rosemary group showing intracytoplasmic swelling (black arrow) and the boundary of hepatocytes are intact (H&E stain-X 500).



**Figure 11:** Section of rat liver from curative blackberry group showing preserved hepatocytes with a great maintenance of normal hepatocellular architecture (H&E stain-X 500).



**Figure 12:** Section of rat liver from prophylactic rosemary group showing a moderate sinusoidal dilation (black arrow) and many tinny spaces are present around central vein ★ as well as between hepatocytes (H&E stain-X 500).



**Figure 13:** Section of rat liver from prophylactic blackberry group showing a mild sinusoidal dilation between intact cord of hepatocytes (black arrow) (H&E stain-X 500).

## DISCUSSION

Matrix metalloproteinase-9 (MMP-9) is found in liver, but the principal cellular source is the Kupffer cell. MMP-9 expression is significantly increased with Kupffer cell activation and involves degradation and accumulation of basement membrane collagen during the early stage of hepatic fibrosis, so it closely relates to hepatic fibrosis and the serum level of MMP-9 considered as a key fibrogenic factor in early stage of liver disorders.<sup>48,49</sup>

The present study demonstrated that the serum level of MMP-9 in the CCL<sub>4</sub> treated rats was markedly increased compared to the normal healthy control rats. This data are coincide with previous studies that reported a significant increase in MMP-9 serum level after CCL<sub>4</sub> treated rats compared to normal healthy control animals. This increase is because MMP-9 can degrade type IV collagen in the basement membrane, which play an important role in maintaining normal function of hepatic cell. Thus, the action of MMP-9 activates the hepatic stellate cells (HSCs), which promote the development of hepatic fibrosis.<sup>49,50</sup> On the other hand, Yongping et al.,<sup>51</sup> and Zhang et al.,<sup>52</sup> reported that CCL<sub>4</sub> causes either liver fibrosis or hepatic cirrhosis resulting in an imbalance between the process of the extracellular matrix protein production and degradation, which in turn resulting in

accumulation of this matrix protein in the hepatic cells by the help of MMP-9 as one type of matrix degrading enzyme. The authors also stated that the serum level of MMP-9 in CCL<sub>4</sub> treated rats was significantly increased than that of normal healthy control animals, which being in accordance with our data and indicated that MMP-9 is a sensitive measuring marker detecting the liver disorders.

As seen in the present study, the prophylactic and curative treatment with blackberry juice significantly reduced the level of MMP-9 in serum compared to CCL<sub>4</sub> treated animals. The hepatoprotective effect of blackberry might be attributed to its highly content of antioxidant components and their ability to prevent liver damage through their high affinity to scavenge ROS and maintaining integrity of plasma membrane, so counteract the degradation role of MMP-9 on the hepatic basement membrane.<sup>53-55</sup> These findings are in agreement with Bowen-Forbes et al.,<sup>23</sup> who reported that polyphenolic blackberry content have highly antioxidant activity and used as hepatoprotective agent against hepatotoxins.

Supplementation of rosemary extract to CCL<sub>4</sub> treated animals show significant decrease in serum level of MMP-9 in both prophylactic and curative group of animals. The hepatoprotective role of rosemary is due to its ability to

prevent HSCs activation, which in turn prevent extracellular matrix accumulation and alleviate the action of MMP-9 as one of extracellular matrix degrading enzymes, also this effects of rosemary is due to its high content of antioxidant components that maintain cellular structure of hepatic cells.<sup>56</sup> Moreover Žegura et al.,<sup>57</sup> also reported that rosemary has antioxidant and antigenotoxic effect through the modulation of cellular antioxidant responses and the effects on bioactivation and detoxification of xenobiotics such as CCL<sub>4</sub>. Recently Domitrović et al.,<sup>58</sup> reported that Rosemary extract significantly ameliorated oxidative/nitrosative stress and inflammatory response in liver tissue induced by CCL<sub>4</sub>.

The present study demonstrated that apoptosis was increased in CCL<sub>4</sub> treated rats compared to normal control healthy rats. This data was illustrated in figure 1 which show a marked increase in the apoptotic bands in CCL<sub>4</sub> treated rats compared to normal control healthy rats. This is due to DNA cleavage and multiple fragmentation of DNA strand as a result of hepatotoxic activity of CCL<sub>4</sub>. This data was in a harmony with the Meng et al.,<sup>59</sup> who reported that the group of animals treated with CCL<sub>4</sub> show increased mortality and enhanced hepatocyte apoptosis and necrosis compared to normal control healthy animals. Also, Srilaxmi et al.,<sup>17</sup> and Xagorari et al.,<sup>60</sup> reported that CCL<sub>4</sub> induced apoptosis in the liver hepatocytes and consequent oxidative stress and cellular degeneration in rat liver .

Both curative and prophylactic groups of rats treated with blackberry show a highly significant reduction in apoptotic bands compared to normal healthy control rats, which illustrated in figure 1. This data is in accordance with Tavares et al.,<sup>61</sup> who reported that the high antioxidant contents of the wild blackberry were found to reduce levels of reactive oxygen species (ROS) , on the other hand treatment with blackberry juice arrests apoptosis as a result of highly polyphenolic and flavonoid antioxidant contents of blackberry. On the other hand, Yu et al.,<sup>62</sup> also reported that ellagic acid, which is one of the major blackberry constituent has the ability to reduce the oxidative stress and apoptosis in hyperlipidemic rabbits through down regulation of both caspase-8 and caspase-9.

Supplementation of rosemary to both curative and prophylactic groups of animals show a highly significant reduction in apoptotic bands compared to normal healthy control rats, which illustrated in figure 1. This data was in harmony with Domitrović et al.,<sup>58</sup> who reported that rosmarinic acid, which is one of the major polyphenolic component of rosemary extract arrests apoptosis through decrease the activation of caspase-3 in case of liver fibrogenesis induced by carbon tetrachloride in mice. Moreover our results are coincide with Zunino and Storms<sup>63</sup> who reported that carnosol, another constituent of the herb rosemary, has been shown to prevent apoptotic cell death in high-risk acute lymphoblastic leukemia.

In our study we also documented the apoptotic pathway in all studied groups of animals by determination of caspases cascade associated with apoptosis. Caspases are an endogenous family of intracellular aspartate-specific cysteine proteases that participate in critical steps of the apoptotic cascade in numerous pathologic processes. Caspases cascade are responsible for the biochemical and morphological changes associated with apoptosis<sup>64</sup>, and the activation of caspase-3 and caspase-7 is considered to be the final step in many apoptotic pathways and caspase-3/7 activity reflects the net contributions of several activators of apoptosis because of their downstream location in both the intrinsic and extrinsic pathways of apoptosis.<sup>51,64,65</sup>

The present study demonstrated that the serum levels of the apoptotic markers caspase-3 and caspase-7 in the CCL<sub>4</sub> treated rats were markedly increased compared to the normal healthy control rats. This data was in accordance with previous studies of Schrieber et al.,<sup>65</sup> Yongping et al.,<sup>51</sup> and Sun et al.,<sup>64</sup> who reported a significant increase in caspase-3 and caspase-7 serum level after CCL<sub>4</sub> treated rats compared to normal healthy control animals, the elevated serum levels of both caspases indicated that apoptosis will enhanced as a result of hepatotoxic activity of CCL<sub>4</sub>. Also, Schrieber et al.,<sup>65</sup> and Kukner et al.,<sup>7</sup> reported that CCL<sub>4</sub> induced massive necrosis, apoptosis in the liver tissues and consequent oxidative stress. On the other hand, Srilaxmi et al.,<sup>17</sup> reported that CCL<sub>4</sub> induced oxidative stress, cellular degeneration apoptotic cell death enhanced by caspase-3 activation .

In our study, we found that both curative and prophylactic treated rats with blackberry juice significantly decreased serum level of both caspases-3 and caspase-7 compared to the CCL<sub>4</sub> treated animals. This results indicated that blackberry juice can counteract the effect of CCL<sub>4</sub> as a powerful hepatotoxin and also can scavenge the ROS through its high antioxidant content. This was in agreement with Tavares et al.<sup>61</sup> who reported that the high antioxidant contents of the wild blackberry were found to reduce levels of reactive oxygen species (ROS), on the other hand they reported that the increased serum level and activity of caspase-3 and caspase-7 decreased after treatment with blackberry juice, so arrest apoptosis as a result of highly polyphenolic and flavonoid antioxidant contents of blackberry.

In addition, Hseu et al.,<sup>66</sup> revealed that the ellagic acid, which is one of the major blackberry constituent protects human keratinocyte cells against oxidative stress and also prevent apoptosis and decrease cell death execution through the inhibition of Caspase-3 activity. This data was in harmony with our results and stated that blackberry constituent have the ability to prevent the liver fibrosis through inhibition of apoptotic markers, which in turn arrest apoptosis.

Supplementation of rosemary extract show significant decrease in serum level of the two apoptotic markers,

caspase-3 and caspase-7 in both curative and prophylactic treated rats compared to CCL<sub>4</sub> treated animals. This evidence indicates the hepatoprotective effect of rosemary against CCL<sub>4</sub> induced hepatic damage. This is coincide with a previous study of Kontogianni et al.,<sup>67</sup> who reported that the rosemary extract developed more pronounced antioxidant, cytotoxic and immunomodifying activities, probably due to the presence of betulinic acid and a higher concentration of carnosic acid in its phytochemical profile. On the other hand, our present results are in agreement with Wang et al.,<sup>68</sup> and Kar et al.,<sup>69</sup> who reported that carnosic acid, which is the major polyphenolic component of rosemary extract arrests apoptosis through decrease the activation of both caspase-3 and caspase-7 in case of prostate cancer. Moreover our results are coincide with Zunino and Storms,<sup>63</sup> who reported that carnosol, another constituent of the herb rosemary, has been shown to prevent apoptotic cell death in high-risk acute lymphoblastic leukemia and also decrease the activation of caspase-3.

Cytokines are small secreted proteins which regulate immunity, inflammation and haematopoiesis in connection with liver disease progression, which is associated with an imbalance between pro- and anti-inflammatory cytokines. Therefore, elevated serum cytokines could be a risk factor for the occurrence of liver disorders and used as biomarkers for early detection of liver diseases.<sup>70</sup> IL-2 is a cytokine produced by T cells in response to inflammatory stimuli and could be possibly a useful marker in chronic liver diseases (CLD) due to its close association with inflammatory processes, given the fact that chronic inflammation is believed to be the key driver for disease progression.<sup>71</sup>

The present study demonstrated that the serum level of IL-2 in the CCL<sub>4</sub> treated rats was markedly increased compared to the normal healthy control rats. The elevated serum level of IL-2 indicated that there is an inflammatory process occurred. This is in line with Seidler et al.,<sup>71</sup> who reported that IL-2 could be possibly a useful marker in chronic liver diseases (CLD) due to its close association with inflammatory processes.

Data of the present work revealed that administration of blackberry and rosemary water extracts before or after CCL<sub>4</sub> treatment markedly decreased the highly elevated level of IL-2 in serum of rats. The literature survey revealed that there is no experimental evidence of the current plants on IL-2, therefore, the present work was the first one in studying the effect of these plants on this cytokine.

In the normal liver, the rate of extracellular matrix (ECM) production equals that of its degradation, resulting in no net accumulation of matrix. Hepatic fibrosis is a process where production of ECM protein surpasses its degradation. Collagen is the main component of extracellular matrix. Abnormal accumulation of collagen in chronic liver injury is a direct index that indicates the

hepatic fibrosis.<sup>72</sup> Hydroxyproline, which is a major component of the protein collagen, is produced by hydroxylation of the amino acid proline, and helps provide stability to the triple-helical structure of collagen by forming hydrogen bonds. Changes in hydroxyproline content in the liver are considered as an index for collagen metabolism and provide valuable information on the biochemical and pathological states of liver fibrosis.<sup>73,74</sup> Therefore the hydroxyproline assay was used as a method for collagen measure.

The present study showed that the tissue level of hydroxyproline content in the CCL<sub>4</sub> treated animals was significantly elevated than that of normal control rats, which is in agreement with the previous study of Zhao et al.,<sup>75</sup> who stated that CCL<sub>4</sub> causes a significant increase in hydroxyproline content in CCL<sub>4</sub> treated rats compared to normal control animals as a result of hepatic damage caused by the hepatotoxic activity of CCL<sub>4</sub>. On the other hand these results are in accordance with Li et al.,<sup>56</sup> and Tao et al.,<sup>76</sup>

The curative and prophylactic treatment of the experimental rats with blackberry juice show significant decrease in the tissue hydroxyproline content compared to the tissue content of hydroxyproline in CCL<sub>4</sub> treated animals. This data was in accordance with Devipriya et al.,<sup>77</sup> who reported that the ellagic acid, which is one of the phenolic component of the blackberry extract can reduce the tissue hydroxyproline content in the group of rats with alcohol induced liver injury compared to the normal healthy control ones.

Supplementation of rosemary extract to both curative and prophylactic groups of animals show a marked decrease in hydroxyproline content in the liver tissue compared to that of CCL<sub>4</sub> treated rats. This is in agreement with Li et al.,<sup>56</sup> who reported that the rosmarinic acid, which is the major component of rosemary content, has powerful anti-fibrotic effect against CCL<sub>4</sub> through its high antioxidant activity and can ameliorate the toxic effect on the liver tissue by decreasing the elevated hydroxyproline content in CCL<sub>4</sub> treated rats compared to the normal healthy control animals.

Oxidative stress is a redox imbalance between pro-oxidants and antioxidants leading to potential cellular damage. Most cells can tolerate a mild degree of oxidative stress, because they have sufficient antioxidant defense capacity and repair systems, which recognize and remove molecules damaged by oxidation. The imbalance can result from a lack of antioxidant capacity caused by disturbances in production and distribution, or by an overabundance of reactive oxygen species (ROS) from other factors.<sup>78</sup> The excessive ROS accumulation will lead to cellular injury, such as damage to DNA, protein and lipid membrane. Because of their potential harmful effects, excessive ROS must be promptly eliminated from the cells by a variety of enzymatic antioxidant and the non-enzymatic antioxidant such as Glutathione (GSH).<sup>79</sup>



Paraoxonase (PON) arylalkyl phosphatase is an ester hydrolase that catalyzes the hydrolysis of some xenobiotics, such as organophosphates, unsaturated aliphatic esters, aromatic carboxylic esters and, possibly, carbamates. The paraoxonase gene family contains at least three members, PON-1, PON-2, and PON-3. PON-1 and PON-3 mRNA are predominantly expressed in liver, whereas PON2 mRNA is found in different tissues including human endothelial and aortic smooth muscle cells. The enzyme PON-1 is circulating in serum and tightly bound with serum HDL, and several studies suggested that this association contributes to the protection conferred by HDL against LDL oxidation.<sup>80</sup> PON-1 protects against liver impairment and attenuates the production of the pro-inflammatory monocyte chemoattractant protein-1 (MCP-1).<sup>81</sup> Also, it was suggested that there is an active role of PON-1 in the regulation of oxidative stress, fibrosis and hepatic cell apoptosis in chronic liver diseases.<sup>82</sup> The liver plays a key role in the synthesis of PON1 and the gene expression has been confined only to the liver. Several previous studies indicated that PON1 protects hepatocytes against inflammation, fibrosis and liver diseases, so serum PON-1 activity would be useful as an index of liver function status.<sup>81, 83</sup>

The present study demonstrated that paraoxonase activity in CCL<sub>4</sub> treated group is significantly decreased than that of normal healthy control rats. The decreased serum level of the paraoxonase is due to the oxidative stress induced by CCL<sub>4</sub>, which results in production of reactive oxygen species ROS that cause cellular damage and imbalance between the pro-oxidants and antioxidants in the liver tissue. This data was coincide with the previous study of Hussein et al.,<sup>80</sup> who stated that CCL<sub>4</sub> has a powerful hepatotoxic activity and has highly oxidative stress power, that is resulting in lipid peroxidation and liver injury with decrease in liver microsomal PON-1 activity. On the other hand, Bindu et al.,<sup>84</sup> also stated that serum PON-1 levels decline in various types of liver diseases as a result of peroxidative changes that occur in the hepatocytes due to the oxidative stress induced by certain hepatotoxins such as CCL<sub>4</sub>.

In the current study, we found that administration of blackberry and rosemary water extracts before or after CCL<sub>4</sub> treatment significantly increased the observed decreased activity of PON-1 in serum of rats. The literature survey revealed that there is no experimental evidence of the current plants on PON-1, therefore, the present work was the first one in studying the effect of these plants on this antioxidant enzyme.

The MDA is a major reactive aldehydic end product produced from the peroxidation of polyunsaturated fatty acids present in the biological membranes and has been recognized to cause some cell alterations by modifying protein structure and serves as an oxidative damage index. The reaction between MDA and the primary amino groups of proteins forms Schiff base compounds, which give rise to intra and intermolecular linkages which can

lead to polymerization and inactivation of enzymes.<sup>82</sup> Now MDA is widely used as one of the most reliable indices of oxidative stress to evaluate the oxidative damage in diseased status including liver injury, hepatic fibrosis and hepatocellular carcinoma.<sup>75</sup>

Data presented in this study indicated that CCL<sub>4</sub> administration resulting in augmentation of oxidative stress in livers of treated rats accompanied by impaired anti oxidative defense, as indicated by a significant elevation in the level of oxidative stress marker MDA. This data are in accordance with<sup>17</sup> who reported that, the oxidative stress in livers of CCL<sub>4</sub> treated rats may be attributed to the metabolic biotransformation of CCL<sub>4</sub> by the cytochrome P-450 system to two free radicals. The first metabolite, a trichloromethyl free radical (CCl<sub>3</sub>•) has been formed from the metabolic conversion of CCL<sub>4</sub> and reacts very rapidly with O<sub>2</sub> and forms a second metabolite, a trichloromethyl peroxy free radical (CCl<sub>3</sub>OO•) or abstract hydrogen atoms to form chloroform. These free radicals initiate the peroxidation of membrane poly-unsaturated fatty acids and covalently bind to microsomal lipids and proteins.

Quenching lipid peroxidation and enhancing endogenous enzymatic and non-enzymatic antioxidant status by the hepatoprotective compounds represent an effective strategy in the prevention of hepatic disorders.<sup>85</sup> Supplementation of the extracts under investigation (Blackberry and Rosemary) to CCL<sub>4</sub> treated animals effectively modulate the deterioration in MDA.

The current investigation revealed that the treatment of both curative and prophylactic groups of rats with blackberry was significantly reduced the level of MDA in the treated animals compared to that of CCL<sub>4</sub> administered group. This hepatoprotective effect of blackberry could be attributed to its ability to scavenge the free radicals that induce lipid peroxidation.<sup>23,55</sup> The current results are in line with previous studies that reported depletion in lipid peroxides level after administration of blackberry to CCL<sub>4</sub> treated rats.<sup>25,86,87</sup>

Another explanation of the anti-lipid peroxidative role of blackberry is that, the ellagic acid and anthocyanine phenolic contents of blackberry have the ability to decrease activities of cytochrome P450 enzymes and hence, decrease the production of reactive metabolites that cause lipid peroxidation.<sup>77</sup>

The current investigation revealed a significant decrease in MDA level in both curative and prophylactic treated groups of animals with rosemary compared to the CCL<sub>4</sub> treated ones. This might be attributed to the protective effect of rosemary extract on the liver against chemically induced oxidative injury.<sup>32,57</sup> These effects are closely related to the rosemary active constituents such as rosmarinic acid, carnosic acid, carnosol, rosmanol, carotenoids and α-tocopherol, which act as a powerful antioxidants with an excellent ability to scavenger the peroxy radicals and are able to block the formation of hydroxyl radical generated in non-lipid system. In addition



these compounds have been shown to inhibit the lipid peroxidation in liver microsomes.<sup>67</sup> The current results are also in accordance with Domitrović et al.,<sup>58</sup> who reported that rosmarinic acid, a major component of rosemary was significantly ameliorated oxidative/nitrosative stress and inflammatory response in liver tissue in animals treated with CCL<sub>4</sub> to induce liver damage and fibrogenesis. In addition, Gutiérrez et al.,<sup>16</sup> also reported that the administration of rosemary was significantly decrease the elevation of MDA level caused by CCL<sub>4</sub> in the experimental animals; this enlightened the possible way of anti-lipid peroxidative activity of rosemary. These beneficial effects of rosemary could be explained by its ability to scavenge (Cl<sub>3</sub>COO\*) and (OH\*) radicals and converting them into a less toxic substances or acting as antioxidants, in addition to inhibition of CYP2E1 that metabolizes CCL<sub>4</sub> inducing ROS generation and lipid peroxidation.

The body has an effective defense mechanism to prevent and neutralize the free radical induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as GR, SOD and CAT and non-enzymatic antioxidants such as (GSH, vitamin C, and vitamin E). All of them constituting a mutually supportive team of defense against reactive oxygen species (ROS) and considered as index of cellular damage.<sup>18</sup>

Data presented in this study indicated that CCL<sub>4</sub> administration resulting in augmentation of oxidative stress in livers of treated rats accompanied by impaired antioxidative defense, as indicated by a significant depletion of free radical scavenging non-enzymatic antioxidant; GSH and enzymatic antioxidants including CAT, SOD, GR, compared to normal healthy control group.

The observations of current study are in accordance with previous studies that revealed a significant decrease in the level of GSH and the activities of GR in CCL<sub>4</sub> treated rats compared to the healthy control animals. This might be attributed to the reduction in their biosynthesis during hepatocellular damage or their excessive utilization in scavenging the free radicals formed during the metabolism of CCL<sub>4</sub>. It has been also reported that the detoxification pathway of CCL<sub>4</sub> reactive metabolites involves GSH conjugation of the trichloromethyl radical, a cytochrome P450 mediated CCl<sub>4</sub> metabolite and forms adducts with these toxic metabolites, which led to depletion of GSH.<sup>82</sup> It has been also reported that the decreased concentration of GSH in liver may be due to NADPH reduction or GSH utilization in the exclusion of peroxides. The decreased level of cellular GSH caused a reduction in the activities of GSH dependant enzymes; GR and GPx; as GSH act as a vital co-factor for these enzymes.<sup>88</sup> Similarly, Acharya et al.,<sup>89</sup> demonstrated that the decreased activity of SOD and CAT in CCL<sub>4</sub> treated rats could be due to the over utilization of these antioxidant enzymes to scavenge the products of lipid peroxidation.

Supplementation of the extracts under investigation (Blackberry and Rosemary) to CCL<sub>4</sub> treated animals

effectively modulate the deterioration in the antioxidant indices; GSH, GR, SOD, and CAT implying the beneficial antioxidant abilities of these extracts.

The current investigation revealed that the treatment of both curative and prophylactic groups of rats with blackberry was significantly increased the level of GSH and the activities of antioxidant enzymes; GR, SOD, and CAT in the treated animals compared to that of CCL<sub>4</sub> administered group. The current results are in line with previous studies that reported elevation of GSH level and the activities of GR, SOD, and CAT after administration of blackberry to CCL<sub>4</sub> treated rats.<sup>86,87</sup> This might be attributed to the ability of blackberry content to affect CAT, SOD, and enzyme systems associated with GSH enhancing their activities. Blackberry has been reported to maintain GSH homeostasis in CCL<sub>4</sub> treated animals and this might be the reason for elevated GSH level observed during blackberry treatment and this will lead to similarly to maintenance of the GR activity.<sup>90</sup>

Another explanation of the role of blackberry and its ability to improve the activities of the liver antioxidant enzymes is that, the ellagic acid and anthocyanine phenolic contents of blackberry have the ability to scavenge the free radicals such as; (O<sub>2</sub>\*), (OH\*), peroxy radical and peroxy nitrite by donation of their phenolic hydrogen atoms and therefore, prevent depletion of the antioxidant enzymes in scavenging the ROS.<sup>25</sup>

The current investigation revealed a significant increase in GSH level and GR, SOD, and CAT activities in both curative and prophylactic treated groups of animals with rosemary compared to the CCL<sub>4</sub> treated ones. This might be attributed to the protective effect of rosemary extract on the liver against chemically induced oxidative injury.<sup>32, 57</sup> The rosemary is well known for its antioxidant properties through its ability to scavenge the free radicals and modulation of cellular antioxidant responses by enhancing the activities of antioxidant enzymes.<sup>91,92</sup> These effects are closely related to the rosemary active constituents such as rosmarinic acid, carnosic acid, carnosol, rosmanol, carotenoids and  $\alpha$ -tocopherol, which act as a powerful antioxidants with an excellent ability to scavenge the peroxy radicals and are able to block the formation of hydroxyl radical generated in non-lipid system.<sup>67</sup>

The activities of serum AST, ALT and ALP are considered as hepatic marker enzymes and used as diagnostic indicators of hepatic injury since they are related to the function of the hepatic cells. Alterations in the activities of these enzymes may be due to cellular impairment and dysfunction<sup>93</sup>, thus analysis of these marker enzymes reflects mechanisms of cellular damage and subsequent release of protein, their extracellular turnover and also reflect mechanisms of neoplastic process.<sup>94</sup>

The present study demonstrated that the activities of ALT, AST, and ALP in the sera of CCL<sub>4</sub> treated rats were markedly increased compared to the normal healthy control rats. This data was in harmony with previous

studies that reported a significant increase in ALT, AST, and ALP activities after CCL<sub>4</sub> injection compared to control animals. This increase is due to the production of free radicals during the course of CCL<sub>4</sub> metabolism, which led to lipid peroxidative degradation of the hepatocellular membrane and as a result, these cytoplasmic enzymes are released into the systemic circulation.<sup>95,96</sup>

As seen in the present study, treatment with blackberry significantly reduced serum AST, ALT, and ALP activities in both curative and prophylactic groups of animals, compared to CCL<sub>4</sub> treated rats. In agreement with these results, previous studies showed that blackberry treated animals showed a significant decrease in the activities of AST, ALT, and ALP in the serum compared to CCL<sub>4</sub> treated rats. The hepatoprotective effect of blackberry as well as its active constituents might be attributed to its antioxidant properties and its ability to prevent the liver damage by maintaining the integrity of plasma membranes, leading to the restoration in the activities of these marker enzymes.<sup>87</sup>

Rosemary supplementation of both curative and prophylactic groups of rats cause a highly significant decrease in serum activity of ALT, AST, and ALP, which elevated as a result of hepatotoxic activity of CCL<sub>4</sub>. This data are met with the published results of Xiang et al.,<sup>32</sup> who reported that Carnosic acid, one of the major active constituents of rosemary, attenuates lipopolysaccharide-induced liver injury in rats and decrease the elevated serum activities of ALT, AST, and ALP as a result of liver damage, via fortifying cellular antioxidant defense system, counteract lipid peroxidation and enhance the free radical scavenging activity, thus preventing the formation of MDA, which causes peroxidation and denaturation of cell membrane leading to leakage of cytoplasmic enzymes into the blood circulation. Moreover, Domitrović et al.,<sup>58</sup> reported that rosmarinic acid, another major active component of rosemary, can decrease the high serum activities of ALT, AST, and ALP induced by CCL<sub>4</sub> due to its protective effect against necrosis of hepatocyte induced by CCL<sub>4</sub>.

All of the drastic changes occurring in the biochemical parameters after intoxication with CCL<sub>4</sub> were confirmed by the histopathological observations of livers of CCL<sub>4</sub> treated rats which showed a perivascular fibrosis with cytoplasmic edema, obvious cellular damage and death. The hepatocytes loss their normal shape and arrangement and showed a necrotic areas with faint cytoplasm, vacuoles with different shapes and sizes, and the nuclei became hyperchromatic and lost their vesicular appearance. These changes might be attributed to that CCL<sub>4</sub> is primarily metabolized in the liver generating excessive free radicals that cause damage to the hepatocytes.<sup>17</sup>

The current results are in agreement with previous studies which reported that the histopathological pictures of liver tissue of CCL<sub>4</sub> treated rats showed loss of normal hepatocellular architecture indicated by centilobular

hepatic necrosis, apoptosis of hepatocytes, edema, acute inflammatory infiltration, and hepatocyte degeneration characterized by cytoplasmic swelling and nuclear pyknosis.<sup>7,97</sup> Additionally, Li et al.,<sup>56</sup> also reported that histopathological observations of livers of CCL<sub>4</sub> treated rats showed appearance of fibrous tissues and extended into the hepatic lobules to separate them completely, a large number of inflammatory cells infiltrated in the intralobular and interlobular regions, the liver structure was disordered and become necrotic, and fatty degenerated liver cells with more collagen deposition were also seen.

Histopathological examination of the liver tissue of both curative and prophylactic groups of rats treated with rosemary showed an improvement in the hepatocytes exhibiting less disarrangement and degeneration of hepatocytes compared to CCL<sub>4</sub> treated rats. The liver sections of rats treated with rosemary in both curative and prophylactic groups showed a significant improvement in the liver architecture indicated by lesser degree of vacuolated hepatocyte with minimal nuclear vesiculation compared to CCL<sub>4</sub> treated rats. In agreement with these results, Li et al.,<sup>56</sup> and Gutierrez et al.<sup>16</sup> reported that animals treated with CCL<sub>4</sub> and rosemary showed improved hepatocellular architecture with decrease in hepatocyte degeneration, necrosis, and infiltration of inflammatory cells were all apparently ameliorated and collagen deposition was also markedly reduced with signs of recovery compared to animals treated with CCL<sub>4</sub> alone. This improvement may be attributed to that rosemary is well known for its antioxidant properties through its ability to scavenge the free radicals and modulation of cellular antioxidant responses by enhancing the activities of antioxidant enzymes, thus preventing oxidative stress-induced cellular damage.<sup>91,92</sup>

Supplementation of blackberry to both curative and prophylactic groups of rats was beneficially ameliorated the histopathological deteriorations induced by CCL<sub>4</sub>. Liver sections of rats treated with blackberry showed a highly significant improvement in the hepatic cells architecture with slight deviation from normal structure of the liver. The improved liver architecture was indicated by lesser degree of vacuolated hepatocytes and less disarrangement and degeneration of hepatocytes compared to CCL<sub>4</sub> treated group. In accordance with these results, Srigopalrama et al.,<sup>87</sup> also reported that ellagic acid, which is one of the major active constituent of blackberry can ameliorate the degenerative effect of diethylnitrosamine (DEN) induced hepatocellular carcinoma through maintenance of the liver architecture with preserved cytoplasm and decrease the vacuolated hepatocytes with slight appearance of neoplastically-transformed cells compared to DEN treated rats. The hepatoprotective effect of blackberry might be attributed to its highly content of antioxidant components and their ability to prevent liver damage through their high affinity



to scavenge ROS and maintained integrity of plasma membrane.

## CONCLUSION

This study demonstrated that matrix metalloproteinase-9 (MMP-9) found to be an excellent diagnostic marker for early detection of liver disorders induced by CCL<sub>4</sub>. Additionally, supplementation of either rosemary or blackberry extracts to CCL<sub>4</sub> induced liver fibrosis in rats, has been found to possess a beneficial protective effect against liver fibrosis through their antioxidant, anti-inflammatory, anti-apoptotic, and anti-fibrotic activities, but the best results were obtained from the curative groups rather than the prophylactic groups of both rosemary and blackberry, and data obtained from blackberry was better than that obtained from rosemary.

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