ASSOCIATION BETWEEN EFFICIENCY OF CERTAIN MEDICINAL PLANTS AND SEVERITY OF RENAL DISORDERS IN RATS

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ABSTRACT
The objective of this study was to evaluate the protective and curative actions of Rosemary and Black mulberry against renal injury induced by CCl4 in rats. The evaluation was done through measuring oxidative stress markers; malondialdehyde (MDA) and nitric oxide (NO) levels. Kidney function indices; creatinine, urea and serum protein were also estimated. Inflammatory mediators; heat shock protein-70 (HSP-70) and transforming growth factor- β1 (TGF-β1) as well as cytotoxicity biomarker; cytochrome p450-2E1 (CYP2E1) were also evaluated. The histopathological analysis of kidney was done for results confirmation. The prophylactic and therapeutic effects of Rosemary and Black mulberry were achieved by the observed decrease in MDA, NO, creatinine, urea, HSP-70, TGF-β1, and significant increase in CYP2E1. In conclusion, both Rosemary and Black mulberry recorded variable degrees of protection and treatment against renal injury induced by CCl4 in rats. Further studies are needed to identify the molecules responsible for their pharmacological effects.

Keywords: Renal injury, Carbon tetrachloride, Rosemary, Black mulberry.

INTRODUCTION
Carbon tetrachloride (CCl4), a clear, heavy, and nonflammable liquid is most widely used for experimental induction of hepatic cirrhosis3. It is known to be nephrotoxic as well as hepatotoxic to humans4. In addition, it has been identified as a probable human carcinogen based on evidence of tumors in animals4. Administration of CCl4 causes an increase in lipid peroxidation products and a decrease in the activity of enzymes protecting lipid peroxidation in the kidney5. These detrimental effects of CCl4 have been attributed to conversion of CCl4 to highly toxic trichloromethyl and trichloromethyl peroxyl free radicals by cytochrome P450 enzyme, resulting in cell injury1.

The general strategy for prevention and treatment of organ damage includes reducing the production of reactive metabolites by using antioxidants5. Antioxidants appear to act against diseases by raising the levels of endogenous defense [e.g., by up-regulating gene expressions of the antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and lipid peroxidase6].

People all over the world are becoming more conscious of the nutrition value, health benefits and safety of their food and its ingredients6. In addition, there is a preference for natural functional food ingredients that are believed to be safer, healthier and less subject to hazards than their artificial counterparts. Evaluation of the functional properties of naturally occurring substances, especially those that are present naturally in human diets, has been of interest in recent years6.

Black mulberry (Morus nigra L., Family Moraceae-often called the mulberry family or fig family) contain large amounts of flavonoid pigments (anthocyanins) that give black mulberries their characteristic red to blue color5. The total pigment extract which is deposited at Therapeutic Chemistry Department, National Research Center, Egypt. Voucher specimen (RM-2011) was used in the present work to evaluate the protective and therapeutic effects of the aqueous extract of Rosemary leaves and juice of Black mulberry fruits on renal injury induced by CCl4 in rats.

The aim of the present work is to evaluate the protective and therapeutic effects of the aqueous extract of Rosemary leaves and juice of Black mulberry fruits on renal injury induced by CCl4 in rats. The evaluation was done through measuring oxidative stress markers, kidney function indices, inflammatory mediators and the cytotoxicity biomarker. Kidney histopathological analysis was also taken into consideration.

MATERIALS AND METHODS

Chemicals
All chemicals used in the present study were of analytical grade, product of Sigma (US), Merck (Germany) and BDH (England).

Plants collection
Rosemary was purchased from a local market; Harraz Market for Medicinal Herbs, Cairo, Egypt. Voucher specimen (RM-2011) was deposited at Therapeutic Chemistry Department, National Research Center, Egypt, as a reference. Dried leaves were ground in a grinder with 2 mm diameter mesh. The dry powder was kept in tightly closed container until needed.

Black mulberry fruits purchased from local district market (Hyper One Market, 6th October City, Cairo). Fresh fruits were mixed with bidistilled water in a blender, and the resulting juice is filtered through natural muslin cloth in a screw press to separate any impurities. The juice was kept in a dark bottle at -20°C until used.

Plants Extraction
The dried leaves of Rosemary were extracted in a Soxhlet apparatus using hot water (40-60°C) for 72 hours. Excess water of Rosemary extract and Black mulberry juice were dried under vacuum at 40°C, producing semisolid residues.

Animals
Male Wistar albino rats (100 to 120 g) were selected for this study. They were obtained from the Animal House, National Research Center, Egypt. All animals were kept in controlled environment of air and temperature with access of water and diet ad libitum.

Ethics
Anesthetic procedures and handling with animals complied with the ethical guidelines of Medical Ethical Committee of National Research Centre, Egypt.

Doses of administration
Rats were subcutaneously injected with a dose of 0.15 ml CCl4/100g body weight three times for one week.
Administration regimes for prophylactic and therapeutic effects were trice a week for five consecutive weeks. Black mulberry was orally administered at a dose of 1.5 g/kg body weight\(^{24}\). Rosemary was orally administered at a dose of 500mg/kg body weight\(^{25}\).

Experimental design

60 male rats were used in this study. Animals were divided into 6 groups (10 rats each). Group 1 served as normal healthy control rats. Group 2 was subcutaneously injected with CCl\(_4\). Groups 3 and 4 were received each of plant extract three times/week for five consecutive weeks before injection with CCl\(_4\) (three times/one week) (prophylactic groups). Groups 5 and 6 were forced with CCl\(_4\), and then treated with each of plant extract as the same administration regimens described above. These groups served as the therapeutic groups.

Sample preparations

Serum sample: Blood was collected from each animal by puncture of sublingual vein in clean and dry test tubes, left 10 minutes at room temperature to clot and centrifuged at 3000 rpm for serum separation. The separated serum was stored at -80°C for further determinations of kidney function tests, total protein, inflammatory mediators and anti cytotoxicity biomarker.

Tissue sample: kidney tissue was homogenized in cold 0.9% NaCl (1:10 v/v) solution, centrifuged at 3000 rpm for 10 minutes, separated the supernatant and stored at -80°C for further determination of oxidative stress markers.

Biochemical assays

Malondialdehyde (MDA) was estimated as the product of lipid peroxidation process. Its concentration was calculated using the extinction coefficient value 1.56 x 10\(^{5}\) M\(^{-1}\) cm\(^{-1}\) and read at 535 nm by the method of Buege and Aust\(^{25}\).

Nitric oxide (NO): as vasodilatory chemokine was assayed by the method of Mosshage et al.\(^{26}\), where Promega's Griss Reagent System is based on the chemical reaction between sulfanilamide and N-1-naphthylurea (NED) under acidic phosphoric acid condition to give colored azo-compound which can be measured colorimetrically at 520 nm.

Creatinine was measured by the method of Bartels and Bohmer\(^{27}\). Creatinine in the sample reacts with picrates in alkaline medium forming a colored complex at 500 nm. Urea was determined by the method of Tabacco et al.\(^{28}\), where the conversion of urea in the sample by urease enzyme provide a colored complex that can be measured by spectrophotometry at 600 nm.

Serum total protein was assayed according to Bradford\(^{29}\). Coomassie Brilliant Blue dye reacts with Bradford reagent to give a blue complex which is measured colorimetrically at 595 nm.

Heat shock protein-70 (HSP-70) measurement was carried out using enzyme-linked immunoassay (ELISA) kit (Kamiya Biomedical Company, USA). The microtiter plate provided in this kit has been pre-coated with an antibody specific to HSP-70. Test procedures were the same as heat shock protein-70. The developed color is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of HSP-70 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Histopathological analysis

Kidney tissues were excised from sacrificed animals, individually weighed, and from them, 5 μm thickness slices were cut, fixed in 10% paraformaldehyde and embedded in paraffin wax blocks. Tissue sections of 5 μm thick were stained with haematoxylin and eosin (H&E) and Masson's trichrome, then examined under light microscope for determination of pathological changes\(^{30}\).

Statistical analysis & calculations

All data were expressed as mean ± SD of ten rats in each group. Statistical analysis was carried out by one-way analysis of variance (ANOVA), Costat Software Computer Program accompanied with least significance difference between groups at p<0.05.

\[
\%\text{change} = \frac{\text{Mean of control} - \text{mean of treated}}{\text{Mean of control}} \times 100
\]

RESULTS

Potency of Rosemary and Black mulberry on oxidative stress markers of CCl\(_4\) treated rats

Injured kidney by CCl\(_4\) recorded significant increase in malondialdehyde and nitric oxide levels by 45.87 and 68.19%, respectively as compared with control group (Table 1). The prophylactic action of both Rosemary and Black mulberry recorded improvement in malondialdehyde level by 33.42 and 29.79%, respectively, while nitric oxide showed improvement by 36.85 and 54.28%, respectively. The therapeutic action of both plant extracts showed improvement by 33.83 and 26.05% for Rosemary and Black mulberry, respectively, while nitric oxide showed improvement by 40.6 and 48.62% (Fig.1).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Crop & Control & CCl\(_4\) & CCl\(_4\) prophylactic with Rosemary & CCl\(_4\) prophylactic with Blackberry & CCl\(_4\) treated with Rosemary & CCl\(_4\) treated with Blackberry \\
\hline
Malondialdehyde & 17.35±0.27a & 25.31±0.83b & 20.14±0.72b & 19.44±0.86a & 20.79±0.63a & 20.04±0.05a \\
Nitric oxide & 6.54±0.27a & 11.0±0.16a & 7.45±0.66a & 8.38±0.45a & 7.82±0.40a & 7.54±0.45a \\
\hline
\end{tabular}
\end{table}

Values are mean ± SD of five rats in each group. Data are expressed as n mole/g tissue for malondialdehyde and µmole/g tissue for nitric oxide. Statistical analysis are carried out using one way analysis of variance (ANOVA) accompanied with least significance difference between groups at p<0.05. Unshared super script letters are significant values between each group at p=0.0001. Values between brackets are percentages change over control.
Effect of Rosemary and Black mulberry on kidney function indices of CCl4 treated rats

Rats treated with CCl4 showed significant increase in creatinine and urea levels by 149.41, 86.01 respectively, while serum protein recorded significant decrease by 43.16% as compared to control group (Table 2). Rosemary and Black mulberry showed prophylactic improvement for creatinine, urea and serum protein by 55.90 ± 2.55%, 15.78% and 15.78% for Rosemary and 144.70, 69.32 and 15.78% for Black mulberry, respectively. Treatment with either Rosemary or Black mulberry recorded significant decrease by 43.16% as compared to control group (Table 2). Rosemary and Black mulberry showed prophylactic improvement for creatinine, urea and serum protein by 135.29, 80.98 and 17.89% for Rosemary and 144.70, 69.54 and 11.57% for Black mulberry, respectively. Treatment with either Rosemary or Blackberry extracts. Rosemary recorded prophylactic improvement by 49.88, 46.66 and 14.46% for TGF-β, HSP-70 and CYP2E1, respectively, while Black mulberry showed prophylactic improvement by 40.32, 62.85 and 19.57% for Rosemary and 69.32 and 15.78% for Black mulberry, respectively. Treatment of CCl4 injured rats with Rosemary showed improvement by 24.33, 67.14 and 13.67% for TGF-β, HSP-70 and CYP2E1 respectively, while treatment with Black mulberry enhanced the levels of TGF-β, HSP-70 and CYP2E1 by 26.55, 36.19 and 15.42%, respectively (Fig.3).

Effect of Rosemary and Black mulberry on kidney histopathology

Kidney histopathological features of normal rats showed normal appearance of tubes, glomeruli and tubulointerstitial cells (Fig. 4 a). Collagen deposition was of normal range in control group (Fig. 4 b). Kidney section of CCl4-treated rats showed significant morphological damage especially in the renal cortex. Glomerular and tubular degenerations were observed varying from glomerular basement membrane thickening, mild dilatation or congestion of space of Bowman, interstitial inflammation, tubular cell swelling or congestion, tubular brush border loss and tubular dilatation (Fig. 4 c). Marked collagen deposition was recorded (Fig. 4 d).

Rats treated with aqueous extract of Rosemary and Black mulberry showed almost normal morphology and normal architecture of the kidney (Fig. 5 a and c). Group of CCl4 prophylactic with aqueous extract of Rosemary and Black mulberry showed normal morphology with the exception of only few swollen glomeruli and rare vascular congestions that were present in both cortical and cortico-medullar regions (Fig. 5 e and g). In all prophylactic and treated groups, mild collagen deposition was observed (Fig. 5 b, d, f and h).

Table 2: Effect of Rosemary and Blackberry on kidney function indices

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CCl4</th>
<th>CCl4 prophylactic with Rosemary</th>
<th>CCl4 prophylactic with Blackberry</th>
<th>CCl4 treated with Rosemary</th>
<th>CCl4 treated with Blackberry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>0.85±0.20</td>
<td>2.12±0.15</td>
<td>0.97±0.04</td>
<td>0.89±0.05</td>
<td>0.88±0.06</td>
<td>0.89±0.05</td>
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<tr>
<td>Urea</td>
<td>31.03±2.92</td>
<td>57.72±2.20</td>
<td>36.21±5.71</td>
<td>46.79±7.17</td>
<td>32.59±4.47</td>
<td>36.14±1.77</td>
</tr>
<tr>
<td>Serum protein</td>
<td>23.75±2.21</td>
<td>13.50±1.39</td>
<td>17.25±1.25</td>
<td>17.00±1.80</td>
<td>17.75±1.70</td>
<td>16.25±2.06</td>
</tr>
</tbody>
</table>

Values are mean ± SD of five rats in each group. Data are expressed as mg/dl for creatinine, g/dl for urea and mg/ml for serum protein. Statistical analysis are carried out using one way analysis of variance (ANOVA) accompanied with least significance difference between groups at p<0.05. Unshared super script letters are significant values between each group at p<0.0001. Values between brackets are percentages change over control.

Table 3: Effect of Rosemary and Blackberry on inflammatory mediators and cytotoxicity biomarker

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CCl4</th>
<th>CCl4 prophylactic with Rosemary</th>
<th>CCl4 prophylactic with Blackberry</th>
<th>CCl4 treated with Rosemary</th>
<th>CCl4 treated with Blackberry</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>3.96±2.51</td>
<td>7.19±2.84</td>
<td>52.11±4.34</td>
<td>55.90±2.55</td>
<td>62.24±2.07</td>
<td>61.36±2.30</td>
</tr>
<tr>
<td>HSP-70</td>
<td>2.10±0.91</td>
<td>5.79±0.44</td>
<td>4.81±0.44</td>
<td>4.7±0.48</td>
<td>4.38±0.28</td>
<td>5.03±0.17</td>
</tr>
<tr>
<td>GYP2E1</td>
<td>18.80±0.90</td>
<td>10.38±0.62</td>
<td>13.10±1.16</td>
<td>14.06±1.11</td>
<td>12.95±0.75</td>
<td>13.28±0.75</td>
</tr>
</tbody>
</table>

Values are mean ± SD of five rats in each group. Data are expressed as pg/ml for TGF-β1 and ng/ml for HSP-70 and GYP2E1. Statistical analysis are carried out using one way analysis of variance (ANOVA) accompanied with least significance difference between groups at p<0.05. Unshared super script letters are significant values between each group at p<0.0001. Values between brackets are percentages change over control.

Fig. 1: Improvement percentages of malondialdehyde (MDA) and nitric oxide (NO).
Fig. 2: Improvement percentages of creatinine, urea and serum protein.

Fig. 3: Improvement percentages of heat shock protein-70, transforming growth factor-β and cytochrome P450-2E1.

Fig. 4: Photomicrography sections (100X) of control (a,b) and CCl4 injured kidney (c,d) stained with haemtoxyline & eosin and Masson’s trichrome. Arrows indicate normal glomeruli and normal interstitial spaces with normal collagen deposition. Small arrows indicated dilatation in glomeruli and its interstitial spaces and marked collagen deposition.
DISCUSSION

The mechanism of CCl₄ hepatotoxicity is well documented in the rat model. According to these reports, CCl₄-induced liver injury is due to the conversion of CCl₄ to CCl₃ and CCl₃O₂ by the cytochrome P450 enzyme. These highly reactive free radicals cause cell damage. However, the pathogenesis of CCl₄-induced renal injury has not been clearly clarified. Rincon et al. showed that effects of CCl₄ on kidney structure and function depended on the functional state of the liver. Ogawa et al. suggested etiologic independence of the renal and hepatic events. The oxidative damage to the lipids and proteins of the rat kidney resulting from chronic toxicity of CCl₄ inhalation was reported by Abraham et al. It has also been reported that systemically administered CCl₄ in rats was distributed at higher concentrations in the kidney than in the liver. Since the kidney has an affinity for CCl₄ and contains cytochrome P450 predominantly in the cortex, therefore the mechanism of CCl₄ nephrotoxicity is probably the same as that of the liver and also independent from the diminished functionality of the liver.

In the present study, CCl₄ injured kidney recorded significant increase in NO and lipid peroxidation process. The reductive dehalogenation of CCl₄ by the P450 enzyme system to the highly reactive trichloromethyl radical initiates the process of lipid peroxidation which is considered to be the most important mechanism in the pathogenesis of renal damage. Although NO was described initially as a vasodilatory chemokine, it plays a major role as antioxidant. The observed increase of NO level permitted vasodilatation in the kidney which contributed by disturbance in Na⁺-K⁺-ATPase. The activity of renal Na⁺-K⁺-ATPase varies in parallel with sustained changes in Na⁺ or K⁺ transport, indicating the participation of this enzyme in the chronic adaptation of the kidney to altered Na⁺ reabsorption or K⁺ secretory load. Not only these hemodynamic effects, but also alterations in membrane lipid composition that influence membrane fluidity, cation transport and Na⁺-K⁺-ATPase activity can predispose renal tubular cells to injury.

CCl₄ treated rats recorded also significant increase in urea and creatinine levels. This was in agreement with Khan et al. who reported that chronic renal injuries by CCl₄ intoxication was associated with urea and creatinine elevation and considered as indicators of kidney injury, where the serum creatinine level does not rise until at least half of the kidney nephrons are destroyed. Renal injuries may contribute to low level of serum protein that might have resulted from remarkable leakage into urine due to injuries in glomeruli and tubules.
We also observed significant increase in heat shock protein-70, TGF-β1, and significant decrease in GYP2E1 in CCl4 treated rats. This was in accordance with Fink et al. who postulated that thermal, oxidative, hemodynamic, osmotic, and hypoxic stresses induce HSP. The same authors added that this stress response results in cytoprotection. Specifically, HSP prevent nonspecific protein assembly, assist in denatured protein refolding, and interfere with proapoptotic pathways. Glomerular capillary hypertension imposes cellular stresses on renal target cells and they are thus potential inducers of a stress response that may counterbalance the deleterious effects of these insults.

The transforming growth factor β1 (TGF-β1) family of cell signaling molecules contains 11 members that play a role in the development, the homeostasis, and the repair of most tissues. The transforming growth factor β1 (TGF-β1) family of cell signaling molecules can be subdivided into TGF-β1 sensu stricto, bone morphogenetic proteins (BMP) and activins, all of which appear to share common features in their downstream signaling mechanisms. TGF have recently been suggested to be involved in the mechanism of compensatory renal growth. Thus, using immunohistochemical staining, TGF-β1 in rat proximal tubule cells appears to increase and act as a modulator of compensatory renal hyperplasia.

Microsomal cytochrome P450 monoxygenases play important roles in the biotransformation of numerous endogenous and xenobiotic compounds. While the liver is regarded as the richest source of P450s and other drug-metabolizing enzymes, the P450s expressed in various extra-hepatic tissues can also contribute to target tissue toxicity induced by tissue-selective toxicants. The kidney is a major target organ for chemical-induced toxicity. A number of P450 isoforms are expressed in the kidney of rodents, including members of the CYP1A, 2B, 2C, 2E, 2J, 3A, 4A and 4F subfamilies. The P450 content in kidney microsomes is 10% of the concentration in liver microsomes in rats. Within the kidney, the renal proximal tubule has the highest concentrations of P450s and cytochrome P450 reductase (CPR). It is also the primary target for xenobiotic-induced renal toxicity. Chemical-induced nephrotoxicity can be caused either by the parent compounds or by their reactive metabolites generated through biotransformation. The nephotoxic metabolites can be produced by local P450s in the kidney, or else they can be generated in the liver or other organs and then transported into the kidney through systemic circulation.

Numerous experimental studies have demonstrated the beneficial effects of antioxidant treatment on CCl4-induced tissue injury. In the present study, we hypothesized that Rosemary and Black mulberry would effectively protect kidneys by their antioxidant, anti-inflammatory and anti-cytotoxic effects against CCl4-induced kidney injury. Our results demonstrate that Rosemary and Black mulberry will be able to reduce the damage to the rat kidney induced by chronic CCl4 poisoning. This was verified by the improvement occur in MDA, NO, urea, creatinine, HSP-70, TGF-β, CYP2E1 and the histopathological observations. Carnosic acid and carnosol found in Rosemary as well as phenolic compounds, including flavonoids, anthocyanins and carotenoids in Black mulberry recorded highly antioxidant activity against free radical species and oxidative stress, which give an additional support of the observed protection.

CONCLUSIONS

Rosemary and Black mulberry succeeded to protect the kidney against injury induced by CCl4. Therapy with either Rosemary or Black mulberry showed more potent effect than the probiotic action through the observed improvement in kidney architecture. Further studies are needed to identify the molecules responsible for these pharmacological effects.

REFERENCES


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