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Synergistic Effect of Milk Thistle and Grape Seed Extracts on the Recovery of Fumonisin b1 Toxicity in Rats.

El-Shafeey M [1,*]; El-Adawi H[1]; Al-Azhari D.[2]; Abd El-Wahab A [1] and Abdel-Mohsen M[3]

1- Medical Biotechnology Dept., GEBRI institute, City of Scientific Research and Technology Applications, New Borg El-Arab City, Alexandria, Egypt
2- Zoology Dept., Faculty of Science, Minia University, Minia, Egypt
3- Applied Medical Chemistry Dept., Medical Research Institute, Alexandria, Egypt
* (e.mail: mohamedshafeey@gmail.com)

ABSTRACT

Fumonisin B1 (FB1) is a mold metabolite produced by Fusarium species that is frequently found in corn worldwide. It is toxic to both liver and kidney. Hepatotoxicity was induced in rats by feeding them FB1 contaminated corn. Evidence of hepatotoxicity was observed after 60 days by an increase in the plasma activity of alanine aminotransferase (ALT), where that elevation reached 78 % ($p = 0.000$), in comparison with the control group. Treatment with milk thistle (S), or grape seeds (G) extracts or both (S+G) was found to return the ALT level back to normal. FB1, drastically depleted glutathione peroxidase (GpX) to 48%, while treatment with S,G, and S+G could elevate the GpX by 76%, 76% and 35% respectively. Lipid peroxidation represented by malondialdehyde was elevated significantly to 137%. On the other hand, the treated groups (S, G, and S+G) have altered the levels down to 47%, 42%, and 29% respectively. In addition to the hepatotoxicity of FB1, the kidney function was investigated too, where the creatinine level was elevated to 65%. The treatment by S, G and S+G lowered the level down to 16%, 15%and 2%. Serum activity of urea was significantly elevated to 30%, the treated group G could significantly reduce it to 23% while the treated groups S and S+G could not reduce that elevation in urea level. Histological examination of liver sections confirmed the serum analysis, where significant improvements were observed in all treated groups in comparison with the liver sections of rats fed on FB1. These improvements might be due to their ability to lower serum total cholesterol and low- density lipoprotein cholesterol levels as well as slowing the lipid peroxidation process by enhancing antioxidant enzyme activity.

Keywords: Milk Thistle - Grape Seed Extracts- Toxicity - Rats

INTRODUCTION

Fumonisins are recently discovered mycotoxins in 1988, their chemical structure and biological activity were elucidated in South Africa (Gelderblom *et al*., 1988; Marasas, 2001). Fumonisin B1 is a mycotoxin produced by the fungus *Fusarium verticillioides*, is known to be the causative agent of several diseases in animals (Marasas, 1996), it causes equine leukoencephalomalacia (ELEM) (Marasas, 2001) and porcine pulmonary edema (Haschek *et al*., 2001; Marasas, 2001). Consumption of food containing *F. verticillioides* and/or fumonisins has been linked epidemiologically to the high incidence of cancer (Sydenham *et al*., 1991; Rheeder *et al*., 1992). Fumonisin B1 has the ability to alter sphingolipid metabolism by inhibiting the enzyme ceramide synthase, an enzyme responsible for the acylation of sphinganine and sphingosine. Structurally, fumonisin resembles sphinganine and sphingosine, free

sphingoid bases that play critical roles in cell communication and signal transduction (Wang *et al*., 1996; Desai *et al*., 2002). The disruption of the sphingolipid biosynthetic pathway leads to increased levels of sphingolipid precursors and decreased levels of complex sphingolipids, since fumonisins disrupt sphingolipid biosynthesis, the resulting elevation in the sphinganine/sphingosine ratio in serum, plasma, or urine has been used as a biomarker for estimating dietary exposure to fumonisins in animals (Shephard *et al*., 1996a, b; Shephard and van der Westhuizen, 1998; Marasas, 2001).

Silymarin, an extract from seeds and fruits of milk thistle (*Silybum marianum*), is a mixture of flavonoid isomers such as silibinin, isosilibinin, silidianin, and silichristin. The seeds of this plant have been used in Europe for many centuries for the treatment of liver and gall bladder dysfunctions (Schulz *et al*., 2004; Wichtl, 2004; Blumenthal, 2003). Silymarin has liver regenerative effects by stimulating the enzyme known as RNA polymerase in the nucleus of liver cells. This result in increase of ribosomal protein synthesis helps to regenerate hepatocytes (Gruenwald, 2004). The ability of silymarin to protect against oxidative stress-induced hepatocellular damage (such as lipid peroxidation of membranes and subsequent membrane degradation) is associated with its free radical scavenging properties and its ability to enhance endogenous antioxidant defences, such as those mediated by SOD or the glutathione system (Schuppan *et al*., 1999; Pascual *et al*., 1993)

Grape (Vitis vinifera) is one of the most widely consumed fruits in the world. Grape is known as the "queen of fruits" because of cleansing properties. A "grape cure" or grape fast involves eating 3–6 pounds of grapes to detoxify and improve liver function (Grieve, 1971; Bown, 2001). Additionally, 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 (Celik *et al.*, 1998)

Grape Seed Extract (GSE) is a medical herb used primarily for its high proanthocyanidin content. GSE is a naturally occurring plant substance that contains a concentrated source of antioxidant nutrients known as oligomeric proanthocyanidins (OPCs), which are more powerful antioxidants than vitamins C, E and beta-carotene. GSE has been found to be the richest source of (OPCs).

MATERIALS AND METHODS

Chemicals: All chemicals were purchased from Sigma-Aldrich Co., Germany. ALT, Albumin, Total protein, Uric acid, Urea, Lipid peroxide and GPx Kits were purchased from Bio-Diagnostic Co., Cairo-Egypt, creatinine and lipid profile kits were purchased from Diamond Co., Cairo-Egypt.

Animals and experimental design

Fifty male Sprague Dawley rats average weight (100-120g) were used. Animals were caged in groups of five and given water *ad libitum*. After one week of acclimatization, animals were divided into two main groups. Group 1 of 10 rats were used as control (C-gp), where they were fed on uncontaminated corn; the second group of 40 rats served as the treated group where they were fed on contaminated corn with at least 250mg/ kg FB1 for 60 days. After that this group was divided into four groups: The first group (FB1-gp) of 10 rats served as positive control, the second one (Sp-gp) of 10 rats were fed on milk thistle extract (S-750 mg/kg b.wt.), the third one (G-gp) of 10 rats were fed on grape seed extract (G- 3g/kg b.wt.) and the fourth one (G+S-gp) of 10 rats were fed on mix of both extracts. At the end of

experimental period, the animals were sacrificed. Blood samples were collected from animals in clean sterilized test tubes and serum samples were separated and used for analysis parameters.

Extraction of GSE and Silymarin

Pre-extraction sample preparation

Red grape (Vitis vinifera) seeds were obtained from Ganaclise Veinyard factory (Abu-Elmatameer). Grape seeds were handily separated from grape skin and stem (waste), then washed with tap water and then left to dry in open air away from direct sunlight. Seeds were crushed in a coffee grinder for two min, but at 15 s on & off intervals to avoid heating of the sample. The crushed seeds were wrapped and stored at -18°C until the extractions were performed (Palma *et al.,* 1999)

Milk thistle:

(Silybum marianum) seeds were obtained from a local market at Alexandria, Egypt. The seeds were handily separated from waste, then were washed with water, dried in open air and crushed in a coffee grinder as explained previously.

Extraction process:

The crushed samples (Grape and Milk thistle) seeds were subjected to preliminary treatment (defatting); crushed seeds were soaked in suitable volume of hexane overnight. Defatted seeds were extracted by pressurized hot water and lyophilized using lyophilizer (Telstar, Spain).

Determination of phenol content

The classic technique employed in phenol analysis is the 4-aminoantipyrine colorimetric procedure (Dannis 1951; Ettinger *et al*., 1951). The absorbance of the samples was read against blank at 500 nm using spectrophotometer (PerkinElmer Lambda EZ 201, USA). The concentration of the sample was calculated from the standard curve prepared previously.

Determination of antioxidant activity

The antioxidant activities of the herbs water extracts were determined using the ferric thiocyanate (FTC) method (Osawa and Namiki, 1981) with slight modification. The absorbance at 500 nm of the resulting red solution was measured, and it was measured again every 24 h until the time when the absorbance of the control reached the maximum value. The percent inhibition of linoleic acid peroxidation was calculated as:

(%) inhibition = 100 - [(absorbance increase of the sample/absorbance increase of the control) × 100].

All tests were run in duplicate, and analyses of all samples were run in triplicate and averaged.

HPLC analysis:

The analysis for milk thistle seed extract was done using a Beckman-C18 column (100 x 4.6 mm, 5 μm particle size), equipped with an autosampler, pump and UV/visible multi wavelength detector

Serum analysis

Blood samples for liver function, kidney function and lipid profile assays were centrifuged at 3000 rpm for 15 min. using centrifuge (Heraeus, Germany), and the resultant serum samples were stored at -20 °C till analysis (Oser, 1965).

Preparation of crude liver homogenate

Liver tissue homogenate was prepared for total glutathione peroxidase (GPx) determination according to Paglia and Valentine (1967). The protein concentration of the clarified homogenate was calculated to determine the required volume which should be added on this assay.

Measurement of liver function markers

Total proteins were determined by means of the biuret reaction as described by Gornall *et al*., (1949). In the presence of an alkaline cupric sulfate, the protein produces a violet color, the intensity of which is proportional to their

concentration. Albumin was assayed according to the method of Doumas *et al*., (1971) where, a green complex of albumin/bromcresol was formed at pH 4.1 and measured spectrophotometerically at 630 nm. Alanine aminotanseferase was determined following the method of Reitman and Frankel (1957). The catalytic activity was measured by spectrophotometery at 505nm.

Measurement of kidney function markers

Creatinine level was determined by colorimetric kinetic method as described by Bartels *et al*., (1972) and Larsen (1972), where Creatinine in alkaline solution reacts with picric acid to form a colored complex. Urea in the serum originated, by means of the coupled reactions described by Fawcett and Soctt (1960).The blue dye indophenol product reaction absorbs light between 530 nm and 560 nm proportional to initial urea concentration. Uric acid level was determined as described by Barham and Trinder (1972), where the uric acid was hydrolyzed enzymatically to release hydrogen peroxide which reacts with 4-aminoantipyrine in the presence of 3,5, Dichloro-2- hydroxybenzensulphonate to form a quinoneimine.

Lipid profile

The cholesterol was determined after enzymatic hydrolysis and oxidation according to Richmond, (1973) and Allain *et al*., (1974). The quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. The HDL-cholesterol was determined by enzymatic colorimetric method as described by Burstein *et al*., (1970) and Lopez–Virella *et al*., (1977) where phosphotungstic acid and magnesium ions selectively precipitating all lipoproteins except the HDL fraction – cholesterol present in the supernatant can be determined by the same method used for total cholesterol. LDL-cholesterol was computed mathematically according to Friedwald's equation (Friedwald, 1972): LDL=TC-(HDL+TG/5)

Measurement of malondialdehyde (MDA) and glutathione peroxidase (GPx)

Malondialdehyde level was determined by the method described by Ohkawa (1979). Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature of 95°C for 30 min. to form thiobarbituric acid reactive product, the absorbance of the resultant pink product can be measured at 534 nm. The assay is an indirect measure of the activity of c-GPx. Oxidized glutathione (GSSG), produced upon reduction of organic peroxide by c-GPx, and is recycled to its reduced state by the enzyme reductase (GR).

Histological examination

Liver specimens of each group were sliced and immediately fixed in 10% formalin. A rotator microtome was used to cut tissue into 4 μm thick sections and mounting was carried out on clean glass slides. Finally, staining with conventional hematoxyline and eosin (H&E) stain for examination of any histopathological changes was carried out

Statistical analysis The data was given as individual values and as mean ± standard error. Comparisons between the means of various treatment groups were analyzed using one way ANOVA. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using the statistical software SPSS, version 10.

RESULTS

Antioxidant activity

Fig. (1) and (2) show the inhibition of linoleic peroxidation by Silymarin and GSE respectively in comparison with ascorbic acid as a standard. From fig.(1), it was evident that the silymarin water extract could inhibit the peroxidation of

linoleic more than ascorbic acid especially in the third and fourth days.

On the other hand, from Fig. (2) the grape seed extract almost has the same effect of ascorbic acid on the inhibition of linoleic acid peroxidation.

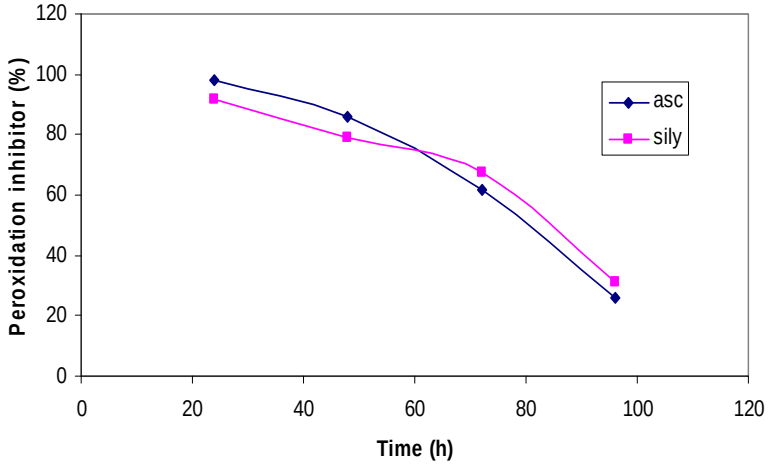


Fig. 1: Inhibition of linoleic peroxidation by ascorbic acid as a standard and silymarin as measured by the FTC method. Absorbance values represent means of triplicates of different samples analyzed.

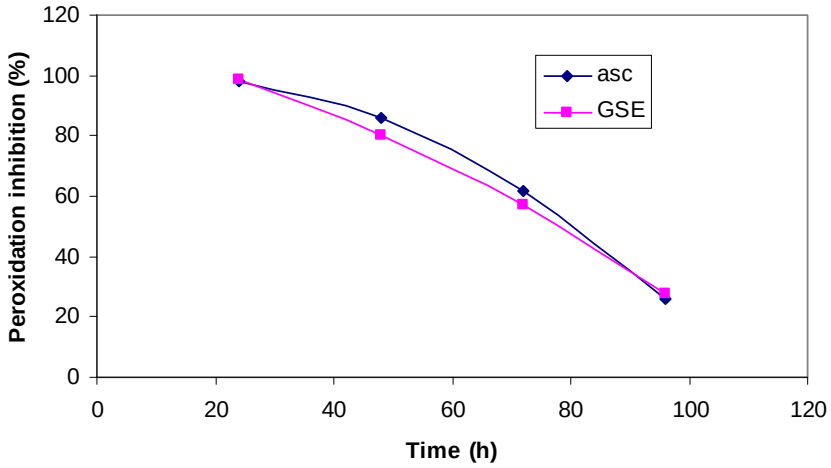


Fig. 2: Inhibition of linoleic peroxidation by ascorbic acid as a standard and GSE as measured by the FTC method. Absorbance values represent means of triplicates of different samples analyzed.

1. HPLC Results

1.1. Resulting chromatogram and gradient elution schedule for HPLC-UV analysis of Silymarin Extract

From the resulting chromatogram that is shown in Fig (3), the main compound in this extract was taxifolin and its derivative, the silybinin A and silybinin B were present in significant amounts. These compounds were identified by their retention times against standard samples.

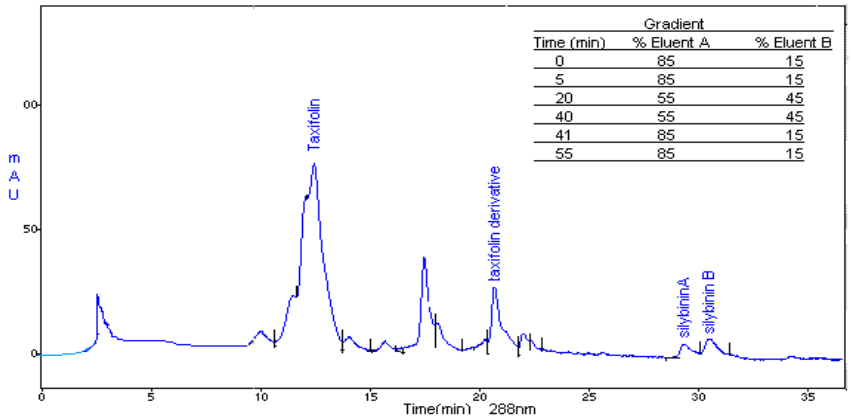


Fig. 3: Resulting chromatogram for HPLC-UV analysis of Silymarin Extract.

1.2. Resulting chromatogram and gradient elution schedule for HPLC-UV analysis of Grape Seed Extract

HPLC-UV analysis: Due to the high polarity of compounds presences in the extract, it was analyzed by HPLC-UV and the resulting chromatogram is shown in Fig. (4). The two main compounds in this extract were gallic acid and epicatechin. Catechin is also present in a significant amount, as well. These compounds were identified by their retention times against standard samples.

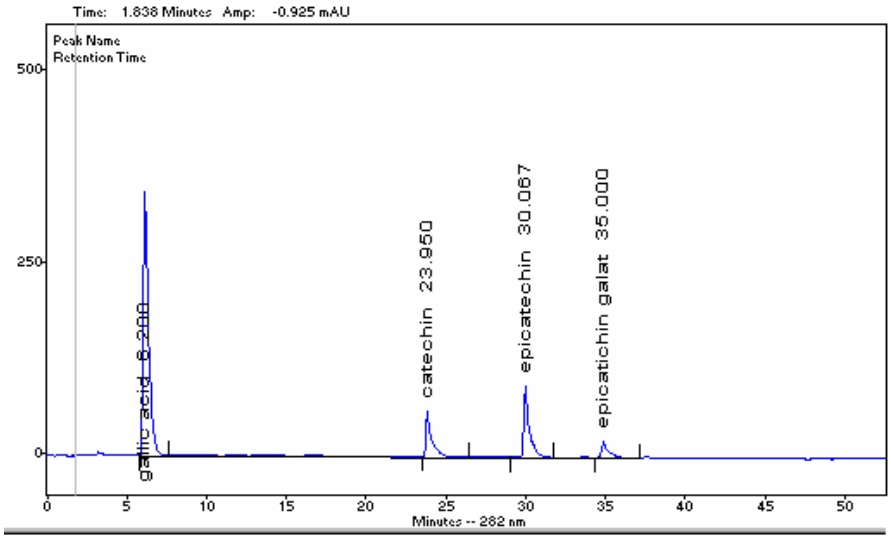


Fig. 4: Resulting chromatogram for HPLC-UV analysis of Grape Seed Extract.

2. Biochemical Results

2.1. Treated results

2.1.1. Serum levels of alanine aminotransferase (ALT)

The treated group (FB1-gp) on which the rats fed on fumonisin B1 contaminated corn, has recorded a very significant elevation in the mean serum activity level of alanine aminotransferase (ALT), that elevation reached to 78 % (p = 0.000), in comparison with control group (C-gp). S-gp and G-gp could significantly lower the mean serum activity level of ALT by 25 % (p = 0.001), and 26.7 % (p = 0.000), respectively in respect to that of FB1-gp, but significantly higher than that of C-gp by 33 % (p = 0.016), and 30 %, (p = 0.022) respectively. In case of S+G-gp where the rats fed diet supplemented with both silymarin and grape seed extracts, they could return back the mean serum

activity level of ALT to control level that it was significantly lower than that of FB1-gp by 32 % (p = 0.000), (Fig.5).

2.1.2. Serum levels of total protein

There was no significant change in the mean serum level of total protein in the FB1-gp, when compared to that of C-gp, (p > 0.05). In the S-gp, there was no significant difference in the mean serum level of total protein when compared to that of C-gp or FB1-gp, (p > 0.05). The G-gp has been recorded a significant elevation in the mean serum level of total protein by 27 % (p = 0.000), 15 % (p = 0.006) and 22.8 % (p = 0.000) when compared to that of C-gp, FB1-gp and S-gp, respectively. In case of S+G-gp, the mean serum level of total protein significantly increased by 14 % (p = 0.014) when compared to that of C-gp, (Fig. 5).

2.1.3. Serum levels of Albumin

There was no significant difference in the mean serum level of albumin in the (FB1-gp) in comparison with that of C-gp (p > 0.05). G-gp showed a significant increase in the mean serum level of albumin by 16 % (p = 0.048) and 25 % (p = 0.005) in respect to that of C-gp and FB1-gp respectively. The S-gp showed a significant increase in the mean serum level of albumin by 16% (p=0.028) when compared to that of FB1-gp. On the other hand, the S+G-gp showed a significant reduction in the mean serum level of albumin by 23% (p = 0.001), 16% (p = 0.011), 28% (p = 0.001), and 33% (p = 0.000) in comparison with C-gp, FB1-gp, S-gp, and G-gp, respectively, (Fig. 5).

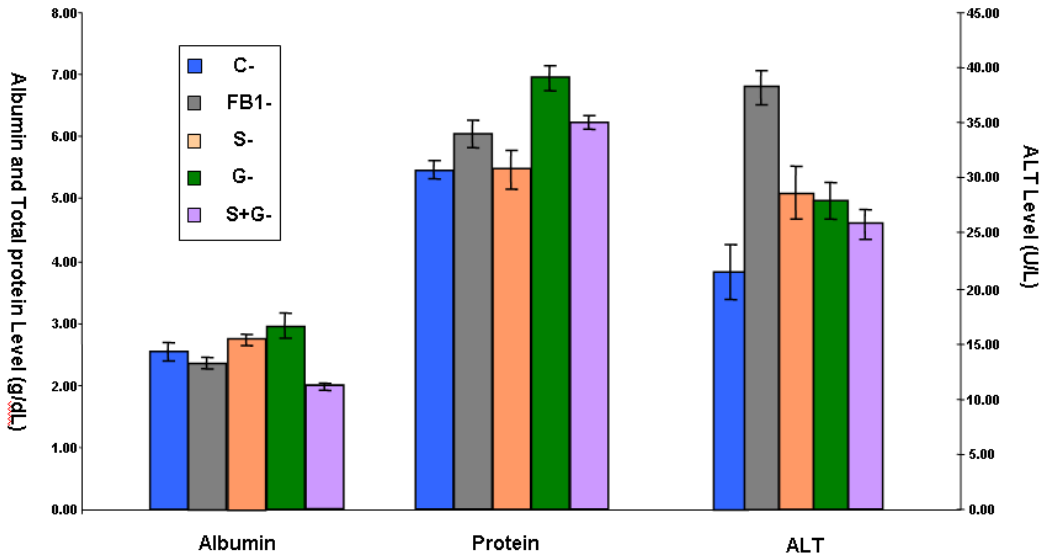
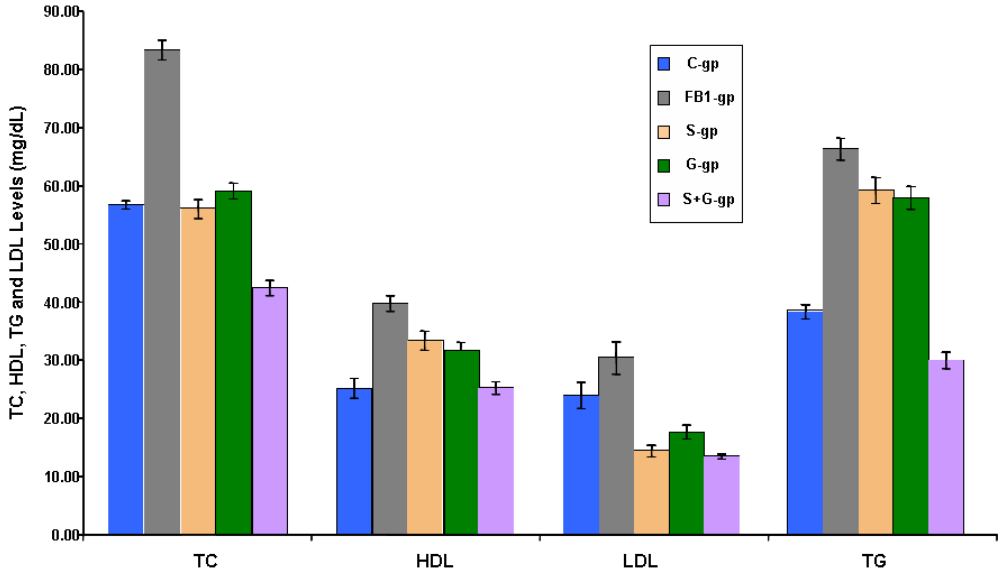


Figure (5): Serum Activity Levels of ALT, Albumin, and Total Protein in Treated groups

2.1.4.Serum Total Cholesterol (TC) level

The FB1-gp has recorded a significant increase in the mean serum level of total cholesterol (TC) by 51 % (p = 0.000) when compared to that of C-gp. The treatment of rats with S after FB1 significantly decreased the mean serum level of TC by 32.6 % (p = 0.000) in respect to that of FB1-gp that it returned back to the normal level. The administration of G after FB1 in the G-gp significantly decreased the mean serum level of total cholesterol by 27.5 % (p = 0.000) when compared to that of FB1-gp and showed no significant difference when compared to that of S-gp (p > 0.05). On the other hand the mean serum level of TC of the group of rats that fed on both S+G after FB1 administration significantly lower than that of C-gp, FB1-gp, S-gp, and G-gp by 23 % (p = 0.000), 49 % (p = 0.000), 24.5 % (p = 0.000), and 30 % (p = 0.000) respectively, (Fig. 6).

Figure (6): Serum Activity Levels of TC, HDL,TG and LDL in Treated Groups



2.1.5. Serum High Density Lipoprotein Cholesterol (HDL-C) level

Feeding the rats with FB1 contaminated corn significantly increased the mean serum level of HDL-C in the FB1-gp by 58% (p = 0.000) when compared to that of C-gp. The treatment of rats after FB1 with S significantly decreased the mean serum level of HDL-C by 16% (p = 0.003) when compared to that of FB1-gp. The mean serum level of the G-gp significantly reduced by 20% (p = 0.000) when compared to that of FB1-gp, but still higher than that of C-gp by 25% (p = 0.000). The S+G-gp could return back the mean serum level of HDL-C to normal level when compared to that of C-gp, on other words could significantly decrease the mean level of HDL-C by 36% (p = 0.000 in respect to that of FB1-gp, (Fig. 6).

2.1.6. Serum Low Density Lipoprotein-Cholesterol (LDL-C) level

A significant increase in the mean serum level of LDL-C was observed in the FB1-gp, this elevation reached to 27% (p = 0.019) in comparison with that of C-gp. The S-gp has been recorded a significant decrease in the mean serum level of LDL-C by 40% (p = 0.001) and 52% (p= 0.000) in respect to that of C-gp and FB1-gp. The G-gp has been recorded a significant reduction in the mean serum level of LDL-C by 25% (p= 0.027) and 42% (p = 0.000) when compared to that of C-gp and FB1-gp. The S+G-gp have been recorded a significant reduction in the mean serum level of LDL-C by 43% (p = 0.001) and 55% (p= 0.000), when compared to that of C-gp and FB1-gp, but showed no significant difference when compared to that of the other groups (p > 0.05), (Fig. 6).

2.1.6. Serum Triglycerides (TG) level

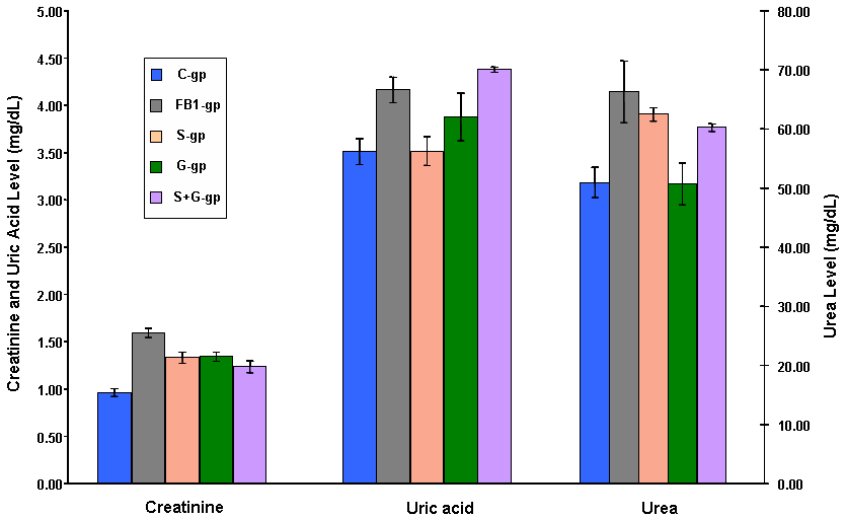
The FB1-gp have been recorded a very significant elevation in the mean serum level of triglycerides (TG) by 72 % (p = 0.000) when compared to that of C-gp. The treated groups S-gp and G-gp recorded a significant decrease in the mean serum level of TG by 10% (p = 0.009) and 12% (p = 0.002) respectively in comparison with that of FB1-gp, but showed a significant increase in the mean serum level of TG by 54% (p = 0.000) and 50% (p = 0.000) respectively when compared to that of C-gp. The S+G-gp have been recorded a significant reduction in the mean level of TG by 21% (p = 0.003), 54% (p = 0.000), 49% (p = 0.000), and 48% (p = 0.000) when

compared to that of C-gp, FB1-gp, S-gp, and G-gp, respectively, (Fig. 6).

2.1.7. Serum Creatinine level

A significant increase in the mean serum level of creatinine was observed in the FB1-gp by 65% (p = 0.000) when compared to that of C-gp. The mean serum level of creatinine in S-gp and in G-gp recorded a significant reduction by 16% and 15% when compared to that of FB1-gp but recorded a significant increase by 38% (p = 0.000) and 39% (p = 0.000) respectively when compared to that of C-gp. The S+G-gp showed a significant reduction by 22% in the mean serum level of creatinine in respect to that of FB1-gp (p=0.000) but recorded a significant increase by 28% in comparison with that of C-gp (Fig. 7).

Figure (7): Serum Activity Levels of Creatinine, Urea and Uric Acid in Treated Groups



2.1.8. Serum Uric Acid level

The FB1-gp in which the rats fed on FB1 contaminated corn showed a significant increase in the mean serum level of uric acid by 18% (p=0.006) when compared to that of C-gp. The S-gp showed a significant decrease in the mean serum level of uric acid by 15% (p= 0.006) in respect to that of FB1-gp. No significant difference in the mean serum level of uric acid of the groups of rats that fed on S and G separately after FB1 administration when compared to that of C-gp ($p > 0.05$). On the other hand the group of rats that fed on both S+G after FB1 administration significantly increased the mean serum level of uric acid by 24% (p = 0.001), 24% (p = 0.001), and 13% (p = 0.034) when compared to that of C-gp, S-gp, and G-gp respectively. No significant difference in the mean level of uric acid in the S+G-gp when compared to that of FB1-gp ($p > 0.05$), (Fig. 7).

2.1.9. Serum Urea level

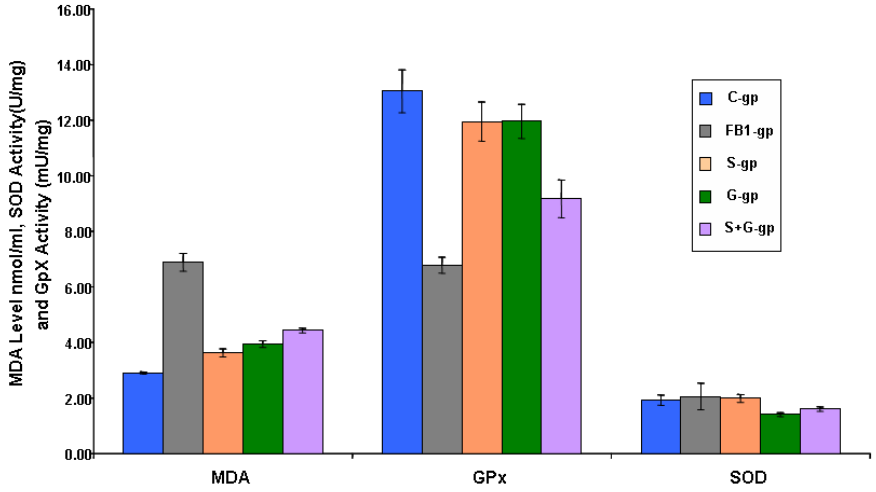
The FB1-gp showed a significant increase in the mean serum level of urea by 30 % (p = 0.003) when compared to that of C-gp. The G-gp could return back the mean serum level of urea to normal level when compared to that of C-gp that means could significantly decreased the mean serum level of urea by 23 % (p = 0.002) when compared to that of FB1-gp. It could also significantly decreased the mean serum level of urea by 18% (p=0.013) in comparison with that of S-gp. The S-gp recorded a significant increase in the mean serum level of urea by 22% (p=0.015) in respect to that of C-gp.The S+G-gp showed no significant difference in the mean serum level of

urea when compared to that of FB1-gp ($p > 0.05$), (Fig. 7).

2.1.10. Serum Malondialdehyde (MDA) level

The FB1-gp has been recorded a very significant elevation in the mean level of Malondialdehyde (MDA), this elevation reached 137% ($p = 0.000$), about three folds higher than that of C-gp. A significant decrease in the mean level of MDA was observed in S-gp, G-gp and S+G-gp by 47% ($p = 0.000$), 42% ($p = 0.000$) and 29 % ($p = 0.000$) respectively in comparison with that of FB1-gp, while they have been recorded a significant increase in the mean level of MDA by 24% ($p = 0.006$), 35% ($p = 0.000$) and 52% ($p = 0.000$), respectively in respect to that of C-gp, (Fig. 8).

Figure (8): Serum Activity Levels of MDA, GPx, and SOD in Treated Groups



2.1.11. Liver glutathione peroxidase (GPx) level

A significant decrease in the mean activity level of glutathione peroxidase (GPx) in liver by 48 % ($p = 0.000$) has been recorded by FB1-gp when compared to that of C-gp. The treatment of rats with both S and G separately after FB1 administration significantly increased the mean serum activity level of GPx by 76% ($p = 0.000$) in respect to that of FB1-gp. In case of S+G-gp it has been recorded a significant increase in the mean activity level of GPx in liver by 35% ($p = 0.002$) in respect to that of FB1-gp, but still less than that of C-gp, S-gp and G-gp by 29% ($p = 0.000$), 23% ($p = 0.000$), and 23% ($p = 0.001$) respectively, (Fig. 8).

2.1.12. Serum level of superoxide dismutase (SOD)

Administration of FB1 showed no significant difference in the mean serum activity level of superoxide dismutase (SOD) when compared to that of C-gp, ($p > 0.05$). The treatment of rats with S, G, and S+G after FB1 administration showed no significant difference in the mean serum activity level of SOD when compared to that of C-gp and FB1-gp, ($p > 0.05$) as the same among the treated groups when compared to each other, ($p > 0.05$), (Fig. 8).

Histopathological results

Microscopic investigations of liver sections (stained with H & E) showed the normal and histopathological features of the liver of control and all treated groups. Control animals showed the normal liver lobular architecture which is formed of radiating strands of cells (hepatocytes) around a central vein, hepatocytes are polyhedral in shape with relatively large sizes with prominent centrally located nuclei. Between cell strands there are sinusoids which are narrow blood spaces with irregular boundaries composed essentially of a single layer of fenestrated

endothelial cells (Fig. 25 A). No abnormal changes were seen in any of the livers of the control rats.

In treated groups, feeding of rats with fumonisin B1 contaminated corn (250 mg/kg diet) caused toxic injury in livers of all treated animals, FB1 treatment resulted in extensive hepatocellular damage, as evidenced by the presence of portal inflammation, pyknotic cells, congestion of terminal hepatic venules (central veins) and portal vein branches and prominent sinusoidal dilation. FB1 treatment showed an increase in the intensity of the portal inflammatory infiltrate. Moreover, changes of cell size with less cytoplasmic inclusions and appearance of large vacuoles were observed. Signs of necrosis in some hepatic areas were also marked in FB1 treated animals (Fig. 9 B & C).

On the other hand, the rats fed on GSE, silymarin, and both of them after FB1 administration showed less signs of amelioration. Although mild cloudy cells, pyknotic nuclei, infiltration of lymphocytes, ruptured boundaries of vascular wall and appearance of larger sinusoids are still found; the regeneration of hepatocytes, activation of Kupffer cells, and rearrangement of hepatocytes displaying architecture with less appearance of necrotic areas (Fig. 10).

DISCUSSION

Fumonisins are a group of mycotoxins that cause different diseases in animals. Biochemically, fumonisin B1 inhibits ceramide synthase (sphinganine and sphingosine-N-acetyltransferase), leading to the accumulation of sphingoid bases, sphingoid base metabolites, and depletion of more complex sphingolipids (Merrill *et al*., 1996). In addition, fumonisin B1 produces reactive oxygen species (ROS).

ROS are capable of reacting with proteins, lipids, and nucleic acids leading to lipid peroxidation in biological membranes. This in turn affects enzymatic processes such as ion pump activity and damages the DNA, thereby inhibiting transcription and repair (Chatterjee *et al*., 2000; 1999). The discovery of new antioxidants has witnessed unprecedented interest in recent years owing to their potential applications in the treatment of ROS-induced diseases.

There have been considerable public and scientific interests in the therapeutic benefits of natural antioxidants, such as polyphenols. Epidemiological studies have shown relationships between the consumption of polyphenol-rich foods and the prevention of diseases such as cancer and coronary heart disease (Adlercreutz & Mazur, 1997; Steinmetz & Potter, 1996).

The choice of male rats in the present study was based on previous studies that showed male rats to be more sensitive to the effects of fumonisins than female ones (Voss *et al*., 1998). In the past, Gelderblom *et al*. (1991) and Voss *et al*. (1995) confirmed that the FB1-induced hepatotoxic and nephrotoxic effects which were manifested by a significant ($P< 0.05$) increase in the clinical chemical parameters associated with liver and kidney function.

Liver Function: The data indicated an elevation in the serum ALT activity representing the damage of heptocytes as a result of FB1 toxicity. Orsi *et al*. (2007, 2009) reported that FB1 increased the ALT activity in rabbits confirming that the liver is one of the main target organs. Thus agreeing with Voss *et al*. (1993, 1995) and Bondy *et al*. (1996) who found that ALT activity was significantly increased in the sera of animals fed a FB1 contaminated diet.

On the other hand, the total protein has been elevated in mice due to FB1 toxicity; a study was done by Voss *et al*. (1995) and confirmed by Bondy *et al*. (1995) who recorded an elevation in albumin level too. Those studies

disagreed with Orsi *et al*. (2007), where they reported that FB1 decreased the total protein and albumin levels in rabbits. The current work does not record any change on both levels of total protein and albumin. That contradiction might be due to the sensitivity differences for each animal study in their work and the current work. Where Orsi *et al*., (2009) has reported that rabbit is more susceptible to FB1 than other animals that could lead to complete liver damage.

Kidney function: Riley *et al*. (1994) were the first group who reported that the FB1 disrupted significantly the sphingolipid metabolism in the kidneys of rats receiving FB1 contaminated diet. That result was supported by Gumprecht *et al*. (1995) and Bucci *et al*. (1998), who showed nephrotoxic effects due to significant increase in the creatinine, urea and uric acid concentrations. Recently, Orsi *et al*. (2009) reported an increase in the urea and creatinine concentrations demonstrating that the kidney is the main target organs of FB1, thus confirming our findings where creatinine, urea and uric acid concentrations were significantly elevated.

The Lipid profile: The data indicated an elevation in the Lipid profile including total cholesterol (TC), HDL-C, LDL-C and TG concentrations, that indicating hepatic injury. This is in agreement with similar findings that obtained by previous works. Voss *et al*. (1995), Edrington *et al*. (1995) and Bondy *et al*. (1996) found that a significant elevation on total cholesterol level has been recorded due to FB1 toxicity. Those findings were confirmed by El-Nekeety *et al*. (2007). Likewise, TG concentration was significantly increased in the sera of animals fed a diet containing FB1 (Voss *et al*., 1993; Edrington *et al*., 1995; El-Nekeety *et al*., 2007). As mentioned before, Bondy *et al*. (1995) found an elevation in the cholesterol concentration which agrees with our investigations but the same study reported that TG was unaffected by FB1, this disagrees with the current results which indicated an elevation in the TG concentration.

The elevation in serum cholesterol (HDL-C, LDL-C) has been suggested as an early sign of FB-toxicosis, that increase has been reported in broilers (Ledoux *et al*., 1992), calves (Osweiler *et al*., 1993), and swine (Colvin *et al*., 1993). Elevated serum cholesterol and triglycerides are probably associated with biliary obstruction and acute hepatic injury as explained by Edrington *et al*. (1995). El-Nekeety *et al*. (2007) attributed the increase in LDL and HDL levels to the need for an increase in lipoprotein to carry the water insoluble cholesterol which is elevated in FB-induced toxicity.

Oxidative stress: Another consequence of FB1 toxicity is oxidative stress. A significant elevation in the concentration of the lipid peroxidation product MDA was recorded in the FB1-gp when compared to that of the control gp that indicates that FB1 stimulates the production of ROS. In the FB1-gp a great reduction in the GPx activity was recorded. These findings were characteristic of the hepatic and renal injury. FB caused enhanced lipid peroxides in rats which resulted in free-radical-mediated toxicity (El-Nekeety *et al*., 2007). The targets of oxidative damage are usually critical biomolecules such as nucleic acids, protein and lipids (Hoehler, 1998). Stockmann-Juvala *et al*. (2004) proved that FB evokes oxidative stress, which may contribute at least in part to FB toxicity and carcinogenicity.

Glutathione plays an important role in the detoxification of the reactive and toxic metabolites of mycotoxins. Several studies have reported that liver necrosis begins when the glutathione stores are almost depleted (Mitchell *et al*., 1973; Abdel-Wahhab and Aly, 2003; 2005). Moreover, the reduced level of GPX may be explained by the association of GPX with FB1 or its metabolites (Abel and

Gelderblom, 1998; Abdel- Wahhab *et al*., 2002).

The treatment with silymarin:

The liver function:

Because the liver performs many vital functions in the human body, liver damage causes unbearable problems (Mitra *et al*., 1998; Chattopadhyay, 2003). Thus studying hepatoprotective compounds is important. Dietary polyphenols are thought to be beneficial to human health by exerting various biological effects such as free radical scavenging, metal chelation, modulation of enzymatic activity, and alteration of signal transduction pathways (Singh & Aggarwal, 1995; Stocker, 1999; Yoshioka *et al*., 1995).

In the treated group (S-gp) silymarin dramatically reduced the elevation of ALT activity and increased the albumin level. Silymarin is frequently used in the treatment of liver diseases where it is capable of protecting liver cells directly. It does this through stabilizing the cell membrane by preventing liver glutathione depletion (Valenzuela *et al*., 1989) and inhibiting lipid peroxidation (Mira *et al*., 1994). The stimulatory effect of silymarin on liver regeneration was observed only in damaged livers (Sonnenbichler and Zetl, 1986), indicating that silymarin increases regeneration potency of damaged liver tissues. The pharmacological properties of silymarin involve regulating cell membrane permeability and integrity, inhibiting leukotriene, scavenging reactive oxygen species, suppressing NF-kB activity, depression of protein kinases, and collagen production, silymarin is able to stabilize cellular membrane (Saller *et al*., 2001).

The kidney function:

High values of creatinine, urea and uric acid as results of FB1 toxicity indicate kidney damage. In the treated S-gp, milk thistle extract decreased the creatinine and uric acid levels but could not change the urea level. In animal studies, silymarin has shown protective effect against damage to kidney from acetaminophen, cisplatin (platinol), and vincristine (oncovin) partly by reducing lipid peroxidation (Sonnenbichler *et al*., 1999). It is important that the protective agent is present in renal tissue before damage occurs. Karimi *et al*. (2005) studied the protective effect of silymarin against cisplatin nephrotoxicity. They supposed that complete protection did not result when silymarin was given after administration of cisplatin. Silymarin mechanism for protection against cisplatin toxicity may be due to the inhibition of lipid peroxidation by scavenging free radicals and increasing intracellular glutathione.

The lipid profile:

In the treated group (S-gp), silymarin dramatically reduced the elevated levels of TC, LDL and TG when compared to that of FB1-gp. Silymarin, an antioxidant has long been used in the treatment of liver diseases (Naveau, 2001; Laekeman *et al*., 2003). The antihypercholesterolemic effect of silymarin was associated with liver cholesterol reduction (Krecman *et al*., 1998), which improves cholesterol uptake from blood (Steinberg *et al*., 1989).

The oxidative stress:

In case of treated group (S-gp), silymarin reduced MDA levels and increased the GPx activity as it did in the protective group. Herbal polyphenolic compounds like in milk thistle extract can function as antioxidant and antiprooxidant by scavenging reactive oxygen species via enzymatic and non-enzymatic reactions (Pyo *et al*., 2004; Marja *et al*., 1999; Sakihama *et al*., 2002). Silymarin components inhibit linoleic acid peroxidation catalyzed by lipoxygenase (Fiebrich and Koch, 1979) and also protect rat liver mitochondria and microsomes in vitro against the formation of lipid peroxides induced by various agents (Bindoli *et al*., 1990).

Silymarin can also interact directly with cell membrane components to prevent any abnormalities in the content of lipid fraction responsible for maintaining normal fluidity (Muriel and Mourelle, 1990).

The treatment with Grape seed extract:

The liver function: In the treated group, GSE significantly reduced the elevation of ALT activity, but in contrast to its action in the protected group GSE elevated the albumin and total protein levels suggesting that GSE could partly return the liver to its normal condition. Yousef *et al*. (2009) reported that animals receiving combined cisplatin-GSE treatment showed significant alleviation of the decreased values of proteins compared to cisplatin treated group. Total protein concentration is likely to be decreased if there is inhibition of protein synthesis or if degradation of protein is promoted (Heidenreich *et al*., 1999). GSE significantly attenuated the hepatotoxicity as an indirect target of cisplatin (Yousef *et al*., 2009), this effect of GSE pretreatment supports the idea that it is bioavailable and exhibits potent antioxidant and anti-inflammatory effects (Bagchi *et al*., 2002).

The Kidney function:

The concentrations of urea and creatinine determine renal function and are thus biomarkers for kidneys disease (Levey *et al*., 1999). GSE indirectly corrects body homeostasis through its improvement of kidney function. In addition to those studies our lab studied the nephrotoxocity of GSE and reported that GSE has no nephrotoxicity (Abd El-wahab *et al*., 2008). The current study proved that GSE has nephroprotective effect too, where; GSE could return back the creatinine and urea levels to normal. GSE could not change the uric acid elevated levels in the treated G-gp, it might be effective only when it is administrated before and not after the toxicity.

The Lipid Profile: Oral administration of proanthocyanidins from grape seed produced a hypocholesterolemic effect in a high cholesterol animal feed model; specifically it prevented an increase in total and LDL plasma cholesterol (Fine, 2000, El-Adawi *et al*., 2006). These findings are confirmed by Yousef *et al*. (2009) who showed that administration of GSE combined with cisplatin has been reduced the levels of cholesterol when compared to cisplatin group. GSE significantly decreased the levels of TC, HDL-C, LDL-C, and TG in the treated group. El-Adawi *et al*. (2006) reported that GSE-supplemented diet exhibited an obvious hypolipidemic effect in rats fed on high cholesterol diet. GSE could reduce the TC, LDL and TG in GSE pre and post treated groups. The hypolipidemic effect of GSE may result from increasing the rate of cholesterol catabolism by increasing the activity of hepatic cholesterol 7-a-hydroxylase enzyme. This enzyme is the rate-limiting enzyme of bile acid biosynthesis, thus suggesting that GSE could stimulate the conversion of cholesterol to bile acids, an important pathway of elimination of cholesterol from body (Del Bas *et al*., 2005). The water-soluble antioxidant, proanthocyanidins in the GSE might trap ROS in aqueous series such as plasma thereby inhibiting oxidation of LDL.

Oxidative stress:

Increased lipid peroxidation is thought to be a consequence of oxidative stress. This occurs when the dynamic balance between prooxidant and antioxidant mechanism is impaired (Kumari and Menon, 1987). ROS may attack any type of molecules, but their main target appears to be polyunsaturated fatty acids, which is the precursor of lipid peroxide formation (Gutteridge, 1982). ROS are highly toxic byproducts of aerobic metabolism; react unfavorably with surrounding macromolecules

resulting in severe cell and tissue damage. The antioxidant and free radical scavenging ability of GSE may be to the ability of polyphenols in the extract, especially flavonoids, to modulate the expression of gamma-glutamyl cystein synthetase which catalyses the rate limiting step in the production of endogenous antioxidant in cells, specifically glutathione. Thus, this enzyme plays a crucial role in cellular antioxidant mechanism and xenobiotics' detoxification (Moskaug *et al*., 2005). Consequently, the protective effects of the gamma-glutamyl cysteine synthetase administered in this study may be due to the elevated GPx activity when compared to that of the FB1-gp.

The current work reveals that the activity of SOD was not affected suggesting that the formation of superoxide is not a prominent feature during FB1-induced hepatotoxicity as was suggested previously (Sahu *et al*., 1998; Marnewick *et al*., 2009). GSE significantly reduced the level of MDA and elevated the GPx activity in comparison with FB1-gp. It is proposed that the consumption of flavonoid-rich foods and beverages helps to limit oxidant damage in the body (Van Acker *et al*., 1996; Yamanaka *et al*., 1997). In vitro experimental results have demonstrated that proanthocyanidins have specificity for the hydroxylradical (Fine 2000; Zayachkivska *et al*., 2006). Also, in the treated group (G-gp) GSE significantly decreased the levels of MDA with an elevation of the GPx activity means that GSE post treatment could have the same effect of pretreatment. Tebib *et al*. (1997) reported that plasma tissue MDAs in rats fed polymer grape seed tannins were reduced, it can be expected that plasma LDL would be less oxidized, strengthens the beneficial effect of decreased LDL-C concentration.

The biochemical function of the selenium containing enzyme glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Ran *et al*., 2007). So, any increase in the GPx activity results in a decrease in lipid peroxide levels, thus improving the lipid profile. This may explain the reduced level of MDA and the near normal levels of TC and LDL-C in the group receiving GSE when compared to that of the FB1-gp. The chemical properties of proanthocyanidins in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers and singlet oxygen quenchers predict their antioxidant activity (Bagchi *et al*., 2000) which has been proven to be significantly more potent antioxidants than vitamins C and E or beta-carotene (Joshi *et al*., 2001).

The water extract of grape seeds contains a plethora of constituents. HPLC analysis of the used extract showed gallic acid, catechin, epicatechin, and other constituents. Although some flavonoids have pro-oxidant activity, most are potent antioxidants. Catechins for example are stronger antioxidants than ascorbic acid and tocopherol in aqueous and lipid phase models, respectively (Cao *et al*., 1997). The catechins,(-)-epigallocatechin 3-o-gallate (Matsuzaki and Hara, 1985; Uchida *et al*., 1992), and procyanidolic oligomers from grape seeds (Masquelier, 1988; Meunier *et al*., 1989) are very active antioxidants in aqueous systems. This suggests that in vivo they may act as radical trappers.

Flavonoids have been shown to inhibit lipid peroxidation formation in rat tissues and also inhibit the free radical production in the cells at various stages. In this context Karthikeyan *et al*. (2007) reported that GSE treatment reduced the levels of heart TBARS in isoproterenol-induced lipid peroxidation. This indicates that the flavonoids constituents of GSE are responsible for both its antioxidant property and its ability to improve lipid profile.

Halliwell (2007) concluded that dietary polyphenols were typical xenobiotics, metabolized as such and rapidly removed from the circulation. They may be beneficial in the gut in the correct amounts, but too much may not be good. It was for the first time using mixture of milk thistle and grape seed extracts in the recovery of FB1 toxicity. Both extracts showed a great amelioration in the liver status that was evident from the histopathological results which confirm the biochemical results. In the treated group (S+G-gp), although both extracts could reduce the activity levels of ALT, they significantly reduced the albumin level and did not change the total protein in comparison to that of FB1-gp, suggesting that both extracts could not return back the injured hepatocytes to some extent. We suggest that in case of treated group, the presence of the same concentration of both extracts represents an overload of polyphenol on the treated rats which could not give the promising results as we hope. That suggestion was supported with the statement of Rucinska *et al*. (2007). Although polyphenols have beneficial antioxidant, anti-inflammatory and anticancer effects, but at higher doses or under certain conditions these compounds may exert toxic prooxidant activities.

Both extracts also reduced the levels of TC, HDL-C, LDL-C, and TG. Thus, both extracts administration before or after FB1 toxicity could partly account for the recovery of toxicity in rats.

Regarding the kidney function, when S+G administrated after induction of FB1 toxicity, a significant reduction in the level of creatinine only was recorded, but the levels of urea or uric acid remained at high levels, suggesting that both extracts could not return the damaged kidneys to their normal condition. Those results confirm again the fact that protection is better than treatment (El-Adawi *et al*., 2011), where the male rats might need longer time to recover and overcome the complications due to toxicity.

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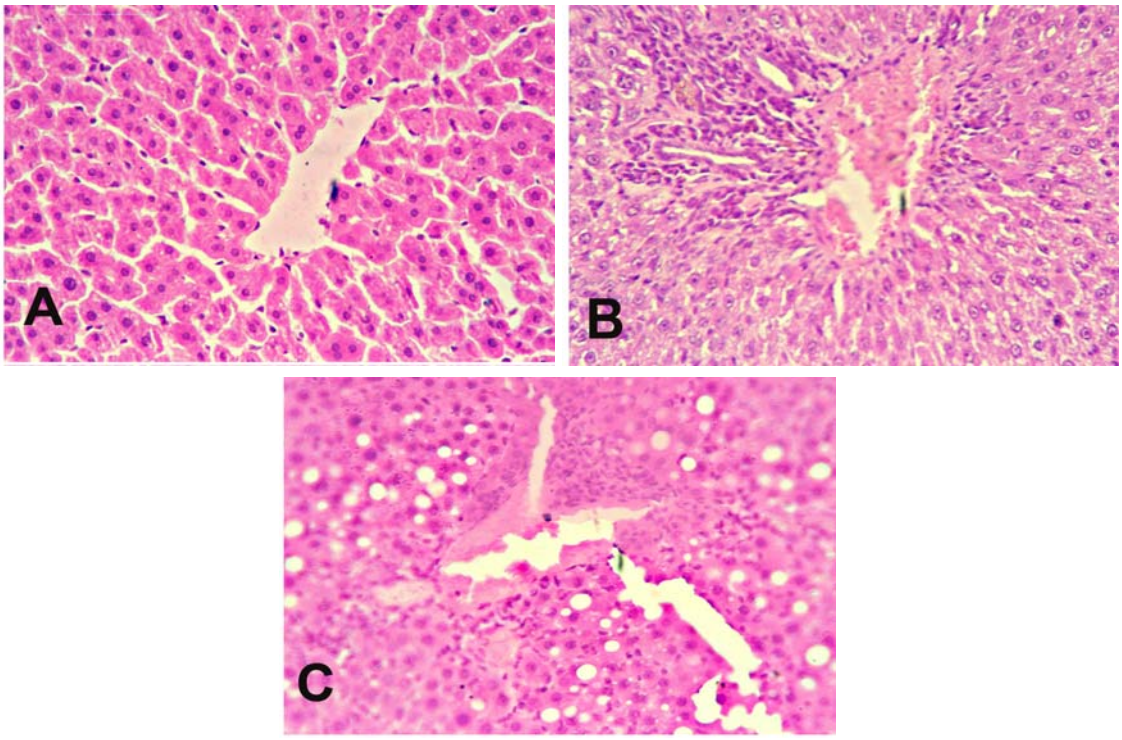

Fig. 9: Section of liver of (A) a control rat, fed on uncontaminated corn, showing normal architecture of hepatocytes, (B) a rat fed on FB1 contaminated corn showing increase of lymphocyte infiltration in portal area (blue arrow), apoptotic bodies (small arrow), pyknotic nuclei and vascular congestion, and (C) a rat in the same FB1-gp showing lymphocyte infiltration, moderate and large sized vacuoles (arrows) and lysis of some hepatocytes (H & E stain, X 400).

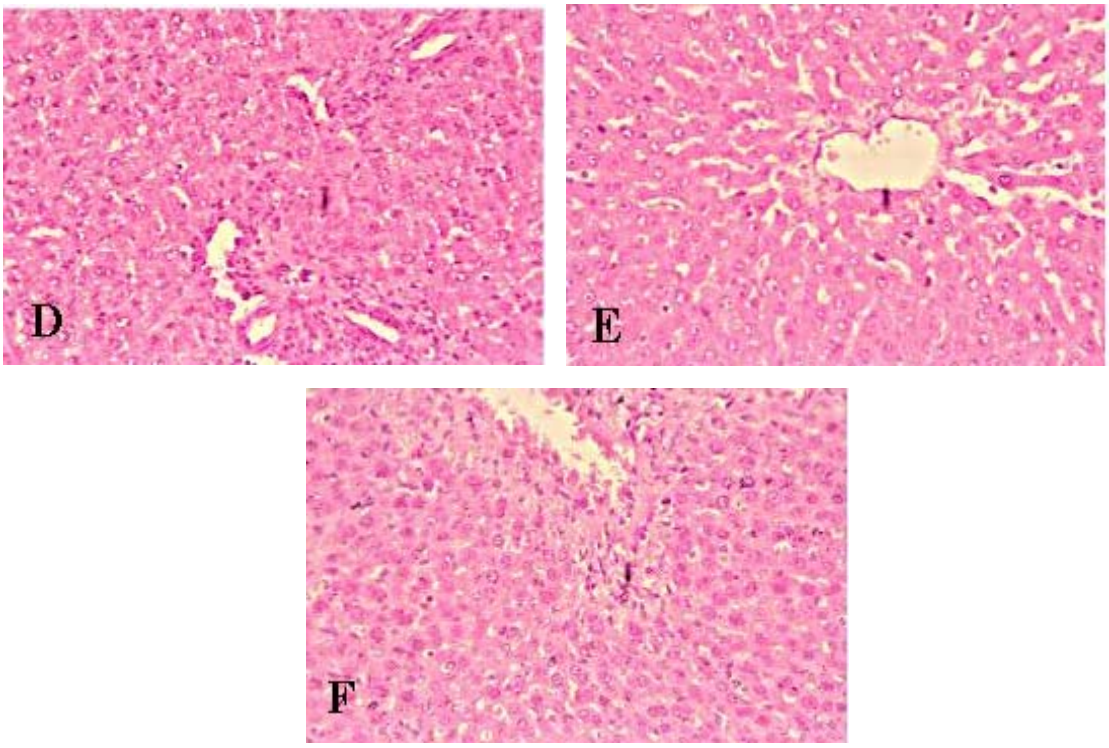

Fig. 10: Section of liver of (D) a rat fed on GSE after FB1 administration, showing cloudy cells, pyknotic nuclei and infiltration of lymphocytes, (E) a rat fed on silymarin after FB1 administration showing regeneration of hepatocytes, activation of Kupffer cells, appearance of pyknotic cells and larger sinusoids, and (F) a rat fed on both S+G after FB1 administration, showing rearrangement of hepatocytes, decrease of pyknotic cells, and appearance of degenerative cells within and surrounding vascular wall (H & E stain, X 400).

ARABIC SUMMARY

التأثير المتآزر لمستخلصات كل من نبات شوك الجمل وبذر العنب على امكانية الشفاء من سمية الفيومينيزين ب1 فى الجرذان

محمد الشافعى أحمد[1*]، هالة إبراهيم العدوى[1]، ضياء الدين بشر الأزهرى، عبير عبدالوهاب[1]، محمد أحمد عبدالمحسن

قسم التكنولوجيا الحيوية الطبية، معهد الهندسة الوراثية، مدينة الأبحاث العلمية والتطبيقات التكنولوجية، برج العرب، الإسكندرية، مصر.

قسم علم الحيوان، كلية العلوم، جامعة المنيا، المنيا، مصر.

قسم الكيمياء الطبية التطبيقية، معهد البحوث الطبية، الإسكندرية، مصر.

(E.mail: mohamedshafeey@gmail.com)

يعتبر الفيومينيزين ب1 منتج ايض ثانوى ينتج من جنس فطر الفيوزاريوم الذى ينمو على الذرة فى مناطق واسعة من العالم، ويعتبر سام على كل من الكبد والكلى. تم استحداث السمية الكبدية بواسطة تغذية 50 من الجرذان بواسطة الذرة المعدية بالفيومينيزين ب1 لمدة 60 يوم. وقد لوحظت سمية الكبد فى الجرذان بعد التغذية بالذرة المعدية لمدة 60 يوم عن طريق زيادة فى نشاط انزيم الألانين امينو ترانسفيريز حيث وصلت الزيادة إلى 78 % مقارنة بالمجموعة الضابطة. وقد أدت المعالجة بواسطة مستخلصات شوك الجمل وبذر العنب ومخلوط منهما بالرجوع بنشاط انزيم الالانين امينو ترانسفيريز الى المستوى الطبيعى. أيضا أدت سمية الفيومينيزين ب1 الى النزول بمستوى نشاط إنزيم الجلوتاثيون بيروكسيديز الى 48 % بينما المعالجة بالمستخلصات فى المجموعات المعالجة أدت الى زيادة نشاط انزيم الجلوتاثيون بيروكسيديز فى مجموعة شوك الجمل ومجموعة بذر العنب ومخلوط منهما الى 76 % و 76 % و 35 % على التوالى. تتضح أكسدة الدهون عن طريق المالوندالدهيد والذى ارتفع بفرق واضح الى 137 % فى مجموعة الفيومينيزين ب1. من ناحية أخرى فى المجموعات المعالجة فقد انخفضت نسبة المالوندالدهيد الى 47 % و42 % و 29 % فى مجموعات شوك الجمل وبذر العنب ومخلوط منهما على التوالى. وكما تم التعرف على سمية الفيومينيزين ب1 على الكبد فقد تم فحص وظائف الكلى أيضا حيث ارتفع نسبة الكرياتينين بنسبة 65 % فى مجموعة الفيومينيزين، وبعد المعالجة بالمستخلصات فى مجموعة شوك الجمل وبذر العنب ومخلوط منهما بنسب 16 %، 15 % ، و22 % على التوالى. أيضا ارتفعت نسبة اليوريا فى مصل الجرذان فى مجموعة الفيومينيزين الى 30 %، وانخفضت بشكل ملحوظ فى مجموعة بذر العنب فقط الى 23 % بينما فى مجموعة شوك الجمل ومخلوط منهما لم يتغير مستوى اليوريا فى مصل الجرذان فى هاتين المجموعتين. أكدت دراسة الأنسجة من خلال القطاعات المأخوذة فى الكبد ماتوصلت إليه النتائج فى تحاليل مصل الجرذان حيث كان هناك تحسن واضح لوحظ فى المجموعات المعالجة بالمستخلصات مقارنة بتلك الموجودة فى مجموعة الفيومينيزينك; هذه التحسينات يمكن أن تكون نتيجة لقدرة المستخلصات على إنزال مستويات الكوليستيرول الكلى وكذلك الكوليستيرول قليل الكثافة وأيضا تقليل أكسدة الدهون عن طريق تحسين نشاط الإنزيمات المضادة للأكسدة.