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Grape (*Vitis vinifera*) Seed Extract Inhibits the Cytotoxicity and Oxidative Stress in Liver of Rats Treated with Carbon Tetrachloride

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Abstract: The present study examined the curative action of Grape seed extract (GSE) on experimentally induced hepatic damage in rats by Carbon tetrachloride (CCl_4). Rats were divided into six groups (8 rats/ group) treated for 21 days and included the control group, Group 2 the group treated orally twice a week with CCl_4 (1.0 mg/kg b.w) in corn oil, Groups 3 and 4 treated orally with GSE at low dose (100 mg/kg b.w) or high dose (200 mg/kg b.w) and Groups 5 and 6 treated with CCl_4 plus GSE at the two tested doses. At the end of experimental period, blood and liver samples were collected from all groups for the biochemical, histological, histochemical and cytogenetic analysis. The results indicated that CCl_4 induced hepatic damage in the rats as evidenced by a significant increase in serum AST, ALT, ALP, triglycerides, MDA, nitric oxide, LDH, CEA, total lipid, cholesterol, DNA fragmentation and NO accompanied with a significant decrease in total proteins, GPX, SOD, DNA and RNA content in the liver and Fas and TNF α gene expression in the liver. Treatment with GSE reversed the values of the biochemical parameters to near normal values and improved the content of nucleic acids in hepatic tissues, the gene expression and the histopathological and histochemical picture of the liver. It could be concluded that GSE may be used in the protection against and/or the treatment of liver disease.

Key words: Liver · Grape seed extract · Cytogenetic · Gene expression · Oxidative stress · Antioxidant

INTRODUCTION

Liver diseases are amongst the most serious health problems in the world today and their prevention and treatment options still remain limited despite tremendous advances in modern medicine. In Egypt, liver cancer is the second cause of deaths from cancer after breast cancer and it is third frequent occurring cancer after bladder and breast cancer [1]. Hepatocellular carcinoma (HCC) is a major health problem, with more than 500.000 cases diagnosed annually. The burden of hepatocellular carcinoma (HCC) has been increasing in Egypt with a doubling in the incidence rate in the past 10 years [2]. Egypt has possibly the highest hepatitis C virus (HCV) prevalence worldwide [3], estimated among the general population to be around 14% [4]. Moreover, hepatitis B virus (HBV) accounts for 10-30% of chronic liver diseases and there is likewise a large occult reservoir of HCV caused chronic liver disease.

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Current liver mortality, including liver cirrhosis and cancer, is over 40,000/year and is increasing annually. This represents more than 10% of total mortality. The conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects [5]. In view of severe undesirable side effects of synthetic agents, there is growing focus to follow systematic research methodology and to evaluate scientific basis for the traditional herbal medicines that are claimed to possess hepatoprotective activity [6]. Most liver disorders cause some degree of hepatocellular injury and necrosis, resulting in various abnormal laboratory testes results and sometimes symptoms. Symptoms may be due to liver disease itself (e.g, jaundice due to Hepatitis) or to complications of liver disease.

As the liver is the first organ to metabolize all foreign compounds so it is susceptible to almost as many different diseases. Some are rare but there are a few, including hepatitis, cirrhosis, alcohol related disorders and liver cancer. A major cause of these disorders is due to exposure to different environmental pollutants and xenobiotics e.g., paracetamol, carbon tetrachloride, thioacetamide, alcohol, etc. These toxicants mainly damage liver by producing reactive oxygen species (ROS). Reactive oxygen free radicals have been known to produce tissue injury through covalent binding and lipid peroxidation and have been shown to augment fibrosis as seen from increased collagen synthesis [7]. Scavenging of free radicals by antioxidants could reduce the fibrosis process in the tissues also free radicals may act as a contributory factor in a progressive decline in the function of immune system [8]. Cooperative defense systems that protect the body from free radical damage include the antioxidant nutrients and enzymes [9-11].

Plant origin polyphenolic compounds are intensely studied in the recent years thank to their potent antioxidant, anti-inflammatory and immunomodulatory properties [12]. Grapes (*Vitis vinifera*), which are one of the most widely consumed fruits in the world have enormous health benefits. They contain a great variety of polyphenolic antioxidants with preventive and also therapeutic effects in several cancers, 60-70% of their content being represented by proanthocyanidins, composed mainly of dimers, trimers, tetramers and oligomers of monomeric catechins [13]. Grape seed extract can be considered among the most powerful natural nutrients efficient in the protection of body health, proanthocyanidins from grape seeds being stronger antioxidants and free radical scavengers than ascorbic acid or vitamin E [14]. It has been reported that grape has important role in controlling of some liver diseases, high blood pressure and anemia. Also fibers and fruit acids in grape have vital role in cleaning blood functions of digestive system and kidney [15]. Flavonoid component of grape seed extract, especially proanthocyanidin has antioxidant activity and it used in treatment of liver disease [16]. The aims of the current study were to evaluate the protective role of aqueous extract of grape seed against the cytotoxicity, DNA damage and oxidative stress in liver tissue in rats treated with CCl_4.

MATERIAL AND METHODS

Chemicals and Kits: Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were purchased from Spectrum-diagnostics Co. (Cairo, Egypt). Triglycerides, total proteins, glutathione peroxidase (GPx), superoxide dismutase (SOD), lipid peroxidase, alkaline phosphatase, nitric oxide, lactic dehydrogenase (LDH), carcinoembrionic antigen (CEA), total lipid and cholesterol kits were purchased from Biodiagnostic Co. (Giza, Egypt). All other chemicals used throughout the experiments were of the highest analytical grade available.

Plant Extract: Grape seed extract (GSE) was obtained from Mepaco Arabian Pharmaceutical Company Cairo, Egypt).

Experimental Animals: Three-month old female Sprague-Dawley rats (100-150g) purchased from Animal House Colony, National Research Centre Dokki, Giza, Egypt. Animals were maintained on the specified diet and housed in filter-top polycarbonate cages in a room free from any source of chemical contamination, artificially illuminated (12h dark/light cycle) and thermally controlled (25 ±1°C) at the Animal House Lab., National Research Centre. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Centre.

Experimental Design: Animals were divided into six groups (8 rats/ group) and were maintained on their respective diet for 3 weeks as follow: group 1, normal control animals which fed on basal diet and water without any treatment; Group 2, animals treated orally with CCl_4 (1.0 mg/ kg b.w) suspended in corn oil twice a week [17]; Group 3, animals treated orally with low dose of GSE (100 mg/kg) [18]; Group 4, animals treated orally with high dose of GSE (200 mg/kg), Group 5, animals treated orally with low dose of GSE plus CCl_4, rats treated orally with the low dose of GSE plus CCL_4 and Group 6, animals treated orally with high dose of GSE plus CCl_4.

The animals were observed daily for signs of toxicity during the experimental period. At the end of the treatment period (i.e. day 21) all animals were fasted for 12 h, then blood samples were collected from the retro-orbital venous plexus under diethyl ether anesthesia. Sera were separated using cooling centrifugation and stored at-20°C until analysis. The sera were used for the determination of ALT, AST, ALP, LDH, NO, CEA, total protein, cholesterol, triglycerides, total lipid, uric acid and createnine according to the kits instructions.

After the collections of blood samples, animals were sacrificed and samples of the liver of each animal were dissected, weighed and was homogenized in phosphate buffer (pH 7.4) to give 20% w/v homogenate [19]. This homogenate was centrifuged at 1700 rpm and 4 °C for 10 min; the supernatant was stored at-70°C until analysis. This supernatant (20%) was used for the determination of hepatic lipid peroxidation and it was further diluted with phosphate buffer solution to give 2% and 0.5% dilutions for the determination of hepatic glutathione peroxidase (2%) and superoxide dismutase (0.5%) activities.

Other samples of the liver from all animals were fixed in 10% neutral formalin and paraffin embedded. Sections (5µm thickness) were stained with hematoxylin and eosin (H&E) for the histological examination. Other sections from liver were stained with Bromophenol blue for the determination of protein content in liver and kidney tissue [20].

Cytogenetic Analysis: Determination of nucleic acids in hepatic tissue: Nucleic acids were determined in hepatic tissues according to the method described previously [21]. In brief, the hepatic tissues were homogenized and the homogenate was suspended in ice-cold trichloroacetic acid (TCA). After centrifugation, the pellet was extracted with ethanol. The levels of DNA were determined by treating the nucleic acid extract with diphenylamine reagent and reading the intensity of blue color at 600 nm. For quantification of RNA, the nucleic acid extract was treated with orcinol and the green color was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present.

DNA Fragmentation Assays for Apoptosis: Apoptotic changes in the liver were evaluated calorimetrically by DNA fragmentation and by agarose gel electrophoresis according to the procedure of the published method [22]. Liver samples were homogenized in 700 µl hypotonic lysis buffer and centrifuged for 15 min at 11,000 rpm. The supernatants containing small DNA fragments were separated; one-half the volume was used for gel electrophoresis and the other half together with the pellet containing large pieces of DNA were used for quantification of fragmented DNA by the Diphenyl amine (DPA) assay. The samples were treated with equal volumes of absolute isopropyl alcohol and NaCl to precipitate DNA. Extracted DNA was electrophoresed on 1% agarose gels containing 0.71 µg/ml ethidium bromide. At the end of the runs, gels were examined using UV transillumination. The Diphenyl amine (DPA) assay reaction suggested earlier [20, 23]. The colorimetric reaction was measured spectrophotometrically at 575 nm and the percentage of DNA fragmentation was calculated.

RNA Isolation: Hepatic tissue cells were ground in liquid nitrogen and total RNA was extracted from all groups of the experiment (five samples from each group). The extraction of total RNA was performed using Biozol reagent according to the manufacturer's procedures. The concentration and purity of RNA was measured at 260/280 nm using ultraviolet spectrophotometer (ratios fell between 1.75 and 1.9, indicating very pure RNA in all cases). Equal amounts of RNA isolated from individual rat of each group were prepared for the semi-quantitative RT-PCR [24, 25].

Reverse Transcription-polymerase Chain Reaction (RT-PCR): Reverse transcription-PCR was performed in a 20-µl volume that contained 5 µM oligo dT12-18, 2 µg total hepatic RNA, 200 U Superscript TM II reverse transcriptase (Life Technologies) at 42°C for 10 min followed by 42°C for 1 h. In a total volume of 20 µl, the PCR mixture contained 150 µM dNTPs, 1 µM antisense and sense primers for Fas or TNF α, 1 µl reverse-transcribed cDNA and 2 U Taq polymerase (PE Applied Biosystems, Foster City, CA). The sequences of oligonucleotide primers were: 5`-Fas, CGC CTA TGG TTG TTG ACC, 3`-Fas, CTC CAG ACA TTG TCC TTC, 5`-TNF α, ACA GAA AGC ATG ATC CGC, 3`-TNFα, GTA GAC CTG CCC GGA CTC, 5'-β-actin, CGTGACATCAAAGAGAAGCTGTGC-3'-β-actin, CTCAGGAGGAGCAATGATCTTGAT-3'. The expected amplicon lengths were 477 bp for Fas and 692 bp for TNFα. Amplification conditions were (94°C 15 s, 54°C 1 min, 72°C for 30 s) for 15–35 cycles. The expected amplicon lengths were 477 bp for Fas and 692 bp for TNF α [26]. An aliquot of the RT-PCR reactants (10 µl) was separated on a 1.2% agarose gel containing ethidium bromide, visualized under UV light and analyzed using NIH Image software.

Statistical Analysis: All data were statistically analyzed by analysis of Variance (ANOVA) using the General Linear Model Procedure of the Statistical Analysis System [27]. The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio [28]. All statements of significance were based on probability of $P = 0.05$.

RESULTS

The results of the current study revealed that various liver enzymes were seriously affected with CCl_4 treatment while upon treatment with grape seed extract whether in low or high doses, a significant improvement was achieved. Significant increases in AST, ALT, ALP, LDH, total lipids, cholesterol, triglycerides, CEA and NO accompanied with a significant decrease in total protein were detected after CCl_4 treatment (Table 1). Animals received GSE at the two tested doses plus CCl_4 showed a significant improvement in all biochemical parameters toward the normal value of the controls. Moreover treatment with CCl_4 resulted in a significant decrease in GPX and SOD activities in liver accompanied with a significant increase in MDA. Treatment with GSE succeeded to induce a significant improvement in antioxidant parameters and oxidative stress markers (Table 2).

The results also indicated that rats treated with CCl_4 showed a significant cytotoxicity as indicated by the depletion in DNA and RNA content in the hepatic tissue (Table 3). The results also indicated that CCl_4-induced DNA damage was evaluated by measuring the level of fragmented DNA calorimetrically using Diphenylamine (DPA) and by comparing DNA profiles on agarose gel electrophoresis. These results showed that CCl_4 caused marked DNA fragmentation in the liver (39.40 %) compared to control untreated rat (7.80) as indicated by DPA assay (Table 4). Treatment with GSE significantly brought down the percentage of the DNA damage to 15.93% and 10.15% at the low and high doses respectively. The DNA fragmentation in response to exposure to CCl_4 and the other treatments was also detected by gel electrophoresis as a DNA ladder representing a series of fragments that are multiples of 180-200 bp (Fig. 1).

In the current study, bands produced from amplifying cDNA of fatty acid synthesis (Fas), tumor necrosis factor (TNF) and the house keeping gene β-actin as a control were analyzed and the results of gene expression was based on quantifying the signal intensities in each band. The results were expressed as the ratio between maximum optical density (OD max) for each band of the target amplification product and the corresponding OD max of β-actin (Fig. 2). These results indicated that exposure to

Table 1: Effect of grape seed extract (GSE) on serum biochemical parameters in rats treated with CCl_4

Groups Parameters	Control	CCl_4	LGSE	HGSE	CCl_4 + LGSE	CCl_4 + HGSE
AST (U/L)	150.75 ± 5.89^a	230.93 ± 4.79^c	151.38 ± 1.98^a	156.81 ± 3.47^a	212.88 ± 2.15^d	157.35 ± 6.10^a
AST (U/L)	65.83 ±1.42^a	98.73 ± 1.76^b	57.88 ± 1.48^c	66.43 ± 1.51^a	65.75 ± 1.73^a	61.44 ± 1.98^a
ALP (U/L)	160.95 ± 15.16^a	226.53 ± 13.29^b	161.42 ± 13.5^a	160.65 ± 10.98^a	176.09 ± 4.33^c	169.88 ± 4.59^d
LDH (IU/L)	1973.70 ± 162.53^a	7867.18 ± 460.74^b	1842.38 ± 161.52^c	1954.42 ± 181.06^a	4157.26 ± 242.02^d	3441.66 ± 196.71^e
TP (mg/dl)	5.96 ± 0.08^a	3.76 ± 0.27^b	7.09 ± 0.21^c	7.06 ± 0.25^c	5.71 ± 0.22^a	7.45 ± 0.20^c
TL (mg/dl)	145.15 ± 6.06^a	181.71 ± 9.11^b	137.56 ± 8.25^c	134.27 ± 9.10^c	117.73 ± 9.88^d	117.73 ± 9.88^d
ThiG (mg/dl)	33.68 ± 2.80^a	90.24 ± 2.28^b	30.07 ± 2.55^a	28.18 ± 2.30^c	49.27 ± 4.66^d	31.72 ± 2.16^a
Cho (mg/dl)	78.42 ± 5.45^a	154.26 ± 3.96^b	74.37 ± 5.19^a	69.08 ± 8.47^c	73.28 ± 1.10^a	62.23 ± 7.10^c
CEA (ng/ml)	4.99 ± 0.39^a	14.03 ± 0.18^b	4.49 ± 0.28^a	4.74 ± 0.31^a	5.17 ± 0.32^c	5.41 ± 0.56^c
NO (µmol/L)	17.84 ± 0.61^a	28.73 ± 1.07^d	15.57 ± 0.53^b	12.41 ± 0.21^c	14.65 ± 0.97^b	14.52 ± 0.42^b

Within each row, means with different superscripts are significantly different at $P<0.05$.

Table 2: Effect of grape seed extract (GSE) on oxidative stress markers in live of rats treated with CCl_4

Groups Parameters	Control	CCl_4	LGSE	HGSE	CCl_4 + LGSE	CCl_4 + HGSE
GPX (U/mg protein)	23.18 ± 0.72^a	7.91 ± 1.55^d	22.32 ± 1.32^a	24.94 ± 0.75^b	18.53 ± 1.41^e	21.8 ± 0.10^f
SOD (U/mg protein)	2.81 ± 0.13^a	1.5 ±0.11^c	3.07 ± 0.14^b	2.81 ± 0.21^a	2.5 ±0.11^a	2.56 ± 0.16^a
MDA (mol/mg protein)	11.31 ± 1.43^a	27.72 ± 0.38^b	11.24 ± 1.38^a	11.72 ± 0.84^a	12.72 ± 1.51^c	13.17 ± 1.37^c

Within each row, means with different superscripts are significantly different at $P<0.05$.

Table 3: Effects of Grape seed extract on nucleic acid contents in hepatic tissues of rats treated with CCl_4

Treatments	DNA mg/gm	RNA mg/gm
Control	0.356 ± 0.012^{a}	0.189 ± 0.003^{ab}
CCl_4	0.170 ± 0.013^{e}	0.109 ± 0.006^{e}
LGSE	0.285 ± 0.013^{bc}	0.126 ± 0.014^{e}
HGSE	0.279 ± 0.017^{c}	0.135 ± 0.016^{cd}
CCl_4 + LGSE	0.220 ± 0.011^{d}	0.141 ± 0.010^{cd}
CCl_4 + HGSE	0.277 ± 0.012^{c}	0.190 ± 0.020^{ab}

Within each column, means with different superscripts are significantly different at $P<0.05$.

Table 4: Effects of Grape seed extract on DNA fragmentation in liver of rat treated with CCl_4

Treatments	DNA Fragmentation %	Changes
Control	7.80 ± 1.43^{de}	--
CCl_4	39.40 ± 1.91^{a}	+ 31.60
LGSE	7.60 ± 0.89^{e}	- 0.20
HGSE	7.24 ± 0.80^{e}	- 0.56
CCl_4 + LGSE	15.93 ± 0.96^{bc}	+ 8.13
CCl_4 + HGSE	10.15 ± 0.71^{d}	+ 2.35

Within each column, means with different superscripts are significantly different at $P<0.05$.

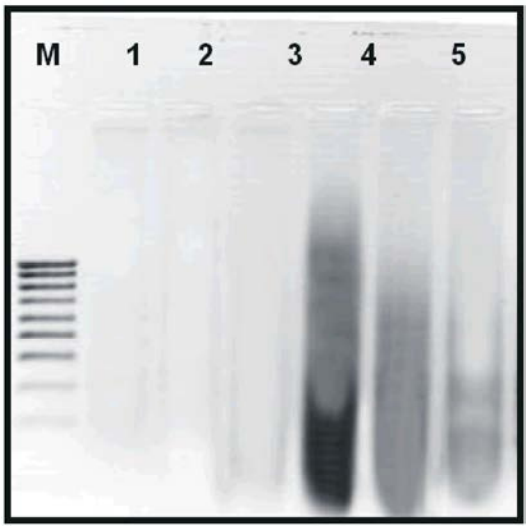

Fig. 1: Effects of GE on DNA fragmentation of hepatic tissue in CCl_4-treated rats. Agarose gel electrophoretic pattern of DNA isolated from liver tissue of different groups. Lane M: phi x marker, Lane 1: Control, Lane 2: low dose of GE, Lane 3: high dose of GE, Lane 4: CCl_4, Lane 5: CCl_4 + low dose of GE and Lane 6: CCl_4 + high dose of GE.

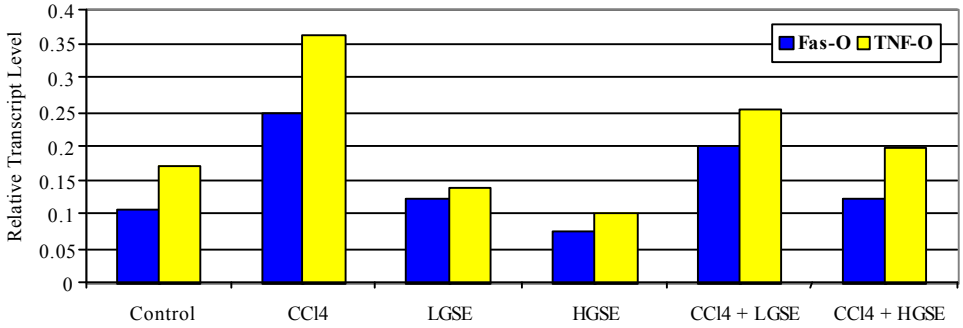

Fig. 2: RNA gene expression of Fas and TNF in the liver of control and treated rats. The results depicted are normalized to levels of β-actin gene. Data are mean of ratios of intensity for each gene divided by that for β-actin.

Ccl_4 resulted in a significant increased in mRNA expression of the Fas gene accompanied with increased in TNF gene expression in the hepatic tissues compared to the other experimental groups. Treatment with GSE alone at the low and high doses induced insignificant increase in the expression of Fas and TNF genes compared to the control group. On the other hand, treatment with GSE at high dose normalized the expression levels of Fas and TNF genes compared to the control level in liver tissues (Fig. 3).

The biochemical results were confirmed by the histological and histochemical examination of the liver and kidney tissues. The histological examination of the liver sections in the control rats (Fig. 4a) or those treated with GSE at the low or high dose (Fig. 4b,c) showed normal structure of hepatocytes with no cellular infiltration around the central vein, portal area or in between the hepatocytes. The microscopic examination of liver sections from CCl_4-treated group showed vacoulation and mononuclear cellular infiltration in between the hepatocytes (Fig. 4d). The liver sections of the animals treated with CCl_4 plus the low dose of GSE showed prominent improve in hepatocytes (Fig. 4e) however; those treated with CCl_4 plus the high dose of GSE showed prominent improve in hepatocytes but the fibrous tissues are still present (Fig. 4f).

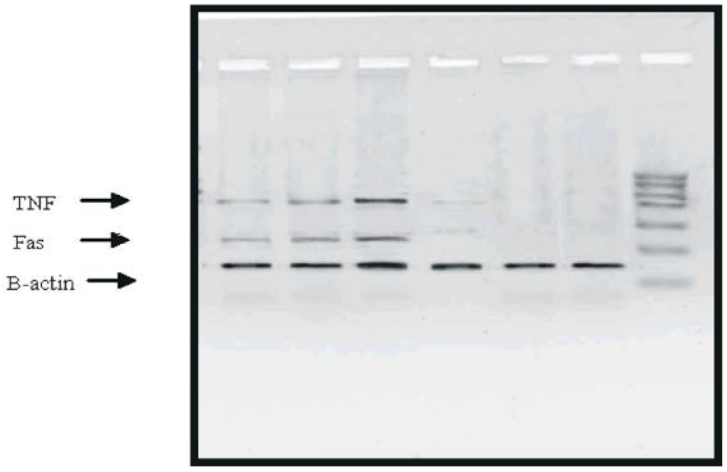

Fig. 3: Effects of GSE on transcript product of hepatic Fas & TNF genes in CCl_4-treated rats. Agarose gel electrophoresis of Fas, TNF and β-actin RT-PCR products of different groups. Lane M: phi x marker, Lane 1: Control, Lane 2: LGSE, Lane 3: HGSE, Lane 4: CCl_4, Lane 5: CCl_4 + LGSE and Lane 6: CCl_4 + HGSE.

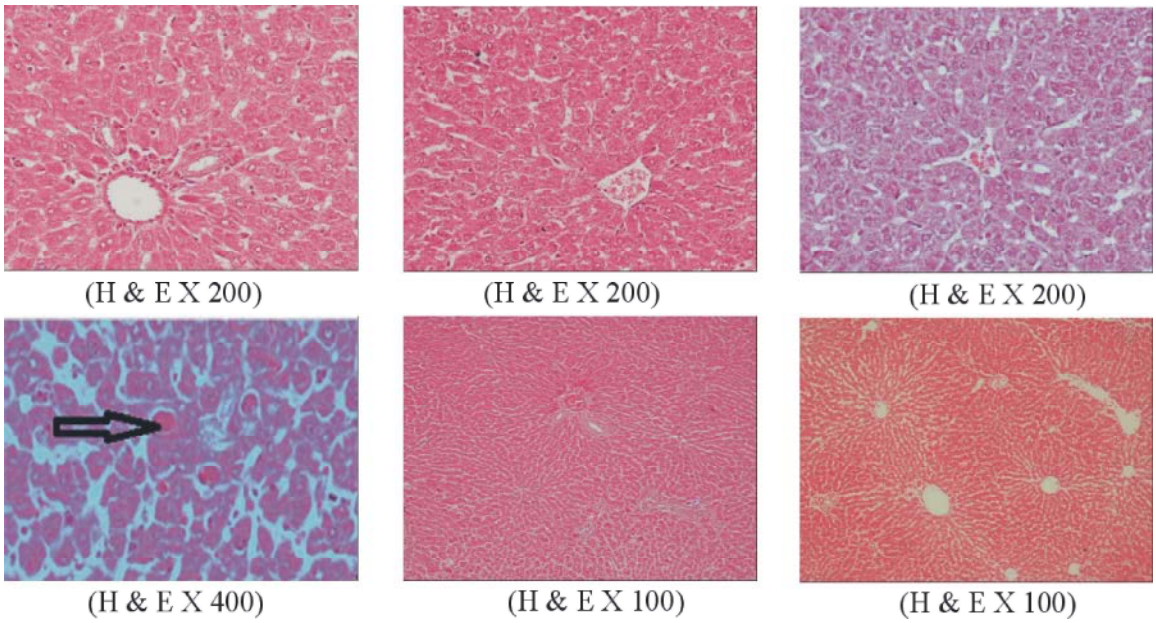

Fig. 4: Photomicrographs of liver sections from (a) control animal showing the normal structure of hepatocytes with no cellular infiltration around the central vein, portal area or in between the hepatocytes, (b) animals treated with low dose of GSE showing the normal structure of hepatocytes with no cellular infiltration around the central vein, portal area or in between the hepatocytes, (c) animals treated with high dose of GSE showing the normal structure of hepatocytes with no cellular infiltration around the central vein, portal area or in between the hepatocytes, (d) animals treated with CCl_4 showing vacoulation and mononuclear cellular infiltration in between the hepatocytes (arrow), (e) rats received low dose of GSE plus CCl_4 showing prominent improve in hepatocytes, (f) rats received high dose of GSE plus CCl_4 showing prominent improve in hepatocytes but the fibrous tissues are still present.

The histochemical examination of the liver section of the control rats (Fig. 5a), low dose of GSE (Fig. 5b) and high dose of GSE (Fig. 5c) stained with bromophenol blue stain for protein evaluation namely in the hepatocytes cytoplasm and nucleus membrane showing normal distribution of blue color. The liver section of rats treated with Ccl_4 showed a decrease in protein reaction in hepatocytes (Fig. 5d). The liver section of rats treated with CCl_4 plus low dose of GSE (Fig. 5e) and high dose of GSE (Fig. 5f) showed obvious improvements in protein reaction.

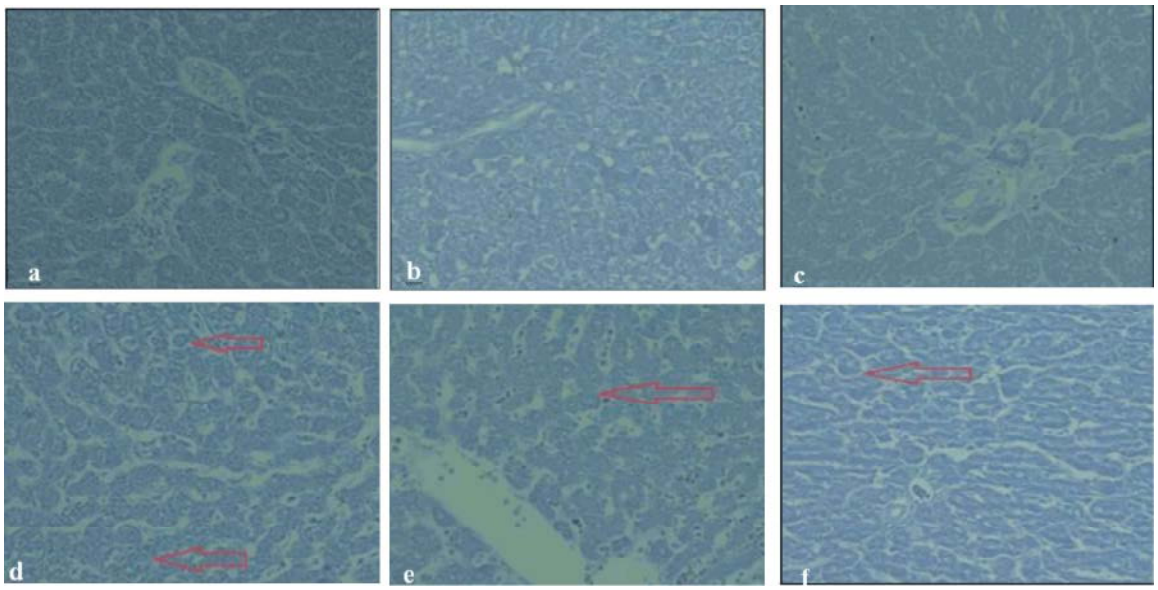

Fig. 5: Hotomicrographs of liver sections from (a) control rats, (b) rat treated with low dose of GSE, (c) rats treated with high dose of GSE showing normal distribution of blue color, (d) rats treated with CCl_4 showing a decrease in protein reaction in hepatocytes, (e) rats treated with CCl_4 plus low dose of GSE and (f) tars treated with CCl_4 plus high dose of GSE showing obvious improvements in protein reaction. (Bromophenol blue stainX400)

DISCUSSION

Ccl_4 is one of the most extensively studied hepatotoxicants. The mechanism by which CCl_4 causes hepatotoxicity is well documented in a series of reports which indicated that its hepatotoxicity undergoes of two phases. The first results from its metabolic conversion to free radical product CCl^-_3 by Cyt P-450 [29]. Once CCl^-_3 has been formed it reacts very rapidly with O_2 to produce CCl_3OO, a much more reactive radical than CCl^- [30]. These free radicals attack microsomal lipids leading to its peroxidation and also covalently bind to microsomal lipids and proteins. This results in the generation of reactive oxygen species (ROS), which includes the super-oxide anion O_2, H_2O_2 and the hydroxyl radical.

In the current study, the results indicated that CCl_4 induced a severe toxicity to the animals and were in agreement with the previous reports. CCl_4 caused significant changes in serum biochemical parameters typical to those reported in the literature [11, 26, 31, 32]. Serum AST and ALT are the simple and well-accepted biomarkers for hepatic dysfunction. Hepatic ALT only locates in cytoplasm, while AST distributes in both cytosolic and mitochondrial fraction. AST release is an important index of relatively severe liver injuries, whereas ALT is more reliable (longer half-life than AST) and liver-specific for acute and moderate liver injuries [33, 34]. Moreover, the liver is considered to be the principal target organ for CCl_4 consequently, the activities of transaminases are sensitive indicators of acute hepatic necrosis [31, 35-37].

The elevated levels of ALT, AST, ALP, LDH, triglycerides, cholesterol and total lipids reported herein indicated severe hepatic parenchymal cells injury [38-40]. Whereas, the decrease in total protein indicated liver necrosis and/or kidney dysfunction [39, 41]. These results clearly showed that CCl_4 has a harmful and stressful influence on the hepatic tissue consistent with those reported earlier [10, 42].

Alteration in the hepatic antioxidant status may therefore manifestation of oxidative stress caused by CCl_4 and its metabolites. Both GPX and SOD are considered enzymatic free-radical scavengers in cells. In the present study, GPX and SOD were found to decline significantly in rats treated with CCl_4. It is well known that SOD plays an important role in the elimination of ROS derived from the peroxidative process in liver tissues [43]. Moreover, SOD removes superoxide by converting it to H_2O_2, which can be rapidly converted to water by CAT [44]. Taken together, the increased level of MDA and the decreased activity of antioxidant enzymes GPX and SOD may be attributed to free radical formation which initiated chain reactions of direct and indirect bond formation with cellular molecules (nucleic acids, proteins, lipids and carbohydrates) impairing crucial cellular processes that may ultimately culminate.

Regarding the elevated serum level of tumor markers (CEA) in CCl_4 administrated rats, previous reports indicated that CCl_4 is a potent hepatotoxicity, enhances reactive oxygen species (ROS) formation and causes oxidative DNA damage, which may play a role in its toxicity and carcinogenicity [26, 31]. CEA are considered specific biomarkers for liver cancer and it is synthesized mainly in the fetal stage; practically no production of this marker occurs in the normal adult. However, when some adult cells are transformed to cancer cells, the synthesis of CEA commences again. Therefore, the current study affirmed that CCl_4 can induce hepatotoxicity in rats as indicated by the elevation of CEA level in serum.

MDA, an end product of lipid peroxidation, is widely used as a marker of lipid peroxidation. Lipid peroxidation (LP) is one of the main manifestations of oxidative damage and has been found that it plays an important role in toxicity and carcinogenicity. It is well documented that CCl_4 enhanced LP [31, 45] that is an indication of free radical mediated toxicity. Free radicals are known to attack the highly unsaturated fatty acids of the cell membrane and induce lipid peroxidation that is considered a key process in many pathological events induced by oxidative stress [46]. In the present study, MDA was found to be significantly higher in the animals treated with CCl_4 alone suggesting that this agent has a significant effect on LP and supported the earlier findings [42, 47, 48].

Nitric oxide (NO) is produced by macrophages and it plays an important role in tumor conditions [49]. The generation of NO by the inducible nitric oxide synthase (iNOS) plays a key role in the cytokine-mediated cell destruction [50]. In the current study, the ingestion of CCl_4 significantly increased NO suggesting that CCl_4 preferentially affects macrophage functions. It is well documented that excess ROS, a condition referred to as oxidative stress, is considered to be a major contributor to cell injury, although many studies have shown that higher levels of ROS can also activate specific genetic programs in various cells [51].

In the current study, treatment with CCl_4 resulted in a significant decrease in hepatic nucleic acid content. It has been reported that cellular DNA damage occurs due to free radicals generated under different conditions and a number of techniques have been developed to measure the oxidatively modified nucleobases in DNA [52]. CCl_4 induced damage in hepatic DNA (strand breaks) and RNA. This demonstrates that oxidation products of hepatic nucleic acids measured in the present study are good biomarkers of oxidative liver damage after CCl_4 exposure. CCl_4-mediated oxidative damage is just one example of the more general phenomenon of oxidative stress. Different forms of oxidative stress may give rise to different oxidation products and the biomarkers that are sensitive to other insults may differ from those that were clearly elevated in this CCl_4 model.

In the current study, bands produced from amplifying cDNA of fatty acid synthesis (Fas), tumor necrosis factor (TNF) and the house keeping gene β-actin as a control were analyzed and the results of gene expression was based on quantifying the signal intensities in each band. These results were expressed as the ratio between maximum optical density (OD max) for each band of the target amplification product and the corresponding OD max of β-actin. The results indicated that exposure to CCl_4 resulted in a significant increased in mRNA expression of the Fas gene accompanied with increased in TNF gene expression in the hepatic tissues compared to the other experimental groups. It is well documented that CCl_4 significantly increased caspase 3 activity and a pro-apoptotic gene (bax) expression as well as decreased anti-apoptotic gene (Bcl-2) gene expression [32].

The significant high level of fatty acid synthesis (Fas) and tumor necrosis factor (TNF) expression found in liver of CCl_4-treated rats indicated that these cells are susceptible to apoptosis. In this concern, Masson *et al.* [53] reported that the proapoptotic proteins Bad and Bax were significantly higher in liver cirrhosis induced by CCl_4 and apoptosis takes place in liver during CCl_4-induced cirrhosis. Moreover, apoptosis lead to DNA damage as indicated by DNA fragmentation and comet formation reported in the current study since a 39.4 % enhancement of DNA fragmentation in liver of rats treated with Ccl_4 compared to the control group. Similar results were observed by Lee *et al.* [54] who reported that CCl_4 induced hepatocyte DNA fragmentation and cytosolic caspase-3 and caspase-8 activity in rats. Moreover, CCl_4 induced DNA strand breaks in hepatocytes measured by single cell gel electrophoresis through the increase in comet tail length in CCl_4-treated group compared to control group. Similar result noticed by Vanitha *et al.* [55] who reported that CCl_4 induced toxicity by comet formation in rats. Moreover, it was reported that CCl_4 increased chromosomal aberrations and SCE's in bone marrow which arise from DNA breaks and reversion of broken fragments at almost homologous loci after their exchange between the two sister chromatids of the same chromosome [56, 57] and hence their formation is dependent on the S-phase of the cell cycle [58] or on DNA replication processes [59].

The biochemical and cytogenetic results of the current study were further confirmed by the histopathological and histochemical study. The

histological and histochemical results showed that liver tissue more or less have significant changes in the histological and histochemical pictures. It is clear that animals treated with CCl_4 showed severe histological changes in liver typical to those reported in the literature. In CCl_4-treated group, the liver showed vacoulation and mononuclear cellular infiltration in between the hepatocytes. Some sections of the same group showed coagulative necrosis in blood sinusoids around the central vein. Moreover, other sections of the animals treated with CCl_4 showed evident fibrosis and cellular infiltration around the portal area accompanied with decrease in protein reaction in hepatocytes. These histological changes were similar to those reported earlier [45, 60]. Similar observations of the liver tissues in rats treated with CCl_4 were also reported previously.

In this study, treatment with GSE at the two tested doses did not induce any significant effect on liver function or the tumor marker (CEA). However, they improve the antioxidant capacity of the body and reduce the oxidative stress as indicated by the reduction of MDA and NO levels as well as DNA fragmentation and up-regulate gene expression. Treatment with GSE to the intoxicated rats could markedly suppress the high serum level of ALT and exerted a resistant effect for the increase of serum AST. These results suggest that the administration of GSE may exert protective effects for further deterioration of the mitochondrial membranes of the hepatocytes [64, 65]. Moreover, these protectives succeeded to improve the histological and histochemical changes resulted in liver tissue in CCl_4-treated rats.

The biological pharmacological and medicinal properties of bioflavonoids have been extensively reviewed [66]. Increasing interest in proanthocyanidins and other polyphenolic compounds in GSE is based on a variety of pharmacological, medicinal and therapeutic potential including inhibition of DNA topoisomerase II, modulation of protein kinase C, angiotensin-converting enzyme and hyaluronidase enzyme activities [66]. Proanthocyanidins has also been demonstrated to exhibit antihypertensive effects [66], anti-peptic activity, monocyte stimulating ability and anti-hepatotoxic activity [67]. In the current study, animals treated with CCl_4 plus GSE at the two tested doses showed significant improvements in the antioxidant enzymes, oxidative stress markers, nucleic acids content and gene expression as well as the significant improvement in the histological and histochemical picture in liver in dose dependent fashion. Previous studies indicated that the chemical properties of bioflavonoids, in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers and singlet oxygen quenchers, predicts their antioxidant activity [66, 68]. Moreover, GSE has also been demonstrated as potent inhibitors of the enzymes phospholipase A2, cyclooxygenase and lipooxygenase [68].

The proanthocyanidins or polyphenolic bioflavonoids may act as antioxidants and/or by other mechanisms, contributing to chemoprotective and health bene?ts. It is well documented that the Bcl-2 gene is an important antagonist of apoptosis, the programmed cell death. Bcl-2 is highly expressed variety of tumor cells including lymphomas leukemia and other solid tumors. These cells particularly the etastatic cells, are resistant to apoptosis. One of the mechanisms of induction of resistance is via increased expression of Bcl-2 [69]. In this regard, the liver cells treated with GSE also had an increased expression of Bcl-2 gene. The other apoptosis related gene p53 is a pro-apoptosis gene [69]. Thus, one of the cellular mechanisms of chemoprevention of GSE appears to be via up-regulation of Bcl-2 gene and down-regulation of p53 gene. The present study demonstrated that GSE signi?cantly normalize Fas and TNF genes in a dose dependent manner. According to Joshi *et al.* [70], one of the possible mechanisms of the action of GSE is via up-regulation of anti-apoptotic gene Bcl-2 expression in these cells which makes the cells resistant to apoptotic cell death.

CONCLUSION

It could be concluded that treatment with CCl_4 resulted in sever biochemical and histological changes accompanied with sever cytotoxic effect in the live. Treatment with GSE resulted in a significant improvement in all tested parameters in dose dependent manner. GSE it self was safe at the tested doses and it may be a candidate for the prevention as well as treatment of liver diseases.

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