Molecular and Anti-oxidant Effects of Wheat Germ Oil on CCl₄-Induced Renal Injury in Mice

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ABSTRACT

The protective effect of wheat germ oil (WGO) as a natural antioxidant was evaluated against carbon tetrachloride (CCl₄)-induced nephrotoxicity, genotoxicity and alterations in gene expression in mice kidney. WGO group: received olive oil orally for 2 days then WGO orally for 8 days, CCl₄ group: received CCl₄ orally for 2 days then olive oil for 8 days, CCl₄ + WGO group: received CCl₄ for 2 days then WGO for 8 days. Administration of CCl₄ induced different abnormal patterns in P53 exons (5,6,7and 8), altered the histological architecture of kidney tissues, down-regulated mRNA expression of Bcl2 gene, and reduced the PCEs/NCEs ratio, the efficiency of kidney function and the concentrations of some antioxidant enzymes. On the other hand, it increased the number of micronucleus, % DNA fragmentation and mRNA expression of BAX gene causing apoptosis that has been proven by DNA ladder assay. Supplementation of WGO has ameliorated these effects of CCl₄ through markedly improving different measured parameters, suggesting that WGO effectively protects kidney against CCl₄-induced oxidative damage in mice. The nephroprotective and free radical scavenging effects of WGO might be due to the presence of bioactive constituents that could be useful against toxic effects of hazardous agents like CCl₄.

INTRODUCTION

Development of renal failure is common in approximately 5% of patients with liver failure. Renal failure may be secondary to the liver failure itself (and is termed the hepatorenal syndrome). Kidney architecture and function are frequently altered in severe liver disease. Renal function is rarely restored in the absence of hepatic recovery (Moore, 1999). Exposure to xenobiotics, either therapeutic drugs or environmental pollutants, especially CCl₄ may lead to renal injury similar to that in human renal dysfunction (Rincón et al., 1999). CCl₄ is a potent environmental hepatotoxin (Sahreen that in addition to persuadinghepatic problems, it also causes dysfunction of kidney and many other tissues as well as generates free radicals in blood (Khan and Ahmed, 2009). The single acute dose of CCl₄ causes liver toxicity that is characterized by hepatocellular necrosis and steatosis while a chronic dose of CCl₄ causes liver cirrhosis. Generation of free radicals has been observed in many tissues; liver, kidney, intestine, heart, lung, brain, and blood after CCl₄ administration (Dashhi et al., 1989). The pathogenesis of CCl₄ induced renal dysfunction may be due to liver functional state or renal injury that may develop independently to hepatic events (Rincon et al., 1999). Moreover, oxidative stress induced by CCl₄ in many situations might be expected to be the reason of nephrotoxicity. The injury induced by CCl₄ is known to decrease GSH of phase II enzyme; and reduces antioxidant enzymes and antioxidant substrates and thus initiates oxidative stress which is an important factor in generating reactive oxygen species (ROS) and resulting in acute and chronic injuries in various tissues (Szymonik-Lesiuk et al., 2003, Weber et al., 2003, Preethi and Kuttan, 2009). ROS causes many harmful effects on the genetic material including oxidative DNA damages, formation of DNA adducts, genetic mutation, strand breakage, and chromosomal alterations (Jia et al., 2002, Khan et al., 2009). Wheat germ oil (WGO) is extracted from the germ of the wheat kernel (Irmak and Dunford, 2005). It is a valuable source of polyunsaturated fatty acids especially the essential fatty acids ω-6 linoleic acid and ω-3 linolenic acid (Mohamed et al., 2005), and is enriched with vitamin E, policosanol, octacosanols and tocopherols which have antioxidant properties (Irmak and Dunford, 2005; Alessandri et al., 2006).

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Experimental studies showed that WGO possesses anti-inflammatory properties (Mohamed et al., 2005), decreases oxidative stress (Alessandri et al., 2006), and improves lipid metabolism (Singh et al., 2006). Recently, WGO has received considerable attention in the treatment of diseases involving platelet aggregation, thrombus formation and oxidative damage of red blood cells membranes (Lass and Sohal, 2000), raised blood sugar and cholesterol levels (Irmax and Dunford, 2005). Moreover, it’s useful in endurance, assisting muscular dystrophies and other neuromuscular disorders, promotes skin cell formation, improves urinary output, prevents rancidity and lower oxygen depletion (Said et al., 2008). WGO stimulates the tocopherol redox-system that may be change the intensity of lipid peroxidation processes (Leenhardt et al., 2008).

This study was carried out to evaluate the role of WGO as a natural antioxidant (ameliorating agent) to alleviate the hazardous effects persuaded by CCl₄ that resulted in DNA damage, apoptosis and oxidative stress in kidney using mouse as a model.

MATERIALS AND METHODS

Experimental animals

Adult male C57 BL/6 mice weighing 22-25 g (6 weeks old) were employed in this study. Animals were obtained from the animal house of the National Research Center (NRC, Giza) and acclimatized for one week prior to the experiments in an environment of controlled temperature (22-25°C), humidity, and light/dark cycle. Mice were housed in stainless steel cages and permitted for free standard laboratory diet ad libitum. All the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals. The animal experimental protocol was approved by the Animal Care and Ethical Committee of National Research Centre.

Chemicals and reagents

Carbon tetrachloride (CCl₄) was purchased from Merck/Schuchardt (Darmstadt, Germany). Kits for all biochemical parameters were purchased from Bio-Diagnostic Company, Diamond Diagnostics Company (Hannover, Germany) and Spectrum Diagnostics Company (Hannover, Germany).

Primers of Bcl-2, Bax and Gapdh genes were obtained from Life Technologies (Grand Island, NY, USA). Wheat germ oil (WGO), all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other molecular kits are mentioned elsewhere.

Experimental design

WGO was given to the animals by oral gavage at dose (1400 mg/kg body weight), according to the previous literature (Karabacak et al., 2011), while CCl₄ (1 mL/kg.b.w) as 50% (v/v) solution was given by oral injection in an olive oil solution. Mice were randomly divided into 4 groups (6 mice/group) and were maintained on their respective diets for 10 days as follows: Control group: untreated control mice received 1 mL oral administration of olive oil for 10 days; (WGO) groups: treated orally with olive oil for 2 days then with WGO for 8 days; (CCl₄) groups: treated with CCl₄ for 2 days then with olive oil for 8 days; (CCl₄+WGO) group: has given CCl₄ for 2 days then WGO for 8 days. At the end of the treatment period (10th day), the animals were kept fasting overnight and sacrificed, then the blood, bone marrow and kidney samples of each animal were obtained for biochemical, micronucleus, and gene expression studies.

Micronucleus assay

Micronucleus assay described by (Schmid, 1975) was used to detect chromosomal damage baseline. The femurs were dissected out and the bone marrow was flushed out with saline, vortex and centrifuged.

The pellet was resuspended in few drops of fetal calf serum. Smears were spread on clean glass slides and left to dry in air. Slides were then fixed in methanol and stained for 5 minutes in May-Grunwald then in 10% buffered Giemsa stain. Finally, slides were rinsed in Sorenson’s buffer two times 2 minutes each. Slides were examined by light microscope at 100x and 2000 intact erythrocytes were counted where polychromatic erythrocytes and normochromatic erythrocytes ratio (PCEs/NCEs) were calculated and micronuclei were counted per 1000 polychromatic erythrocytes. Results were expressed as mean ±SE.

DNA-laddering assay

According to the standard protocol described by (Sriram et al., 2010), kidney cells were homogenized in cold lysis buffer containing 50 mM Tris HCl, pH 8.0, 10 mM EDTA, 0.1 M NaCl, and 0.5% SDS. Then incubated with 0.2 mg/mL proteinase K at 50°C overnight and finally with 0.5 mg/mL RNase A at 37°C for one hour. Phenol extraction was carried out, and DNA in the aqueous phase was precipitated by 25μL (1/10 volume) of 7.5 M ammonium acetate and 250 μL (1/1 volume) isopropanol. Fragmented DNA was detected by running 5 μg of the genomic DNA on a 1.5% ethidium bromide-treated agarose gel (Sigma,UK). The gel run at 80 Volt (power supply Biorad, Model 200/2.0), and visualized under UV transillminator (Statagene, USA).

Quantitative determination of genomic DNA fragmentation

DNA damage was quantified using one kidney from each animal per each treatment group. DNA fragmentation assay was based on the method of (Wu et al., 2006) with some modifications. The whole frozen kidney was homogenized in cold TE solution pH 8.0 (5 mmol Tris–HCl, 20 mmol EDTA) and 0.2% triton X-100. An aliquot of 1.0 ml from each sample was centrifuged at 27,000 × g for 20 min to separate the intact chromatin (pellet, B) from the fragmented DNA (supernatant, T). The pellet and supernatant fractions were examined for DNA content using a freshly prepared DPA (Diphenylamine) solution for reaction. Optical density was read at 620 nm using spectrophotometer (SmartSpec™ Plus Spectrophotometer catalog # 170–2525), and color reaction was
based on the method of (Burton, 1956). The results were expressed as amount of % fragmented DNA using the formula:

\[
\% \text{ fragmented DNA} = \frac{T \times 100}{T + B}
\]

Single strand conformation polymorphism (SSCP) analysis

Isolation of Genomic DNA

Genomic DNA was extracted from mice kidney tissues using genomic extraction kit (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions.

Polymerase chain reaction (PCR) amplification

For SSCP analysis of p53 exons 5–8, four pairs of PCR primers were listed in table 1 based on the published sequence (Gutierrez et al., 1992). Samples were initially denatured at 95°C for 5 min. Then, Thirty five cycles of denaturation (95°C), annealing (58°C for exons 5-8) and extension (72°C) and final extension at 72°C for 7 min were done. PCR products were separated and visualized by electrophoresis through 2% ethidium bromide-treated agarose gel using UV transilluminator and photograph by gel documentation system (UVITEC).

SSCP analysis

5 μl aliquot of the PCR product were mixed with 5 μl of denaturing loading dye (95% formamide, 0.1% bromophenol blue, 0.1% Xylene cyanol FF and 0.5 μl 15% Ficoll) and 5 μl of TE buffer, then itwas heat denatured at 94°C for 5 min, then snap-cooling on a freeze-block (K20 8C) for 5 min (Gasser et al., 2006). 10 μl of the samples were then loaded into 0.5 mm thick well of 3% non-denaturing polyacrylamide gel (acrylamide/bisacrylamide = 29:1, v/v) for exon 5, 6, 8 and exon 7 respectively, prepared using MiniPROTEAN® 3 Cell Set (Bio-Rad, USA). Electrophoresis was carried out at (90V for 2 hr at 4°C), and stained for 5 min in 100 ml of 1x TBE with ethidium bromide to visualize the DNA bands. Finally, gel was examined using UV transilluminator and photograph by gel documentation system (UVITEC).

Expression levels of Bax and Bcl-2 genes by reverse transcriptase polymerase chain reaction (qRT-PCR) analysis:

Transcription levels of Bax and Bcl-2 in the Kidney tissues of the experimental mice were measured using qRT-PCR technique. Total RNA was extracted from the kidney tissues using Gene JET RNA Purification Kit (Thermo scientific, USA) and according to the manufacturer’s instructions. RNA concentrations and purity were determined by measuring the absorbance A260/A280 ratios using Nanodrop spectrophotometer. cDNA was prepared from the extracted RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, USA). qRT-PCR was carried out from the synthesized cDNA using SYBR green-based real-time PCR and was detected with 7500 Fast systems (Applied Biosystem 7500, USA) the amplification profile consisted of an initial denaturation at 94°C for 5 min followed by denaturation at 94°C, annealing at 52°C for Bcl-2 & 58°C for Bax and extension at 72°C for 30 s was carried out.Total volume for each PCR reaction was 20 μL according to the manufacturer’s protocol. Bcl-2 primers sequences are shown in table 2 were designed using NCBI primer blast, while Bax primers were previously published (Exley et al., 1999). Each sample was prepared as duplicate for each gene expression. All signals were normalized to mRNA levels of the house keeping gene, Gapdh, and expressed as RQ=2^{ΔΔCt}. Results were reported as Mean ± Standard Error (SE) of relative change compared to the untreated control.

Biochemical Analysis of Kidney Function Test

The levels of renal function markers including creatinine, urea, and uric acid were determined using the appropriate commercially kits. Serum urea and uric acid were measured according to (Tietz,1995), while serum creatinine was measured according to the method of (Tietz,1986).

Table 1: Oligonucleotide primers used for the p53 gene amplification.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sense</th>
<th>Antisense</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5'-TCTTTCAGTACTCCTC-3'</td>
<td>5'-AGGCCTGTTGAGCGTTAC-3'</td>
<td>214</td>
</tr>
<tr>
<td>6</td>
<td>5'-GGCTTCTGATTTATCTGTGC-3'</td>
<td>5'-CAACTGTCTCTAAGAGGCAC-3'</td>
<td>181</td>
</tr>
<tr>
<td>7</td>
<td>5'-TCACCTGGATCTGTCTCTT-3'</td>
<td>5'-CAGGCTAACCTACCCCTACCA-3'</td>
<td>170</td>
</tr>
<tr>
<td>8</td>
<td>5'-ACTGCCTGTGGCTTGGTCCTT-3'</td>
<td>5'-GGAGAGGCGCTTGGCAGTT-3'</td>
<td>280</td>
</tr>
</tbody>
</table>

Table 2: Sequences of primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense 5-3</th>
<th>Antisense 5-3</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GTATCGGACGCCTGGTTC</td>
<td>CTTGCGCGTGGTAGATCAT</td>
<td>128</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>GGGATGACCTTCTCTGTCGC</td>
<td>CATGACCCTACCAGGAACCCAAA</td>
<td>170</td>
</tr>
<tr>
<td>Bax</td>
<td>CGGCCAATTGGAGATGACTG</td>
<td>GCAAGTGAAGAGGGCAACC</td>
<td>160</td>
</tr>
</tbody>
</table>
Renal oxidative stress biomarkers

Kidney from each experimental group were collected in tubes containing 1.5ml saline and homogenized, centrifuged at 10,000 rpm for 10 min at 4°C. Malondialdehyde (MDA) (Ohkawa et al., 1979), glutathione (GSH) (Beutler et al., 1963), glutathione-S-transferase (GST) (Habig et al., 1974), catalase (CAT) (Aebi, 1984) were determined using Bio-diagnostic assay kits according to the manufacturer’s instructions.

Histopathological examination

For pathological studies fresh portions of kidney were immediately fixed in 10% buffered formalin. Then, embedded in paraffin and sliced to 3 - 4 μm, and then stained with hematoxylin and eosin (Mayer, 1903). The stained sections were evaluated by a histopathologist unaware of the treatments using light microscopy (U-III Multi-point Sensor System; Nikon, Tokyo, Japan).

Statistical analysis

Unpaired student T-test was used to test the significance difference between groups. Statistics were carried out with the statistical analysis systems (SAS) program (SAS, 2005). P<0.05 was considered statistically significant.

RESULTS

Micronucleus assay

The frequencies of MnPCEs in each tested group are presented in figure 2 and indicated that mice treated with CCl₄ alone showed a high frequency of MnPCEs compared to the control group. However, animals treated with CCl₄+WGO showed a significant reduction in the mean of MnPCEs which reached 11 in the CCl₄ treated group and reduced to 7 in the CCl₄+WGO group. Meanwhile; the PCEs/NCEs ratio in CCl₄ group (0.53±0.03) indicated a significant cytotoxicity (P<0.05) when compared to the control group and a marked elevation in this ratio in CCl₄+WGO group (0.83±0.03). Moreover, WGO alone did not induce any significant difference in the frequency of MnPCEs or PCEs/NCEs ratio compared to the control group, which indicates its safety.

Fig. 2: Effects of WGO on (A) CCl₄-induced micronucleated polychromatic erythrocytes (MN-PCEs) and (B) polychromatic to normochromatic (PCEs/NCEs) ratio in mice bone marrow cells. Results are expressed as means± SE, (*) significant difference with negative control at P<0.05 using T-test, (#) statistically compared with WGO, and ($) statistically compared with CCl₄ group.

Qualitative and quantitative DNA fragmentation

The current results showed that CCl₄ induced apoptotic DNA laddering at 150& 250 bp in mice kidney on agarose gel (Fig 3A). Slight smear of DNA with no apoptotic bands were observed in animals given the combined treatments of CCl₄+WGO. Moreover, the administration of WGO did not induce any differences from the control group. On the other hand, the results of % DNA fragmentation in CCl₄ induced apoptotic changes in the kidney are presented in figure (3B) and indicated that the percentage of DNA fragmentation significantly increased (P<0.05) in the group treated with CCl₄ compared to the control group. However, mice treated with CCl₄ and received WGO showed a significant improvement in the percentage of DNA fragmentation towards the control values.

Fig.3: (A)DNA fragmentation showing apoptotic laddering of genomic DNA isolated from mice kidney treated with CCl₄ at ~150 &250bp(indicated by arrows) and slight smear of DNA in CCl₄+WGO groups, compared with their control and WGO group. (B) Graph representing the effect of WGO on %DNA fragmentation induced by CCl₄ in mice kidney. Results are expressed as mean± SE, (*) significant difference with negative control at P<0.05 using T-test, (#) statistically compared with WGO, and ($) statistically compared with CCl₄ group.

SSCP-PCR analysis

Figure (4A) showed PCR products of the isolated DNA from kidney tissues representing different sizes of the four tested P53 exons (5-8) 214, 181, 170 and 280bp respectively, and confirming the successful amplification of the interested bands. Five mice were examined per each exon. SSCP analysis of exons...
5–8 revealed altered banding patterns which were identified as extra bands (e), band shifts (s) and deleted bands (d) that confirming the inductions of mutations in group treated with CCl4. Number and frequency of samples that showed different band patterns compared to their controls detected by SSCP assay were estimated as follow: two mutations in exons 5 (10%), three mutations in each of exons 6 & 7 (15%) out of twenty, and no mutation was observed in exon 8. However, mutation frequencies were highly decreased after WGO administration and reached the negative control level.

Results indicated a significant over expression (P<0.05) in Bax reach 2 folds in the animals treated with CCl4 compared to the control. On the other hand, the expression of Bcl-2 was significantly (P<0.05) down-regulated to be ~0.5 compared to the control in the same group (Fig.5B). Treatment of CCl4 group with WGO reduced the expression of mRNA Bax from 2 to 1.4 folds. Moreover, treatment with WGO increased the level of Bcl-2 expression from 0.5 to 1.0 in CCl4+WGO group and reached nearly the expression level in the control. Furthermore, there was a marked elevation (P<0.05) in Bcl-2 gene expression in WGO group and non-significant expression of Bax gene when referred to the control group.

### Kidney function tests
The nephrotoxicity induced by CCl4 was demonstrated by the significant increase (P < 0.01) in serum creatinine, urea, and uric acid in CCl4-treated mice when compared to the control group. On the contrary, the treatment with WGO has improved the kidney function in CCl4+WGO group by reducing the levels of creatinine, urea, and uric acid significantly (P < 0.01) as compared with the CCl4 group (Table 3).

### Renal Oxidative Stress Biomarkers
The current study revealed an elevation in MDA level significantly (P< 0.05) in CCl4-treated mice as compared to the control group. While a significant reduction (P<0.05) in the levels of GSH, GST and CAT were observed in the same group. In contrast, the level of MDA was decreased significantly in CCl4+WGO group when compared to the CCl4-treated group. On the other hand, a significant increase in concentrations of GSH, CAT and GST (P< 0.05) were observed subsequent to the oral administration of WGO after CCl4 treatment (Table 4). No significant changes of the tested parameters were observed in WGO group except a marked elevation of GST level.

### Table 3: Effect of WGO on serum urea, uric acid and creatinine of mice treated with CCl4.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Creatinine (mg/dL)</th>
<th>Urea (mg/dL)</th>
<th>Uric Acid (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.13 ± 0.007</td>
<td>23.64 ± 0.86</td>
<td>1.32 ± 0.06</td>
</tr>
<tr>
<td>WGO</td>
<td>0.17 ± 0.008</td>
<td>25.20 ± 0.17</td>
<td>1.403 ± 0.20</td>
</tr>
<tr>
<td>CCl4</td>
<td>0.79 ± 0.02*</td>
<td>31.07 ± 0.27*</td>
<td>4.903 ± 0.29*</td>
</tr>
<tr>
<td>CCl4+WGO</td>
<td>0.25 ± 0.02</td>
<td>27.76 ± 0.37*</td>
<td>2.163 ± 0.11</td>
</tr>
</tbody>
</table>

Values are expressed as means ± S E (n = 6). Statistically compared with negative control (*), WGO (#), and with CCl4 group ($) at p<0.05 using Unpaired student T-test.

### Table 4: Effect of wheat germ oil on oxidative stress markers in kidney cells of male mice received CCl4.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/g tissue)</th>
<th>GSH (mg/g tissue)</th>
<th>CAT (nmol/min/ g tissue)</th>
<th>GST (U/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>75.64 ± 0.51</td>
<td>9.4 ± 0.15</td>
<td>3.69 ± 0.17</td>
<td>2.457 ± 0.06</td>
</tr>
<tr>
<td>WGO</td>
<td>76.87 ± 0.73</td>
<td>10.21 ± 0.41</td>
<td>4.23 ± 0.07</td>
<td>3.44 ± 0.17</td>
</tr>
<tr>
<td>CCl4</td>
<td>95.07 ± 2.58*</td>
<td>6.4 ± 0.2*</td>
<td>2.23 ± 0.17*</td>
<td>1.12 ± 0.16*</td>
</tr>
<tr>
<td>CCl4+WGO</td>
<td>65.09 ± 2.66*</td>
<td>8.21 ± 0.22*</td>
<td>3.36 ± 0.13*</td>
<td>2.48 ± 0.28*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE, statistically compared with negative control (*), WGO (#), and with CCl4 group ($) at p<0.05 using Unpaired student T-test.
Histopathological examination

Kidney sections from the control and WGO-treated groups showed normal structure of renal glomeruli and renal tubules. (Figure 6A, B). In contrast, Acute toxicity of CCl₄ has reflected marked deleterious histological changes of kidney including portal tract infiltration with mononuclear cells (arrow) and fibrosis (arrow head), significant glomerular and tubular degenerations, congestion in the glomerular capillary tuft, slight vacuolization in the endothelial cells lining the tuft of the glomeruli, wide congestion in the glomerular capillary mass, inflammatory cells infiltration, absence of epithelial cells of renal tubules and deformation in the renal tissue architecture were observed as well (Fig.6C). On the other hand, administration of WGO after CCl₄ treatment in CCl₄+WGO group restored the healthy morphological and structural shape of the kidney, where the tubules and corpuscles appeared to be alike to those of the control group (Fig.6D).

WGO is a rich source of natural antioxidant tocopherols, sterols and vitamin E which acts as an inhibitor of oxidation processes in different body tissues and protects cells against the effects of free radicals (Olson et al., 1999). Moreover, WGO generates DNA protective properties (Gelmez et al., 2009), as indicated by improving DNA fragmentation, which is in a close agreement with other study (Krings et al., 2006).

It’s known that administration of 0.172 mM CCl₄ to hepatocytes, resulting in loss of Ca²⁺ from mitochondria and endoplasmic reticulum (Albano et al., 1985), because of the inactivation of the mitochondrial unipot system and Ca-ATPase of the endoplasmic reticulum. The loss of ATP supply can result from the interruption of mitochondrial function that contributed to CCl₄-derived reactive oxygen species (Lemasters et al., 1998). The resulting ATP depletion has been considered as a causative factor in CCl₄-induced cell death (Harman and Maxwell, 1995). Obviously, this causes an increased cytosolic Ca²⁺ (Tsokos-Kuhn et al., 1985; Long and Moore, 1986). Cytosolic Ca²⁺ elevation in the cell, breakdown cytoskeletal structures and trigger a number of catabolic enzymes such as proteases, endonucleases, and phospholipases, whose sustained action results in cell death via apoptosis or necrosis (Nicolera et al., 1992, Haozui et al., 2000). We confirmed this speculation by DNA laddering assay and by the marked (P<0.05) elevation in the ratio of mRNA levels of Bax to Bcl-2 genes in CCl₄ group, compared to the control animals. Which indicates its mitochondrial mediated apoptosis pathway (Duan et al., 2005). The altered expression of Bax and Bcl-2 mRNA induced by CCl₄ in mice kidney is similar to that initiating a chain of lipid peroxidation reactions by abstracting hydrogen from polyunsaturated fatty acids (PUFA). These radicals can dramatically change the properties of biological membranes, resulting in a severe cell damage in liver and kidney and play a vital role in pathogenesis of diseases (Weber et al., 2003). Free radicals induced by lipid peroxide rejoin with DNA to form pirimedopurinone (MIG) adduct (Marnett, 2000), and can produce a multiplicity of DNA modifications including base and sugar lesions, strand breaks, DNA protein, cross-links and base-free sites. If left un-repaired, oxidative DNA damage can lead to harmful biological consequences in the organisms, including cell death, transformation of cells to malignant cells, genetic mutation, oxidative DNA damages, point mutations, strand breakage, and chromosomal alterations as revealed in the present study by different p53 mutations (Fig.4) and DNA fragmentation (Fig.3), similar results were previously reported (Jia et al., 2002, Khan et al., 2009, Murugesan et al., 2009, Alkreathy et al., 2014). In addition, DNA fragmentation itself induces p53 gene expression; blocks cell cycle, and gives additional time to repair DNA (van Gijssel et al., 1997). These factors further rationalize the significant increase (P<0.05) in MN in CCl₄-treated group and the elevation in the cytotoxicity ratio, these findings are in sharp contrast with Rossi et al. who documented an increase in the frequency of chromosomal aberrations in bone marrow and liver cells of CCl₄-treated male mice at dose level of 1 ml/kg/b.wt (Rossi et al., 1988, Abdou et al., 2012).

Fig. 6: Photomicrographs of kidney sections of mice stained with haematoxylin-eosin under light microscope. (A) Control mice showing normal mouse kidney with normal tubular brush-borders (T) and intact glomeruli (G). No evidence of congestion or inflammation was observed in the glomeruli (G). (B) WGO-treated mice showing normal morphology of kidney. (C) CCl₄-treated mice showing glomerular degeneration, tubular brush-borders loss, interstitial edema (E), necrosis of epithelium (N), inflammatory cells infiltration, mononuclear cells (arrow) and fibrosis (arrow head). (D) CCl₄+WGO treated group showing attenuated necrosis, reduced inflammatory cells and improved tubule and glomeruli architecture (400X).

DISCUSSION

Metabolic activation of some chemicals including various environmental toxicants and even clinically useful drugs; to highly reactive substances such as free radicals can cause severe cellular damages in different organs of the body. Free radicals induced by CCl₄ during metabolism cause liver, lung damages and acute nephrotoxicity in experimental animals, and thus can be used as a model to study their hazardous effects (Ichi et al., 2009). CCl₄ is metabolized by cytochrome P450 2E1 to trichloromethyl radical (CCl₃*). In the presence of oxygen, (CCl₃O*) radical is converted to the trichloromethylperoxy radical (Cl₃COO*) which is capable of...
reported by (Abdel-Aziem et al., 2011). However, severe DNA damage triggers apoptosis as well. WGO administration caused significant reductions in kidney mRNA levels of Bax, associated with a significant comparable elevation in kidney mRNA level of Bcl-2 compared to the control group. These findings are similar to Mona et al. study showing the ameliorative effect of WGO on Bcl-2 and Bax gene expressions after an acute single dose of irradiation 5Gy (Mohamed and Ahmed, 2014). The main role of Bcl-2 gene product in the apoptosis process is based on its ability to inhibit conductivity of ion channels in mitochondrial membrane, and in most cases to prevent mitochondrial disruption and the release of cytochrome c. Therefore, down regulation of anti-apoptotic Bcl-2 may enhance the postmitotic signaling of apoptosis in CCl₄-treated mice. The Bax/Bcl-2 ratio indicates the relative amounts of pro- and anti-apoptotic proteins of the Bcl-2 family. The increased Bax/Bcl-2 ratio due to WGO administration may be considered as an indicator of enhanced cell death in response to reactive oxygen species as reported by Debatin (Debatin, 2004).

Free radicals produced from the peroxidation of the polyenoic lipids of the endoplasmic reticulum, decrease the activities of antioxidant enzymes (Adewole et al., 2007, Khan and Ahmed, 2009) and reduces glutathione (GSH) content, this is in harmony with our findings (Table 3) as indicated by the significant decrease in CAT, GSH and GST activities. It has been reported that SOD, CAT, GSH, GPx and GST constitute a mutually helpful team of defense against ROS (Bandyopadhyay et al., 1999), thus their depletion would cause an increased accumulation of superoxide radicals, which could further stimulate lipid peroxidation. Therefore, administration of WGO after CCl₄ intoxication protected the antioxidant machineries of the kidney through enhancing the levels of CAT, GSH and GST activities, increasing GSH content and thus decreasing lipid peroxidation.

GST is a cystolic protein that is found in high concentrations in the kidney and very specific for the proximal tubules cells. During renal tubular injury induced by CCl₄ treatment, GST is continuously released into the urine (Harrison et al., 1989). Thus, renal function could directly affected by oxidative stress that enhance renal vasoconstriction or reduction of the glomerular capillary ultrafiltration coefficient; that results in reduction of the glomerular filtration rate as revealed by the marked impairment in renal function alongside with the significant elevation of the oxidative stress and the abnormal histopathological examination in the kidney (Garcia-Cohen et al., 2000). In addition, elevated level of urinary urea, uric acid and creatinine might have resulted from remarkable leakage due to hypercellularity of both glomeruli and tubules.

Lipid peroxidation radicals result in the generation of ROS, like superoxide anion O₂⁻ and hydroxyl radical OH*, from the metabolism of oxygen. ROS are extremely reactive molecules that can cause severe damage to cells and tissues; causes several disorders such as cardiovascular disease, oxidative stress, aging; Alzheimer’s disease, mutations, and cancer (Ames, 1998; Finkel and Holbrook, 2000). Moreover, ROS interact with the renal mitochondrial membranes producing large amounts of oxygen radicals that cause deterioration of the kidney architecture (Tabassum et al., 2007), as indicated by the congestion in the glomerular capillary tuft and absence of epithelial cells of the renal tubules (Fig. 7C). This abnormal architecture reflected the severe pathological effect of CCl₄ and consequently, altered the kidney function tests and the damage the defense system of the kidney. It is thought that the capacity for tubular absorption may have been altered, thus producing functional overload of nephrons with subsequent renal dysfunction. These data are in accordance with Adewole et al., who mentioned that acute dose of CCl₄ is associated with an impairment of kidney function (Adewole et al., 2007). On the other hand, WGO protect kidney tissue against oxidative damages induced by CCl₄. Thus the attenuation of lipid peroxidation and restoration of the healthy architecture of kidney tissues by WGO in CCl₄-treated mice provide a convincing evidence for the involvement of ROS in CCl₄-induced lipid peroxidation.

Administration of WGO markedly ameliorates the toxicity of CCl₄ through suppression of free radicals generation and increased the enzymatic activities of antioxidant enzymes till reaching nearly the normal range. High content of vitamin E in WGO has the potency to deactivate the reactive free radicals, thus avoiding the propagation of the radical chain reaction (Ismael et al., 2014).

WGO contains numerous saturated fatty acids and a high percentage of unsaturated fatty acids (81%) that has anti-inflammatory properties and can decline oxygen free radicals and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (Abd-El-Hameed et al., 2013). In addition, the phenolic compounds in WGO reveal an antioxidant influence that could decrease the pro-oxidative state and give a potent antioxidant protection to different organs in the body (Durak et al., 2010). Taken together, we could speculate that WGO exerted a therapeutic effect on CCl₄-induced acute renal injury in mice, possibly through its antioxidant action.

**CONCLUSION**

This study provides a model of kidney damage similar to that occurs in renal dysfunction patients and brings the possibility to recover CCl₄-induced nephrotoxicity using WGO, the natural anti-oxidant agent, through its marked antioxidant activity coupled with favorable anti-apoptotic effects, that enhancing the function of the kidney and counteract the toxicity action of renal free radicals induced by deleterious agents by upregulation of oxidant defense system.

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