PROTECTIVE EFFECTS OF GRAPE SEED OIL AGAINST CCl₄ INDUCED OXIDATIVE STRESS IN RAT BRAIN
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ABSTRACT

This study aimed to investigate the possible beneficial effects of grape seed oil (GSO) on CCl₄-induced acute brain toxicity in rats. The animals injected with acute dose of CCl₄ (2 ml / kg b. wt.) showed a statistical significant decrease in the blood hematological parameters (WBCs, RBCs, PLT counts, Hb and MCV values), and a significant elevation in serum TNF-α, and IL-6 levels. In addition, damage of the brain DNA, disturbance of the brain antioxidant status; a significant decrease in SOD, GSH-Px and Catalase (CAT) activities as well as GSH level, accompanied with a significant elevation in MDA and NO levels, as well as a high significant elevation in xanthine oxidase and inducible nitric oxide synthase (iNOS) gene expressions have been observed due to the oxidative stress produced after CCl₄ injection. The pretreatment of GSO exerted significant ameliorated the hematologic parameters, and serum TNF-α and IL-6 levels, protected DNA damage, improved SOD, GSH-Px and CAT activities as well as GSH, MDA and NO levels and down-regulation of xanthine oxidase (XO) and inducible nitric oxide synthase (iNOS)gene expression levels in the brain tissues of CCl₄ injected rats. These findings suggest that GSO prevents acute brain damage due to CCl₄ toxicity, which could be attributed to its immuno-modulation and antithrombotic, antiapoptotic, antioxidant, and anti-inflammatory activities. It can be suggested that GSO which containing high level of polyphenolic compounds, essential fatty acids and vitamin E (Tocopherol) revealed to protect the brain from CCl₄ toxicity and/or any other toxicant cause oxidative stress. Keywords: Grape Seed Oil (GSO), carbon tetrachloride (CCl₄), brain damage, antioxidants, cytokines, xanthine oxidase, inducible nitric oxide synthase (iNOS) relative gene expression.

INTRODUCTION

Carbon tetrachloride (CCl₄) is a once-popular industrial that is now strictly regulated in many countries. Acute administration of a large dose of CCl₄ causes severe necrosis, while chronic administration of lower doses is frequently used to induce hepatic fibrosis (Jaeschke et al., 2013). It has been well established that CCl₄ is metabolized in the liver to the highly reactive trichloromethyl radical (CCl₃) and/or CCl₃OO and these free radicals lead to auto-oxidation of the fatty acids present in the cytoplasmic membrane phospholipids and causes functional and morphological changes in the cell membrane (Weber et al., 2003). The lipid solubility of CCl₄ renders it readily available to cells. Hence, it is deposited and mediates injury in several organs, including the brain (Sanzgiri et al., 1997; Basu, 2003 and Karadeniz et al., 2007). Elevated lipid-peroxides (LPO) can lead to oxidative stress when the antioxidant defense system is suppressed. This is particularly
important in the brain that rely its function mainly on aerobic metabolism, in conjunction with its high content of unsaturated lipids which renders the brain highly susceptible to peroxideative damage (Halliwell, 2001; Halliwell, 2006 and Li et al., 2013).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems (Valko et al., 2004 and Lee et al., 2012). Beneficial effects of ROS/RNO involve physiological roles in cellular responses in defense against infectious agents and in the function of a number of cellular signaling systems. One further beneficial example of ROS/RNO at low concentrations is the induction of a mitogenic response. In contrast, at high concentrations, ROS/RNO can be important mediators of oxidative/nitrosative damage to cell structures, including lipids and membranes, proteins and nucleic acids (Poli et al., 2004). The harmful effects of ROS/RNO are balanced by the antioxidant action of non-enzymatic antioxidants in addition to antioxidant enzymes (Halliwell, 1996a).

The Grape seed oil (GSO) contains high amounts of essential fatty acids such as, linoleic acid (69-78%), palmitic acid (5-11%), oleic acid (15-20%), and stearic acid (3-6%), as well as a high amount of phenolic compounds, including gallic acid, catechin, epicatechin and procyanidins, also, a very high level of antioxidant vitamin E (60–120 mg/100 g), which makes the oil very stable, and its antioxidant property and biological activity are 50 times greater than that of vitamins E and C (Natella et al., 2002; Bail et al., 2008; Maier et al., 2009 and Mokhtari et al., 2011). The potent antioxidant property is claimed to be the protective mechanism of GSO (Bagchi et al., 2002, 2003; Uma Maheswari and Rao 2005).

This study aimed to investigate the acute toxicity of CCl₄ in rats’ brain. Furthermore, evaluate the pretreatment protective effects of the dietary GSO on the brain activities.

**MATERIALS AND METHODS**

**Chemicals:**

The GSO, CCl₄ and the all other chemicals and reagents used in this study were of high analytical grade and purchased from Sigma-Aldrich Chemical Co., (USA) (Nasr City, Cairo, Egypt).

**Animals**

Female Wistar rats (weighing 100–120 g) were obtained from the Nile pharmaceutical Co. Cairo, Egypt. Upon arrival, the animals were allowed to acclimatize for one week before starting the experiment, and fed on a standard pellet diet and drinking water *ad libitum*. They were housed at the animal facility at the National Centre for Radiation Research and Technology, at a temperature of 25 °C and humidity of 60 ± 5%. The study was conducted in accordance with international guidelines for animal experiments and approved by the Ethical Committee at the National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt.
Experimental design:

Rats were randomly divided into four groups (n=6). Group I (Control group): untreated control group. Rats of this group were orally administered with an equivalent volume of water during the period of GSO administration. Rats in group II (GSO group): 3.5 g/kg body weight (≈ 4 ml / Kg b. wt. according to oil density = 0.87 gm/ml at 25°C) GSO (Uma Maheswari and Rao 2005), were orally administered to the rats by gastric intubation once daily for seven consecutive days. In group III (CCl₄ group): rats of this group were orally administered with an equivalent volume of distilled water during the period of GSO administration once daily for seven consecutive days. Then they were administered 50% CCl₄ intra-peritoneal (IP) (acute single dose; 2 ml /kg b. wt., 50% CCl₄ was prepared in olive oil) (Cho et al., 2013). In group IV (GSO/CCl₄ group): rats of this group were orally pretreated with GSO (3.7 g/kg b. wt.) by gastric intubation every day for 7 days, then, after 2 hours of the last dose of GSO, animals were administered CCl₄ intraperitoneal as group II. After 16 hours of CCl₄ administration (Cho et al., 2013), rats were fasted overnight, anesthetized by light ether and the blood was collected from the eye of each animal in a glass tube, then, allowed to clot for 30 min at 25°C, centrifuged at 4000 xg and sera were separated for the pro-inflammatory cytokines, TNF-α, IL-6 assays and biochemical parameters determination. Another part of blood was collected in EDTA-containing tube for determination of hematologic parameters. The brain was excised immediately and immersed in physiological saline, dried on filter paper then stored at -20°C.

Determination of hematologic indices and biochemical blood parameters:

The peripheral blood parameters: red blood cells (RBCs) count, platelets (PLT) count, hemoglobin (Hb) concentration, and hematocrit (HCT) levels were determined using Automated Hematology Analyzer (XT-2000i, Sysmex Corporation, KOBE, JAPAN). Biochemical parameters were determined using a Biochemical Blood Analyzer (ALFA WASSERMANN DIGNOSTIC TECHNOLOGIES, LLC, ACE, Alere, USA).

Determination of Iron concentration in the brain tissues:

The brain tissues of different groups were digested in a boiling mixture of conc. HNO₃ and H₂O₂ (5:1 v/v) until complete digestion of the organic materials using Milestone MLS-1200 Mega, High Performance Microwave Digester Unit, Italy. Iron concentrations were estimated in the prepared samples using Atomic absorption spectrophotometer (Thermo Scientific, iCE 3000, AA05130901 v1.30 England).

DNA Fragmentation Analysis

For determination of genomic DNA fragmentation, rat brains were rapidly removed, washed, and homogenized. The homogenized tissue transferred to a centrifuge tube with extraction buffer (10 mmol/L Tris-HCl (pH 8.0), 0.1 mol/L EDTA (pH 8.0) and 0.5% SDS, then incubated for one hour at room temperature and then digested in the same buffer with 200 μg/ml proteinase K (Sigma) at 50°C overnight. An equal volume of phenol
equilibrated with 1 mol/L Tris buffer (pH 8.0) was then added, and the tube was placed on a roller apparatus for 1 hour. After the two phases were separated by centrifugation at 1500 g for 30 minutes at room temperature, the viscous aqueous phase was transferred to a clean tube, and the extraction was repeated with an equal volume of phenol/chloroform. After the second extraction, the aqueous phase was separated and the DNA precipitated by the addition of 0.1 vol 3 mol/L sodium acetate and 2 vol absolute ethanol. DNA precipitate was collected by centrifugation at 15000 g for 20 minutes at room temperature, rinsed with 70% ethanol, and finally resuspended in 0.5 ml extraction buffer in a 1.5-ml microcentrifuge tube until dissolved. To detect DNA fragmentation, 10 μl of each DNA was electrophoretically fractionated on 1.5% agarose gel with 0.5 μg/ml ethidium bromide then visualized and photographed under UV light (Okamura et al., 2000).

**Determination of antioxidants and oxidative stress parameters in brain homogenate:**

The brain was weighed and homogenized (10%) in chilled 50 mmol phosphate buffered saline (pH 7.4), centrifuged at 4000 g, 4°C for 15 minutes, using universal centrifuge (16R, Germany), then the supernatants were used for the determination of the following parameters:

- SOD activity was determined according to Nishikimi et al. (1972). The assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye, which was followed photometrically at 560 nm. The enzyme activity expressed as U/g wet tissue.
- Glutathione-peroxidase (GSH-Px) activity was measured according to Rotruck et al. (1973) that based on indirect determination of GSH-Px, whereas GSH-Px react with known amount of GSH, then the residual glutathione reacted with DTNB (dithionitrobenzoic acid). The color developed was read at 412 nm. The enzyme activity expressed as μ mol of GSH oxidized/g wet tissue/min. Catalase (Cat) activity was assessed according to Aebi (1984). Catalase reacts with a known quantity of H₂O₂ in the presence of horseradish peroxidase (HRP), remaining H₂O₂ react with 3, 5-Dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity measured at 510 nm, which is inversely proportional to the amount of catalase in the original sample. The enzyme activity expressed as U/g wet tissue.
- Glutathione (GSH) concentration was measured according to Beutler et al. (1963) using 5,5'-dithionitrobenzoic acid (DTNB) and expressed as mg/g wet tissue. Lipid peroxides in terms of malondialdehyde (MDA) were measured according to the method of Satoh (1978), using 1, 1, 3, 3-tetraethoxypropane as a standard. MDA concentration expressed as nmol/g wet tissue. Nitric Oxide (NO) determined as nitrite concentration. The method used depends on Griess reactions which convert nitrite into a deep purple azo-compound which photometrically measured at 540 nm according the method of Montgomery and Dymock (1961). NO concentration expressed as μmol/g wet tissue.
Detection of xanthine oxidase (XO) and inducible nitric oxide synthase (iNOS) relative gene expressions by reverse transcription polymerase chain reaction (RT-PCR) (Li et al., 2012):

For the detection of XO and iNOS, RNA was isolated, reverse transcribed into cDNA, and amplified by PCR. About 30 mg of brain tissues was homogenized and then centrifuged at 14000 x g for 10 min. The supernatant was then examined for detection of XO and iNOS expression.

RNA extraction:

RNA was extracted from tissue homogenate by using SV-total RNA isolation system (Promega, Madison, USA) according to the manufacturer's recommendation. The extracted RNA sample was dissolved in Ribonuclease (RNase) - free water and RNA concentration and purity were determined by measurement of absorbance at 260 nm/280 nm, the isolated RNA has an A260/280 ratio of 1.9–2.1. The integrity of the RNA was studied by gel electrophoresis on a 1.2% agarose gel, containing ethidium bromide.

cDNA Synthesis by RT-PCR:

About 5 μg of RNA was reverse transcribed by using 12.5 μL of oligonucleotide primer (oligo(dT)12-18 primer) in a total volume of 0.2 μmol/L, and was denatured at 70°C for 2 min. The denatured RNA was placed on ice for 5 min and 6.5 μL of reverse transcription mixture [containing 50 mmol/L Tris HCl, pH 8.5, 50 mmol/L KCl, 3 mmol/L MgCl2, 0.5 mmol/L of dNTPs, 10 mmol/L dithiothreitol (DTT), 1 U/μL RNAse inhibitor, and 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase] was added. Then the reaction tube was placed at 42°C for 1 h followed by heating to 92°C to stop the reaction. The PCR was performed by adding the PCR mix to about 5 μL of single strand complementary DNA (cDNA). The PCR mix contained 10 mmol/L Tris HCl pH 8.3, 50 mmol/L KCl, 100 mmol/L dNTPS, and 2.5 U of tag polymerase, and about 10 μmol/L of each of sense and antisense primers. Specific PCR primer sequences of XO, iNOS and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is represented in Table 1. The PCR cycling conditions were 94°C for

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Xanthine oxidase</td>
<td>Forward: 5'-CGC AGA ATA CTG GAT GAG CGA GGT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCG GTG GGT TTC TTCTTCTTG AAC-3</td>
</tr>
<tr>
<td>iNOS</td>
<td>Forward: 5'-GGG CCA CCT TTA TGT TTG TG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCGGTGGGTTCTTCTTCTTG AAA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-AGA AGG CTG GGG CTC ATT TG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AGG GGC CAT CCA CAG TCT TC-3'</td>
</tr>
</tbody>
</table>

1 min for denaturation followed by 57°C for 1 min and 72°C for 45 s; for 40 cycles with final extension at 72°C for 12 min.

Gel electrophoresis:

10 μL of PCR product was analyzed on 2% agarose gel with ethidium bromide staining and the product was visualized on ultraviolet
transilluminator, then gel documentation was performed. PCR products were semi-quantified by using a gel documentation system (Bio Doc Analyze) supplied by Biometra, Germany. The relative expression of the studied genes was calculated using the comparative threshold cycle method. All values were normalized to the GAPDH genes (Livak and Schmittgen, 2001).

**Determination of serum Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6) levels**

The separated sera were used for the determination TNF-α and IL-6 using an ELISA kits for rat (Glory Science Co., Ltd, USA). The measurements were done according to the catalogue instruction guidelines. The cytokine levels were calculated after plotting the standard curves and expressed as pg/ml.

**Statistical Analysis**

All statistical analyses were conducted by using the SPSS statistical package for Windows Version 15.0 (SPSS Software, Chicago, IL) according to Greasley 2008. The results for continuous variables were expressed as mean ± standard error or by one-way analysis of variance (ANOVA). P values less than 0.05 (P<0.05) were considered statistically significant.

**RESULTS**

**Hematologic parameters**

Table 2 showed the hematological index results, white blood cells count (WBC), red blood cells count (RBC), platelet count (PLT), hemoglobin (Hb), hematocrit (HCT), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) levels in the experimental rats. GSO treated animals showed non-significant difference (P>0.05) as compared to the healthy control rats in WBCs, RBCs count, Hb, HCT%, MCV, MCH and MCHC values, but showed significant difference (P<0.05) in PLT count. In contrast, CCl4 treated rats (intoxicated control) showed significant decrease (P<0.01) in WBCs, RBCs, PLT count, Hb, MCV and MCH levels, but non-significant difference in HCT and MCHC values as compared to healthy control group. However, pretreatment of GSO for seven days showed amelioration in the harmful of these hematologic parameters as compared with intoxicated control rats.

**Determination of iron concentration in serum and brain tissues:**

Fig. 1 showed the concentrations of iron in serum and brain tissues of the different experimented groups. Iron concentration showed significant decrease (P<0.05) in serum but significant increase (P<0.05) in brain tissues after CCl4 injection as compared to health control group. Non-significant difference was observed in serum or brain iron concentrations in GSO treated group. However, total iron level is significantly elevated in serum due to CCl4 injections were significantly reduced in brain tissues due to pretreatment of GSO.

**DNA fragmentation**

The DNA fragmentation pattern was monitored in the experimental rats brain homogenates by agarose gel electrophoresis (Fig. 2). CCl4-treated
group showed strand breaks/ streaking of the DNA (as opposed to low molecular weight bands of the DNA of 100 bp, specific to apoptosis) which was absent in DNA isolated from brain homogenates in both health control and GSO treated groups that showed the presence of undamaged DNA.

Table 2: Effect of GSO on the hematological parameters in the experimental rats

<table>
<thead>
<tr>
<th>Group</th>
<th>WBCs (x 10^3/ μL)</th>
<th>RBCs (x 10^6/ μL)</th>
<th>PLT (x 10^3/ μL)</th>
<th>Hb (g/dL)</th>
<th>HCT (%)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control</td>
<td>16.67 ± 0.30</td>
<td>8.042 ± 0.21</td>
<td>865.33 ± 14.16</td>
<td>15.33 ± 0.47</td>
<td>40.48 ± 1.06</td>
<td>50.45 ± 1.49</td>
<td>19.08 ± 0.49</td>
<td>37.87 ± 0.28</td>
</tr>
<tr>
<td>2 GSO</td>
<td>16.28 ± 0.24</td>
<td>8.012 ± 0.19</td>
<td>821.83 ± 6.42</td>
<td>15.17 ± 0.32</td>
<td>41.20 ± 0.64</td>
<td>51.57 ± 1.36</td>
<td>18.95 ± 0.39</td>
<td>36.87 ± 0.96</td>
</tr>
<tr>
<td>3 CCl₄</td>
<td>7.60 ± 0.26</td>
<td>6.458 ± 0.19</td>
<td>578.17 ± 17.16</td>
<td>13.10 ± 0.36</td>
<td>39.80 ± 3.60</td>
<td>61.30 ± 4.08</td>
<td>20.30 ± 0.47</td>
<td>33.75 ± 1.30</td>
</tr>
<tr>
<td>4 GSO-CCl₄</td>
<td>11.84 ± 0.46</td>
<td>7.922 ± 0.23</td>
<td>738.17 ± 7.08</td>
<td>14.93 ± 0.32</td>
<td>40.67 ± 0.93</td>
<td>51.43 ± 1.09</td>
<td>18.87 ± 0.29</td>
<td>36.75 ± 0.37</td>
</tr>
</tbody>
</table>

The results were expressed as mean ± SE

WBC: Total white blood cells. RBC: Red blood cell count. HGB: Hemoglobin. HCT: Hematocrit. MCV: Mean cell volume. MCH: Mean cell hemoglobin. MCHC: Mean cell hemoglobin concentration. PLT: Platelet count.

* non-significant to control, at P>0.05, ** Significant to control at P<0.05, *** Significant to CCl₄ at P<0.05, **** non-significant to CCl₄ at P>0.05
Fig. 1. Serum iron (Fe) Concentration of different experimental groups.

- Non-significant difference in comparison with control rats, at $P > 0.05$.
- Significant difference in comparison with control rats at $P < 0.05$.
- Significant difference in comparison with CCl$_4$ intoxicated rats at $P < 0.05$.

Fig. 2. Agrose gel electrophoresis of DNA isolated from brain homogenate. Lane M: DNA marker 100 pb, Lane 1: Normal, Lane 2: GSO, Lane 3: CCl$_4$, Lane 4: GSO/CCl$_4$.

The Antioxidant status of brain tissues:

The effects of CCl$_4$ on the endogenous antioxidant status are shown in Table 3. Administration of CCl$_4$ induced significant decrease in the brain SOD, GSH-Px, CAT activities as well as GSH content accompanied with a significant increase in MDA and NO levels (Table 3) as compared to control group. Pretreatment of GSO (3.7 g/kg body weight) for 7 consecutive days before CCl$_4$ injection resulted in an improvement in the activity of brain SOD, GSH-Px and CAT activities as well as GSH level ($P < 0.05$) accompanied by a significant decrease ($P < 0.05$) in MDA and NO levels.

XO and iNOS relative gene expressions levels in brain tissues:

To investigate the protective mechanism of the GSO on CCl$_4$ intoxicated rats, the level of XO and iNOS relative gene expressions were estimated in the brains of different treated groups (Fig. 3. and Fig. 4.).
results showed that CCl₄ treatment significantly (P<0.01) increased the levels of XO and iNOS by 7.05 and 9.13 folds, respectively, as compared to control group. However, the treatment of GSO (3.7 g/kg b. wt.) improved the relative gene expression levels of XO and iNOS, to 2.88 and 7.08 folds, respectively, in brain of intoxicated rats as compared to control animals. However, a non-significant difference (P>0.05) in the relative XO and iNOS gene expressions as well as in the antioxidant enzymes GSH-Px and CAT activities as well as GSH, MDA, and NO levels in brain tissues were observed in GSO pretreated health rats as compared to control group.

**Serum TNF-α and IL-6 levels:**

As shown in Fig. 5, significant increases (P<0.05) in the serum TNF-α and IL-6 were observed in intoxicated group as compared with the corresponding values of control rats. The pretreatment of GSO into intoxicated animals, significantly decreased the elevation in serum TNF-α and IL-6 levels as compared to the CCl₄ intoxicated group (P<0.05).

| Table 3: Effect of GSO on SOD, GSH-Px and CAT activities as well as GSH, MDA and NO levels in the brain tissues of the experimental rats |
|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| **Group**     | **SOD U/g wet tissue** | **GSH-Px μ mol GSH/g wet tissue/min** | **CAT U/g wet tissue** | **GSH mg/g wet tissue** | **MDA nmol/g wet tissue** | **NO μmol/L** |
| 1 Control     | 9.89 ± 0.33           | 9.79 ± 0.42            | 26.66 ± 0.41           | 9.23 ± 0.192           | 143.61 ± 3.602           | 7.77 ± 0.349   |
| 2 GSO         | 10.37 ± 0.16          | 9.74 ± 0.32            | 26.39 ± 0.35           | 10.53 ± 0.141          | 149.48 ± 3.214           | 7.58 ± 0.379   |
| 3 CCl₄        | 7.08 ± 0.32           | 6.59 ± 0.17            | 15.53 ± 0.80           | 7.08 ± 0.315           | 257.44 ± 7.458           | 26.52 ± 0.758  |
| 4 GSO/CCl₄    | 9.46 ± 0.37           | 9.29 ± 0.12            | 24.37 ± 0.24           | 9.46 ± 0.153           | 153.56 ± 3.729           | 17.80 ± 0.379  |

The results were expressed as mean ± standard error (Mean ± SE).  
*a* non-significant to control group, at P>0.05.  
*b* significant to control group at P<0.05.  
*c* significant to intoxicated group at P<0.05.

![Fig. 3: Real-time PCR of Xanthin Oxidase (XO) gene expression of different experimental groups relative to housekeeping gene.](image)

*a* non- Significant difference in comparison with control rats, at P > 0.05.  
*b* Significant difference in comparison with control rats at P < 0.05.  
*c* significant difference in comparison with CCl₄ intoxicated rats at P<0.05.
Fig. 4: Real-time PCR of iNOS gene expression of different experiential groups relative to housekeeping gene.

- non- Significant difference in comparison with control rats, at $P > 0.05$,
- Significant difference in comparison with control rats at $P < 0.05$,
- Significant difference in comparison with CCl$_4$ intoxicated rats at $P<0.05$.

Fig. 5: Serum TNF-α and IL-6 levels of different treated groups.

- non- Significant difference in comparison with control rats, at $P > 0.05$,
- Significant difference in comparison with control rats at $P < 0.05$,
- Significant difference in comparison with CCl$_4$ intoxicated rats at $P<0.05$.

DISCUSSION

Brain is the highest sensitive organ to oxidative stress due to its high O$_2$ utilization rate, high iron content, polyunsaturated fatty acid content, and low antioxidant capacity as compared to that of other organs (Halliwell, 2001 and Madrigal et al., 2006). Carbon tetrachloride (CCl$_4$) is a well-known environmental biohazard, which can cause free radical generation and toxicity in different tissues, including the brain (Karadeniz et al., 2007 and Ozturk et al., 2003). Detoxification of reactive oxygen species is one of the prerequisites of aerobic life and many defenses have evolved, providing an important antioxidant defense system of prevention, interception, and repair consisting of non-enzymatic scavengers and quenchers, as well as enzymatic systems, including superoxide dismutase and hydroxyl-peroxidases, such as glutathione peroxidase, catalase, and other hemoprotein peroxidases can
become pivotal in antioxidant defense (Sies, 1991). Early studies used 2 ml / Kg b. wt. of CCl₄ (prepared with olive oil 1:1 v/v; ip) used to evoke acute hepatotoxicity (Cho et al., 2013). Whereas, 4 ml / Kg b. wt. of GSO; administered orally that were used as anti hepatotoxicant (Uma Maheswari and Rao 2005). In this study, the protective effect of the mentioned dose of GSO was investigated against the acute dose of CCl₄ that could induce brain damage in rats.

Intra-peritoneal administration of 2 ml CCl₄ /kg b. wt. (Cho et al., 2013) greatly affected the hematological parameters. It cause hematotoxicity occurred as a significant decreases in WBCs, RBCs and PLT counts, Hb, MCV and MCH values. The observed depletion in the RBCs count along with the Hb level is similarly consistent with previous reported of anemia in CCl₄-treated experimental animals (Mortiz and Pankow, 1989 and Adaramoye and Akinloye, 2000). This depletion in RBCs count and Hb level leads to iron deficiency anemia which is characterized by a micro-cytichypochromic blood picture, also hyperactivity of bone marrow, which leads to production of red blood cells with impaired integrity that are easily destroyed in the circulation this could be another reason for decreasing hematological values (Tung et al., 1975 and Ballinger, 2007). As seen in Fig.1, the total iron level in serum of CCl₄-intoxicated rats showed significant decrease that confirm that animals exhibited iron deficiency anemia due to CCl₄-toxicity. Moreover, in the present work, animals exhibited increase in brain iron concentration. The increase of iron levels which were reported in the present study agree with the finding of Wood et al., 2004 and showed that the toxic free radical types are superoxide radical anion(O₂⁻), the presence the latter in high amount leads the releasing of free iron circulatory system because (O₂⁻) attack to ferritin.

The depression in RBCs count and Hb level recorded in the present study could be attributed to disturbed hematopoiesis, destruction of erythrocytes, and reduction in the rate of their formation and / or their enhanced removal from circulation due to CCl₄ toxicity. Also treatment with CCl₄ induced marked leucopenia, as reported previously, exposure to CCl₄ induced decrease in leukocytes count in peripheral blood of experimental animals (Jirova et al. 1996 and Mandal et al., 1998). The present study showed that the pretreatment of GSO could improve these hematologic parameters in animals treated with CCl₄, this protective and improvement effect might be attributed to the antioxidant nature, immuno-modulation and antithrombotic activity of GSO.

DNA fragmentation was markedly observed in CCl₄ intoxicated group as a strand break (Fig.2). The DNA strand break consists of base modifications and the DNA lacking a base (Liu et al., 1996; Chen et al., 1997; Cui et al., 1999 and Huang et al., 2000). The metabolism produces trichloromethyl radicals that can bind to proteins and DNA could cause direct damage to these macromolecules (Weber et al., 2003). DNA fragmentation also, could be attributed to irreversible cell death, apoptosis or necrosis in in the nuclear DNA exposed to CCl₄ (Chen et al., 1997; Lu et al., 2012 and Lee et al., 2012). DNA fragmentation can be activated by proteases (Liu et al.,
Free radicals are known to damage proteins, lipids, and nucleic acids. The enzymes involved in the cell defense against oxygen cytotoxicity have been repeatedly proposed to be superoxide dismutases, catalase and peroxidases. Superoxide dismutases catalyze the dismutation producing hydrogen peroxide (H$_2$O$_2$) whereas catalase or peroxidases (essentially glutathione peroxidase in animal cells) remove it (Mavelli et al., 1982). The result of the present study indicates that CCl$_4$ could inhibit the antioxidant enzymes; SOD, catalase, and GSH-Px activities. In the present study, the decrease in brain SOD activity that observed in CCl$_4$-intoxicated rats could be attributed to the adaptive responses; where SOD play a key role in protecting cells against oxidative stress damage (Halliwell et al., 2001; and 2006). H$_2$O$_2$ was removed through its reduction to water and molecular oxygen, this reaction is catalyzed by catalase and / or GSH-Px. The observed decline in catalase and GSH-Px activities in the brain of CCl$_4$-intoxicated rats is evident that their ability to detoxify H$_2$O$_2$ after administration of CCl$_4$, however, the accumulation of H$_2$O$_2$, inhibits their activities. The resistance of many cells against oxidative stress is associated with high intracellular levels of the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine; GSH), the major non-enzymatic antioxidant (Navarro et al., 1999). GSH acts directly as a free radical scavenger by neutralizing the hydroxyl radical (HO•), restores damaged molecules by hydrogen donation, reduces peroxides, and maintains protein thioles in the reduced state (Sies, 1986). The significant reduction in the brain GSH level was observed in the present study (Table 3), as compared to the control group, could be attributed to an enhanced utilization in large amount to combat the CCl$_4$-induced free radical damage. Moreover, glutathione peroxidase (GSH-Px) acts in conjunction with GSH, which is present in cells in high (micromolar) concentrations, to decompose H$_2$O$_2$ or an organic peroxide (ROOH) to water / or alcohol while simultaneously oxidizing GSH. Significantly, GSH-Px competes with catalase for H$_2$O$_2$ as a substrate and is the major source of protection against low levels of oxidative stress (Valko et al., 2006). The significant decrease in brain GSH-Px activity of CCl$_4$-intoxicated rats could be attributed to its inactivation by lipid peroxidation (LPO) by products (Sies, 1991). Also, as GSH is the substrate of

1997 and Enari et al., 1998) or by neuronal nitric oxide synthase (Huang et al., 2000; Yoshida et al., 1994 and O’Neill et al., 1996). Additionally, evidence suggests that reactive oxygen species, most likely nitric oxide, superoxide ions, and hydroxyl radicals, mediate the nucleic acid damage, which is referred to as oxidative DNA damage. (Epe et al., 1996; Liu et al., 1996; Beckman and Ames, 1997; Cui et al., 1999 and 2000 and Huang et al., 2000). The pretreatment of GSO exerts a significant protection on DNA damage. In this study that might be attributed to its scavenging of ROS, its antioxidant and its anti-apoptotic activities. Grape seed extract (GSE) showed neuroprotective effects achieved by inhibiting DNA damage (Hwang et al., 2004 and Balu et al., 2006). In addition, procyanidins, catechin and gallic acid were reported to be good cellular preventive agents against DNA oxidative damage and apoptosis via induction of endogenous antioxidant enzymes (Bagchi et al., 1998a; and 1998b; Du et al., 2007 and Morin, et al., 2008).
GSH-Px and required for its catalysis, the decrease in GSH concentration could be contributed to the depletion of brain GSH-Px activity, whereas the depletion of glutathione in vitro and in vivo is known to cause inhibition of glutathione peroxidase activity and has been shown to increase lipid peroxidation (Anundi, 1979 and Reiter and Wendel, 1982). The enhanced lipid peroxidation expressed in terms of MDA contents and reduction in the brain GSH level in CCl₄ intoxicated rats as observed in the present study indicated the damage of the brain cells which is confirmed by the earlier reports (Karadeniz et al., 2007). The cleavage of CCl₄ leads to the formation of highly unstable free radicals (\(^{•}\)CCl₃ or \(^{•}\)CCl₃O₂) which initiated peroxidation (Recknagel et al., 1989). The differences in oxidant production and the levels of LPO products observed in the brain may be attributed to the differences in their iron content which influence the generation of reactive oxygen species. Certain brain regions like cortex, striatum and hippocampus are highly enriched with non-heme iron, which is catalytically involved in the production of free radicals (Schenck and Zimmerman 2004 and Zecca et al., 2004). The peroxidation of membrane phospholipids eventually leads to loss of membrane integrity and, finally, to cell death. Intra peritoneal administration of CCl₄ in rats induces LPO and oxidative protein damage in their brain (Dani et al., 2008) and increased the LPO index in liver, kidneys, heart and blood serum (Botsoglou et al., 2008). These changes result in modulation of the enzymatic antioxidant defenses of the tissues (Maier et al., 2009 and Lavrentiadou et al., 2013). The current data display that CCl₄-induced elevation the lipid peroxidation (MDA)and NO levels in the rat brain, which were associated with a decrease in the activities of antioxidant enzymes. This indicates the acute toxic effect of CCl₄ in the brain tissue. Similar results of CCl₄-induced brain toxicity were reported by Szymonik-Lesiuk et al. (2003) and Karadeniz et al. (2007) whereas, the activities of SOD, CAT, GSH-PX and level of GSH were diminished, accompanied by elevated levels of MDA and NO. Both oxidative and nitrosative stresses have been reported to alter lipids and proteins (Yao and Keshavan, 2011). In addition to the phospholipid-rich composition of the brain, the lack of neuronal regeneration renders the brain susceptible to oxidative/nitrosative stress (Yao and Keshavan, 2011 and Lee et al., 2012). Nitric oxide, a free radical of oxygen, appears to increase in brain due to CCl₄ intoxicated rats in the present study. Reactive nitric oxide may combine with superoxide ion to form peroxynitrite, which generates 3-nitrotyrosine in protein. Peroxynitrite is also known to initiate lipid peroxidation, cause direct or indirect oxidative damage in nucleic acidpromote apoptosis (Epe et al. 1996; Halliwell, 1996b; Cui et al., 1999; Huang et al., 2000; Pacher et al., 2007 and Lee et al., 2012). The pretreatment of GSO showed significant amelioration of harmful in SOD, CAT and GSH-Px activities as well as the levels of GSH, MDA and NO. Pretreatment of GSO to CCl₄ intoxicated rats eventually resulted in a fall in peroxidative levels, which highlight the antioxidant property of GSO. The protective effect of GSO against CCl₄-induced oxidant production and LPO is correlated with the direct scavenging activity towards peroxyl radicals both in...
the membrane and in the aqueous phase. Grape seed extract reduced the incidence of free-radical-induced lipid peroxidation in the central nervous system of aged rats and reduced hypoxic ischemic brain injury in neonatal rats (Feng et al., 2005), could reduce reactive oxygen species production, which may be related to the enhancement of the antioxidant status in the central nervous system (Balu et al., 2006). The antioxidant activity of GSO towards hydroxyl radicals is considered to be due to its high amounts of essential fatty acid, which is essential for the production of prostaglandins. The antioxidant activity of the GSO is attributed to its high polyphenols; galic acid, catechin procyanidins and vitamin E contents (Natella et al., 2002; Busserolles et al., 2006; Bail et al., 2008; Maier et al., 2009 and Mokhtari et al., 2011).

Moreover, nitric oxide (NO) is synthesized from L-arginine by a family consisting of NO synthase (NOS) isoenzymes: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). nNOS and eNOS are constitutive enzymes activated by the increase in intracellular Ca$^{2+}$ (Pentyala et al., 1994). The iNOS is expressed calcium-independently by inflammatory cells induced by endotoxic or pro-inflammatory cytokines (Zhou and Zhu, 2009). Therefore, inflammation or neuronal excitation leading to increased intracellular Ca$^{2+}$ may enhance the production of NO and evoke apoptosis (Lee et al., 2012). Additionally, xanthine oxidase is a key enzyme in purine metabolic pathway, catalyzing the oxidation of hypoxanthine to xanthine then to uric acid, liberating superoxide radicals and hydrogen peroxide molecules, so it is a critical source of reactive oxygen species (ROS) in inflammatory disease (Massey et al., 1969; Lacy et al., 1998; Srivastava and Kale, 1999; Fatokun et al., 2007 and Kelley et al., 2010). It is well known that the XO (or its interconvertible and the predominant intracellular isofom: xanthine dehydrogenase-XDH), has been implicated in the generation of the toxic reactive oxygen species (ROS) in a variety of ischemic, neurodegenerative, and inflammatory conditions (Okuda et al., 1996; Harrison, 2002 and Berry and Hare, 2004). In addition, although lower concentrations of free radicals may be beneficial in endothelial adaptation to ensure vasomotion control, their higher concentrations may induce several intracellular pathways such as phosphatases and transcription factors e.g. NF-κB to disrupt endothelial integrity by producing other potent ROS like the hydroxyl radical via Fenton-reaction (Kvietys et al., 1989). NF-κB is activated by oxidative stress induces overexpression of the pro-inflammatory cytokines such as TNF-α, IL-1βand IL-6 (Umezawa et al., 2000 and Lee et al., 2010). Consequently, to investigate the protective mechanism of GSO against CCl$_4$-induced brain acute toxicity in rats, relative gene expression levels of xanthine oxidase (XO) and iNOS in rats’ brain as well as serum concentrations of TNF- and IL-6 were determined in the present study.

The results demonstrated that a significant increase in the relative level of XO gene expression is observed in the present study. As well as, the levels of TNF-α and IL-6 were consistently found to be elevated in CCl$_4$ intoxicated rats than in controls. The increment of the relative gene expression levels of XO and iNOS has been suggested to be a ROS/RNS generating mechanism by CCl$_4$ intoxication. The polyphenolic components,
catechin and procyanidins, could strongly and non-competitively inhibit xanthine oxidase activity, scavenging free radicals and prevent ROS accumulation and cell apoptosis (MaffeiFacino et al., 1994 and Du et al., 2007). Cytokines play important roles during inflammation, they are signaling molecules that mediate inflammation and immune response and have many cellular functions and affect tissue homeostasis (Ao et al., 2009 and Saleh et al., 2013). It is obvious that the pre-treatment of GSO significantly inhibit XO, iNOS expression and strengthen the anti-inflammatory response towards CCl4 toxicity by decreasing their levels. Consequently, GSO significantly reduced the NO production by attenuated iNOS expression, and enhanced the antioxidant status of brain tissues in CCl4 injected rats. These results could be attributed to that XO activity could be attenuated by vitamin E and the phenolic fractions of GSO that may be effective in controlling some mediators of immune response associated with increased production of NO via the effect on XO activity and its production of superoxide anion as well as uric acid (Kahl and Elsasser, 2004). Moreover, GSE decreased the progression of inflammation by down-regulating the iNOS expression (Zhou et al., 2011). The polyphenol-rich GSE may be useful for the inhibition or prevention of inflammatory processes via NF-κB activation (Gessner et al., 2012).

In conclusion, the results of the present study showed that CCl4 exerts hemato- and neurotoxicity in the experimental animals. CCl4 (2 ml / kg b. wt.) showed a statistical significant decrease in the blood hematological parameters (WBCs, RBCs, PLT counts, Hb and MCV values), which are ameliorated by the pretreatment of GSO. However, the results suggested that CCl4 could exert neurotoxicity by induction of XO and iNOS activities then enhanced ROS/RNO. Thus, the brain exhibited an extent of oxidative damage upon exposure to the acute dose of CCl4 that attributed to inhibition of antioxidant enzymes and elevation of GSH, MDA and NO levels accompanied by elevation of serum TNF-α and IL-6. The pretreatment of rats with GSO protected the DNA from oxidative damage and promoted brain LPO and antioxidant status via inhibition of XO, iNOS gene expression and minimizing serum levels of TNF-α and IL-6.

Accordingly, GSO might be used to protect the brain from exposure to CCl4 and/or any type of toxicant cause oxidative stress that could be attributed to the strong antioxidant, anti-inflammatory and antiapototic activities of GSO.
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التأثيرات الوقائية لزيت بذر العناب على الإجهاد التأكسدي الناجم عن رابع كلوريد الكربون في مخ الجرذان

تتناول هذه الدراسة دراسة التأثيرات الوقائية لزيت بذر العناب على الإجهاد التأكسدي الناجم عن رابع كلوريد الكربون (CCl4) في مخ الجرذان. تم استخدام 24 أثني الجرذان حيث قسمت إلى أربعة مجموعات عشوائية في كل مجموعة ستة جرذان وأجريت التجارب كالآتي: الأولى مجموعة حاكمة، الثانية مجموعة تم إعطائها جرعة يومية 4 مللي لكل كيلو جرام من زيت بذر العناب لمدة سبعة أيام متواصلة عن طريق الفم. المجموعة الثالثة تم حقنها برابع كلوريد الكربون في اليوم السابع (2 مللي لكل كيلو جرام) و المجموعة الرابعة تم تغذيتها بزيت بذر العناب لمدة سبعة أيام متواصلة ثم في اليوم الثامن تم حقنها كلوريد الكربون. وفي اليوم الثامن تم سحب الدم من عين الفئران بعد تغذيتها ودراسة كمية الدم وفصل الدم عن طريق الفم. المجموعة الثالثة تم حقنها برابع كلوريد الكربون في اليوم الثامن. في اليوم الثامن تم سحب الدم من عين الفئران بعد تغذيتها ودراسة كمية الدم وفصل الدم عن طريق الفم. المجموعة الرابعة تم تغذية الفئران بزيت بذر العناب لمدة سبعة أيام متواصلة ثم في اليوم الثامن تم تسخين الدم من عين الفئران بعد تغذيتها ودراسة كمية الدم وفصل الدم عن طريق الفم.

وتبلغ هذه الدراسة دراسة تأثيرات الوقائية لزيت بذر العناب على الإجهاد التأكسدي الناجم عن رابع كلوريد الكربون في مخ الجرذان. حيث تم تغذية الفئران بزيت بذر العناب لمدة سبعة أيام متواصلة ثم في اليوم الثامن تم تسخين الدم من عين الفئران بعد تغذيتها ودراسة كمية الدم وفصل الدم عن طريق الفم.

وقد أظهرت النتائج أن زيت بذر العنب برابع كلوريد الكربون أدى إلى زيادة هبوط الدورة الدموية في مخ الجرذان. وتلقى هذا النتائج في العديد من الدراسات السابقة حيث أظهر أن زيت بذر العناب يحتوي على مادة فموية وأحماض دهنية أساسية ونسبة عالية من فيتامين E يمكن استخدامه لوقاية من التأثيرات السامة الناجمة عن الإجهاد التأكسدي الناجم عن رابع كلوريد الكربون في مخ الجرذان.