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Amelioration of paracetamol hepatotoxicity and oxidative stress on mice liver with silymarin and *Nigella sativa* extract supplements

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ABSTRACT

Objective: To evaluate the ameliorator property of silymarin or/and *Nigella sativa* (*N. sativa*) extract against *N*-acetyl-*p*-aminophenol (APAP)-induced injury in male mice at the biochemical, histological and ultrastructural levels.

Methods: The mice were divided into seven groups (10/group). The first group was served as control. While, the second group was treated with dose of APAP. The third and fourth groups were treated with silymarin alone and *N. sativa* extract alone respectively. The fifth and sixth groups were treated with combination of APAP with silymarin and APAP with *N. sativa* extract respectively. The seventh group was treated with combination of both ameliorative compounds (silymarin and *N. sativa* extract) with APAP and all animals were treated for a period of 30 days.

Results: Exposure to APAP at the treated dose to mice led to an alteration of liver functions, increased the alanine transaminase, aspartate aminotransferase and lactate dehydrogenase levels, decreased total protein level as well as the increasing the superoxide dismutase and malondialdehyde while decreased catalase, glutathione peroxidase and glutathione reduced activities. The effects of APAP on the biochemical parameters of mice were dose-dependent. Administration of silymarin or/and *N. sativa* extract to APAP-treated mice attenuates the toxicity of this compound, objectified by biochemical, histological and ultrastructural improvement of liver. But the alleviation was more pronounced with the both antioxidants.

Conclusions: The synergistic effect of silymarin and *N. sativa* extract is the most powerful in reducing the toxicity induced by APAP and improving the liver functions and antioxidant capacities of mice.

1. Introduction

The liver is the most significant organ of our body for detoxification; disorders to this organ remain some of the most serious health problems[1]. Liver diseases remain to be serious health problems and the management of liver disease is still a challenge to the modern medicine. Liver plays an essential role in regulation of physiological processes, involved in several vital functions such as storage, secretion and metabolism. It also detoxifies a variety of drugs and xenobiotics and plays a central role in transforming, clearing the chemicals and is susceptible to the toxicity from these agents[2].

Hepatotoxicity is a common cause of severe metabolic disorders and even death[3]. Hepatic damage occurs due to its multi-dimensional functions, various xenobiotics and oxidative stress leading to distortion of all of its functions[4].

Acetaminophen [paracetamol or *N*-acetyl-*p*-aminophenol (APAP)] is a derivative of para-aminophenol used as an analgesic and antipyretic drug belonging to the para-aminophenol class of the nonsteroidal anti-inflammatory drugs[5].

APAP (paracetamol) produces acute liver damage at very larger dose. The hepatotoxicity of APAP has been attributed to the formation of toxic highly reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) which causes oxidative stress and glutathione depletion[6]. It is a well-known antipyretic and analgesic agent, which produces hepatic necrosis at higher doses[7].

Nanji *et al*. showed that APAP is mainly metabolized in the liver to excretable glucuronide and sulphate conjugates[8]. The hepatotoxicity of APAP has been attributed to the formation of toxic metabolites when part of it is activated by hepatic cytochrome P-450 to form the highly reactive metabolite (NAPQI). Accidental or intentional intake of high doses often causes acute hepatocellular necrosis with high morbidity and mortality[9].

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Nigella sativa L. (Ranunculaceae) (*N. sativa*), commonly known as "black cumin", is an erect herbaceous annual plant. It grows in Mediterranean countries and is also cultivated in the north of Morocco. *N. sativa* seeds have traditionally been used in Middle Eastern folk medicine as a natural remedy for various diseases as well as a spice for over 2 000 years. The seeds of *N. sativa* have been subjected to a range of pharmacological, phytochemical and nutritional investigations in recent years. It has been shown to contain more than 30% (w/w) of a fixed oil with 85% of total unsaturated fatty acid[10]. The protective role of the black seed oil against hepatotoxicity has been investigated in animal experiments[11]. *N. sativa* oil (0.27 g/100 g body weight/day) was administered into adult rats and the treated animals showed decrease in serum (gamma-glutamyl transferase) nuclear DNA content which was elevated in the control group of the animals.

Silymarin contains a number of active constituents called flavolignans which are also used to help protect the liver from poisoning[12]. *Silybum marianum* (milk thistle) has been used to treat liver diseases since the 16th century. Its major constituents are flavonoids, silibinin, silidianin, silichristin and isosilibinin of which silibinin is the biologically most active compound and used for standardisation of pharmaceuti cal products[13].

The pharmacological profile of silymarin has been well defined and hepatoprotective properties of silymarin were investigated both *in vitro* and *in vivo*. Experimental studies demonstrated antioxidant and free radical scavenging properties, improvement of the antioxidative defence by prevention of glutathione depletion and anti-fibrotic activity[13].

Therefore, the present study was undertaken to evaluate the ameliorator property of silymarin or/and *N. sativa* extract on toxic status during APAP-induced injury in male mice at the biochemical, histological and ultrastructure levels.

2. Materials and methods

2.1. Chemicals

APAP was purchased from the Egyptian International Pharmaceutical Industries Company; silymarin was obtained from Sedeco Pharmaceutical Co-6-october City, Egypt. The *N. sativa* seeds were purchased from a local herb store with a fair degree of quality assurance. Seeds were washed to remove sand and other debris and air-dried and finely powdered with an electric microniser according to traditional mode of preparation[14]. Crude extract was obtained by the maceration of 800 g of these seeds by boiling in distilled water (1 200 mL) for 24 h and filtered through muslin[15]. After 24 h, the aqueous extract was filtered and concentrated at room temperature, then the dried extract was stored at 4 ℃ until use[16]. Other chemicals and reagents were of the highest analytical grade and were bought from standard commercial suppliers in Roche (Germany).

2.2. Animals

ICR male mice, weighing approximately 35-40 g were provided by Faculty of Veterinary Medicine, Zagazig University. The animals were maintained in solid bottom shoe boxtype polycarbonate cages with stainless steel wire-bar lids, using a wooden dust-free litter as a bedding material. Animals were located in air-conditioned room and were allowed free access to pellet diet and tap water for a week before starting the experiment. The European Community Directive (86/609/EEC) and national rules on animal care have been followed. After 2 weeks of acclimation, animals were randomly divided into seven groups with 10 animals in each one as following: Group 1 was served as untreated control (1 mL/kg of physiological saline); Group 2 was treated with paracetamol (2 g/kg)[17]; Group 3 was treated with silymarin (50 mg/kg)[18]; Group 4 was treated with *N. sativa* extract (0.25 g/100 g)[14]; Group 5 was treated with paracetamol and silymarin; Group 6 was treated with paracetamol and *N. sativa* and the final Group 7 was treated with combination of both ameliorative compounds (silymarin and *N. sativa* extract) with paracetamol. All the groups were treated orally for 30 successive days.

2.3. Collection of blood samples

At the end of experimental period, blood samples of the fasted mice were collected from the medial retro-orbital venous plexus immediately with capillary tubes (Micro Hematocrit Capillaries, Mucaps) under ether anesthesia[19]. Then, the blood was centrifuged at 3 000 r/min for 15 min and serum was collected for different biochemical analyses.

2.4. Hepatic biomarkers determination

Serum aspartate transaminase (AST) and alanine transaminase (ALT) activities were determined with kits from Human Diagnostic Worldwide, Germany.

2.5. Preparation of tissues for measurement of oxidative/antioxidant parameters

The tissues of liver and kidney were used for the analysis of oxidative stress and antioxidant parameters. Prior to dissection, tissue was perfused with 50 mmol/L sodium phosphate buffer saline (100 mmol/L Na_2HPO_4/NaH_2PO_4) (pH 7.4) and 0.1 mmol/L ethylenediaminetetraacetic acid to remove any red blood cells and clots. Then tissues were homogenized in 5 mL cold buffer/g tissue by a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 10 000 r/min for 20 min at 4 ℃, and the resultant supernatant was transferred into Eppendorf tubes and preserved in a deep freezer until used. The supernatant was used for the determination of some biochemical parameters of liver tissues.

2.6. Lipid peroxidation assay

The extent of lipid peroxidation was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA) by using the method of Ohkawa *et al*.[20]. Malondialdehyde concentrations were determined using 1,1,3,3-tetraethoxypropane as standard and expressed as micromoles per gram of tissue.

2.7. Antioxidant enzymes

Superoxide dismutase (SOD) activity was measured according to the method described by Marklund and Marklund in which pyrogallol underwent autoxidation at 440 nm for 3 min[21]. One unit of SOD activity was calculated as the amount of protein that caused 50% pyrogallol autoxidation inhibition. The SOD activity was expressed as units per gram tissue. Before determination of the catalase activity, samples were diluted 1:9 with 1% Triton X-100 (v/v). Catalase activity was measured according to the method

described by Aebi[22]. The hydrolysis of H_2O_2 and the resulting decrease in absorbance at 240 nm over a 3 min period at 25 ˚C was measured. Catalase activity was expressed as units per gram tissue.

2.8. Measurement of enzymes involved in glutathione metabolism

The glutathione peroxidase (GPX) activity was measured by the method of Hafeman *et al*.[23]. The reaction mixture contained 0.5 mL of 0.4 mol/L sodium phosphate buffer (pH 7.0) and 0.4 mmol/L ethylenediaminetetraacetic acid, supplemented with 0.25 mL of sodium azide (1 mmol/L), 0.5 mL of glutathione (2 mmol/L) and 0.25 mL of distal water. About 0.5 mL of homogenate was added and allowed to equilibrate for 5 min at 37 ˚C. The reaction was initiated by adding 0.5 mL of H_2O_2 (1.25 mmol/L). Absorbance at 340 nm was recorded at 1, 3, and 6 min. The activity of GPX was expressed in terms of nanomoles glutathione consumed per minute per gram of tissue (IU/g).

The glutaredoxin activity was measured by the method of Hafeman *et al*.[23]. The reaction mixture contained 1.2 mL of 67 mmol/L sodium phosphate buffer (pH 7.0), 0.2 mL of sodium azide (1 mmol/L) and 0.1 mL of oxidized glutathione (7.5 mmol/L). Homogenate (0.5 mL) was added and allowed to equilibrate for 5 min at 37 ˚C. Reaction was initiated by adding 0.25 mL of reduced nicotinamide adenine dinucleotide phosphate (2 mmol/L). Absorbance at 340 nm was recorded at 1, 2 and 3 min. The activity of glutaredoxin was expressed in terms of micromoles-mol glutathione produced per minute per gram of tissue (IU/g).

2.9. Histological evaluations

Histological examination of the tissues was conducted after removal of liver tissues from mice. The tissues were gently rinsed with a physiological saline solution (0.9% NaCl) to remove blood and adhering debris. They were then fixed in 5% formalin for 24 h, and the fixative was removed by washing overnight with running tap water. After dehydration through a graded series of alcohols, the tissues were cleared in methyl benzoate and embedded in paraffin. Sections were cut by a microtome at 6 μm thickness and stained with Hematoxylin staining as described by Gabe and counter stained with eosin dissolved in 95% ethanol (Hematoxylin and Eosin stain)[24]. After dehydration and clearing, sections were mounted with digital picture exchange and observed under a microscope. The histopathological examination was performed according to Lobenhofer *et al*.[25].

2.10. Ultrastructure examination

Liver was cut into small pieces of about 1-3 mm in size and immediately fixed in 2.5% glutaraldehyde for 24-48 h. The specimens were then washed in phosphate buffer (pH 7.2-7.4) 3-4 times for 20 min every time and post-fixed in a buffered solution of 1% osmium tetraoxide for 2 h, after that they were washed in the same buffer 4 times for 20 min each. Fixed specimens were dehydrated in ascending grades of ethyl alcohol (30%, 50%, 70%, 90% and 100%), cleared in two changes of propylene oxide, and embedded in Epon resin[26]. Semithin sections (1 μm thick) were stained with toluidine blue for 2 min and examined by the use of a light microscope. The resin blocks were retrimmed to get rid of the undesired tissue. Ultrathin sections (60-90 nm thick) and representative fields of semithin sections were selected and were cut with a diamond knife using a Reichert OMVs ultramicrotome, mounted on copper grids and double stained with uranyl acetate and lead citrate. The grids were examined and photographed using a transmission electron microscope (JEOL JEM-1200 EX II, Japan) operated at 60-70 kV, Faculty of Agriculture (Electron Microscope Unit), Mansora University.

2.11. Statistical analysis

Statistical analysis was performed using SPSS for Windows version 17.0. Data were given in the form of arithmetical mean ± SE. Differences between groups were evaluated by One-way ANOVA according to $P < 0.05$ and *post-hoc* Duncan test. For each histological parameter, a score has to be performed for analyzing a number of different histological sections in each organ for each animal and then the median value has to be calculated for each group; finally a comparison could be done by a semi-quantitative test using SPSS.

3. Results

3.1. Biomarkers of liver assessments

The total protein content decreased in paracetamol treated group depending on the dose, but increased by 36.45 and 38.14 fold in the groups treated with the silymarin and *N. sativa* extract respectively when compared with control group (Table 1). Meanwhile combination of paracetamol, silymarin and *N. sativa* extract has ameliorated total protein content as compared with control group. Serum ALT activity of silymarin and *N. sativa* extract groups was nonsignificantly increased by 5.79% and 1.5%, when compared with control group. In the paracetamol-treated groups, the ALT was increased by 85.71 fold as the dose increase when compared to control. Treatment the mice with 1 000 mg/kg paracetamol with the combination of silymarin and *N. sativa* extract decreased the activity of ALT more than each compound separately. The same observation has been noticed in the AST and alkaline phosphatase (ALP) activities that increased by giving the dose of paracetamol and decreased by the treatment of each antioxidant separately.

To investigate the hepatic cells integrity, we measured lactate dehydrogenase (LDH) activity (Table 1). It represents the marked elevation in serum LDH in paracetamol treated group by 83.85 fold as compared to control group, while groups treated with either silymarin or *N. sativa* showed nonsignificant changes in LDH activity when compared to normal control group. The best ameliorative LDH value was recorded in group treated with both silymarin and *N. sativa* extract with paracetamol.

3.2. Oxidative and antioxidant responses

As shown in Table 2, the data showed that treatment with paracetamol caused a significant decrease in the activity of SOD in liver and kidney. Administration of silymarin caused nonsignificant increase in SOD activity in liver and kidney tissues as compared with those of control mice. In addition, a significant recovery relating to SOD was observed in response to the presence of either silymarin or *N. sativa* extract or both with paracetamol in all tested tissues. Treatment of the mice with silymarin and *N. sativa* extract nonsignificantly increased SOD and slightly decreased the SOD activity of liver and kidney tissues respectively. However, co-

administration of silymarin or *N. sativa* extract with paracetamol increased the SOD activity in tested tissues but still better than administration of paracetamol alone.

The catalase activity decreased after paracetamol treatment in all examined tissues. The administration of either silymarin or *N. sativa* extract increased the catalase activity in liver and kidney tissues as compared with the paracetamol group. The treatment of the mice with silymarin and *N. sativa* extract in combination with paracetamol elevated the catalase activity in liver and kidney tissues as compared with paracetamol + silymarin or paracetamol + *N. sativa* extract.

The activity of GPX was significantly decreased in liver and kidney tissues of mice treated with paracetamol by 75.24% and 71.34% as compared with the control group, respectively (Tables 2 and 3). Treatment of the animals with silymarin or *N. sativa* extract alone caused significant changes in the activity of this enzyme as they elevated this enzyme as compared with control group. While the presence of silymarin or *N. sativa* extract in combination with paracetamol minimized the observed alterations in the examined enzyme activity induced by paracetamol intoxication in all examined tissues. Thus there was elevation in GPX activity after administration of both silymarin and *N. sativa* extract, respectively.

As shown in Tables 2 and 3, treatment of the mice with paracetamol caused a significant ($P < 0.05$) in the activity of glutaredoxin in liver and kidney tissues by 75.24 and 71.34 fold as compared with control animals, respectively. Administration of silymarin or *N. sativa* extract alone caused nonsignificant changes and slight decrease in glutaredoxin activity respectively as compared with control mice. In addition, a significant recovery relating to glutaredoxin was observed in response to the presence of silymarin and *N. sativa* extract in combination with paracetamol which showed amelioration in the enzyme activity as compared with paracetamol treated group only.

3.3. Histopathological investigations of liver tissues

Figure 1 depicts photomicrographs of Hematoxylin-Eosin stained liver tissues of control and experimental groups of mice. Figure 1A shows the section of liver tissue from control mice, demonstrating normal histology with normal central vein and normal hepatic lobule. Similarly, the sections of liver tissues from mice treated with silymarin alone also revealed normal liver tissue formed of central vein surrounded by cords of hepatocytes with normal vesicular nuclei and eosinophilic cytoplasm (Figure 1D) and group treated with *N. sativa* extract showed normal central vein surrounded by cords of hepatocytes (Figure 1E). Mice liver treated with

Table 1

Changes of serum liver functions in male mice treated with silymarin, *N. sativa* extract and paracetamol separately or in combination.

Groups	Total protein (g/dL)	AST (IU/mL)	ALT (IU/mL)	LDH (μIU/mL)
Control group	7.48 ± 0.27^{b}	12.20 ± 0.58^{e}	13.00 ± 0.70^{e}	271.66 ± 47.21^{ef}
Acetaminophen (paracetamol)	4.88 ± 0.40^{e}	167.80 ± 7.39^{a}	91.00 ± 1.18^{a}	$1\,628.40 \pm 35.37^{a}$
Silymarin	7.68 ± 0.44^{b}	13.80 ± 6.02^{de}	13.80 ± 1.82^{de}	270.30 ± 33.34^{ef}
N. sativa extract	7.89 ± 0.63^{ab}	13.10 ± 1.01^{de}	13.20 ± 2.10^{e}	265.32 ± 14.06^{f}
Acetaminophen + silymarin	5.76 ± 0.65^{d}	75.00 ± 1.67^{b}	53.20 ± 0.86^{b}	720.00 ± 30.37^{c}
Acetaminophen + *N. sativa* extract	5.24 ± 0.51^{d}	76.40 ± 3.12^{b}	55.40 ± 0.50^{b}	762.66 ± 50.63^{bc}
Acetaminophen + silymarin + *N. sativa* extract	7.02 ± 0.16^{c}	64.20 ± 4.61^{c}	45.60 ± 2.73^{c}	620.31 ± 51.26^{d}

Means within the same column in each category carrying different letters are significant at $P \leqslant 0.05$ using Duncan's multiple range test, where the highest mean value has symbol (a) and decreasing in value were assigned alphabetically.

Table 2

Changes in SOD, catalase, malondialdehyde, GR and GPX activities of liver in male mice treated with silymarin, *N. sativa* extract and paracetamol separately or in combination.

Groups	SOD (IU/g)	CAT (IU/g)	MDA (nmol/g)	GR (IU/g)	GPX (IU/g)
Control group	12.41 ± 1.79^{e}	4.49 ± 0.01^{b}	1.74 ± 0.10^{f}	6.91 ± 0.75^{a}	8.04 ± 0.74^{b}
Acetaminophen (paracetamol)	25.59 ± 1.22^{a}	1.52 ± 0.03^{f}	12.35 ± 0.26^{a}	1.12 ± 0.48^{e}	1.99 ± 0.51^{f}
Silymarin	12.76 ± 1.51^{e}	4.53 ± 0.03^{b}	1.50 ± 0.66^{g}	6.99 ± 0.58^{a}	8.64 ± 0.44^{ab}
N. sativa extract	11.66 ± 1.18^{f}	4.63 ± 0.04^{ab}	2.91 ± 0.12^{e}	5.30 ± 0.94^{c}	6.50 ± 1.34^{c}
Acetaminophen + silymarin	18.62 ± 1.22^{c}	2.57 ± 0.04^{e}	7.30 ± 0.25^{c}	3.01 ± 0.82^{d}	4.89 ± 0.82^{e}
Acetaminophen + *N. sativa* extract	19.05 ± 1.24^{b}	2.72 ± 0.05^{de}	7.38 ± 0.38^{bc}	3.03 ± 1.56^{d}	4.83 ± 1.00^{e}
Acetaminophen + silymarin + *N. sativa* extract	15.25 ± 1.03^{d}	3.95 ± 0.02^{c}	5.04 ± 0.26^{d}	5.77 ± 1.19^{bc}	5.70 ± 1.13^{d}

GR: Glutathione reductase; CAT: Catalase; MDA: Malondialdehyde. Means within the same column in each category carrying different letters are significant at ($P \leqslant 0.05$) using Duncan's multiple range test, where the highest mean value has symbol (a) and decreasing in value were assigned alphabetically.

Table 3

Changes in SOD, catalase, malondialdehyde, GR and GPX activities of kidney in male mice treated with silymarin, *N. sativa* extract and paracetamol separately or in combination.

Groups	SOD (IU/g)	CAT (IU/g)	MDA (nmol/g)	GR (IU/g)	GPX (IU/g)
Control group	23.32 ± 0.87^{d}	5.49 ± 0.05^{ab}	8.67 ± 0.02^{f}	6.41 ± 0.50^{a}	5.13 ± 0.36^{b}
Acetaminophen (paracetamol)	29.61 ± 1.52^{c}	1.31 ± 0.04^{e}	38.68 ± 0.42^{a}	1.62 ± 0.30^{e}	1.47 ± 0.36^{f}
Silymarin	22.14 ± 2.98^{e}	5.28 ± 0.07^{b}	8.60 ± 0.18^{f}	6.50 ± 0.43^{a}	5.32 ± 0.40^{ab}
N. sativa extract	18.82 ± 0.91^{f}	5.11 ± 0.01^{b}	9.42 ± 0.01^{e}	5.31 ± 0.38^{b}	4.27 ± 0.43^{c}
Acetaminophen + silymarin	34.94 ± 1.66^{a}	4.51 ± 0.07^{d}	14.79 ± 0.03^{c}	3.25 ± 0.92^{d}	2.25 ± 0.92^{e}
Acetaminophen + *N. sativa* extract	34.92 ± 1.59^{a}	4.47 ± 0.07^{d}	18.27 ± 0.15^{b}	3.41 ± 0.29^{d}	2.41 ± 0.29^{e}
Acetaminophen + silymarin + *N. sativa* extract	31.91 ± 0.88^{b}	4.94 ± 0.08^{c}	10.59 ± 0.05^{d}	4.20 ± 0.53^{c}	3.20 ± 0.53^{d}

Means within the same column in each category carrying different letters are significant at ($P \leqslant 0.05$) using Duncan's multiple range test, where the highest mean value has symbol (a) and decreasing in value were assigned alphabetically.

paracetamol showed markedly dilated congested central vein (orange arrow) filled by large number of red blood cells and surrounded by hepatic cords with congested dilated central parts of sinusoids and markedly pyknotic nuclei and focal hepatocytes necrosis with drop off hepatocytes (Figures 1B and 1C). Figure 1F portrays samples of liver tissue from paracetamol-treated mice in combination with silymarin, exhibiting swollen hepatocytes with foamy finely granular cytoplasm. Group treated with paracetamol and *N. sativa* extract showed cords of hepatocytes with mild congested sinusoids (Figure 1G). Treatment of the mice with paracetamol with silymarin and *N. sativa* extract showed mild lobular inflammation with few scattered intra lobular aggregates of chronic non-specific inflammatory cells (Figure 1H).

3.4. Ultrastructure investigations of liver tissues

Figure 2 depicts electron micrographs of liver tissues of control and experimental groups of mice. Figure 2A shows the section of liver tissue from control mice, demonstrating part of the nucleus, mitochondria, rough endoplasmic reticulum (RER), glycogen inclusions and lipid droplet (scale bar 10 μm). Figure 2B shows mice liver treated with paracetamol showing irregular nuclei (red arrow) with some pyknosis in nuclei with appeared nucleolus, appeared mitochondria, fragmented RER and showing nearly disintegration of most of cellular contents (black arrow) except few number of mitochondria, (scale bar 5 μm). Figure 2C shows liver of silymarin treated group displayed normal cell organelles: nucleus, mitochondria, RER and normal cell membrane (scale bar 2 μm). Figure 2D shows the section of liver tissue from *N. sativa* group, demonstrating normal appearance of nucleus, RER, mitochondria and glycogen rosettes (scale bar 2 μm). Figure 2E portrays samples of liver tissue of paracetamol-treated mice in combination with silymarin, exhibiting irregular nucleus but with ameliorative appearance than acetaminophen treated group, depris

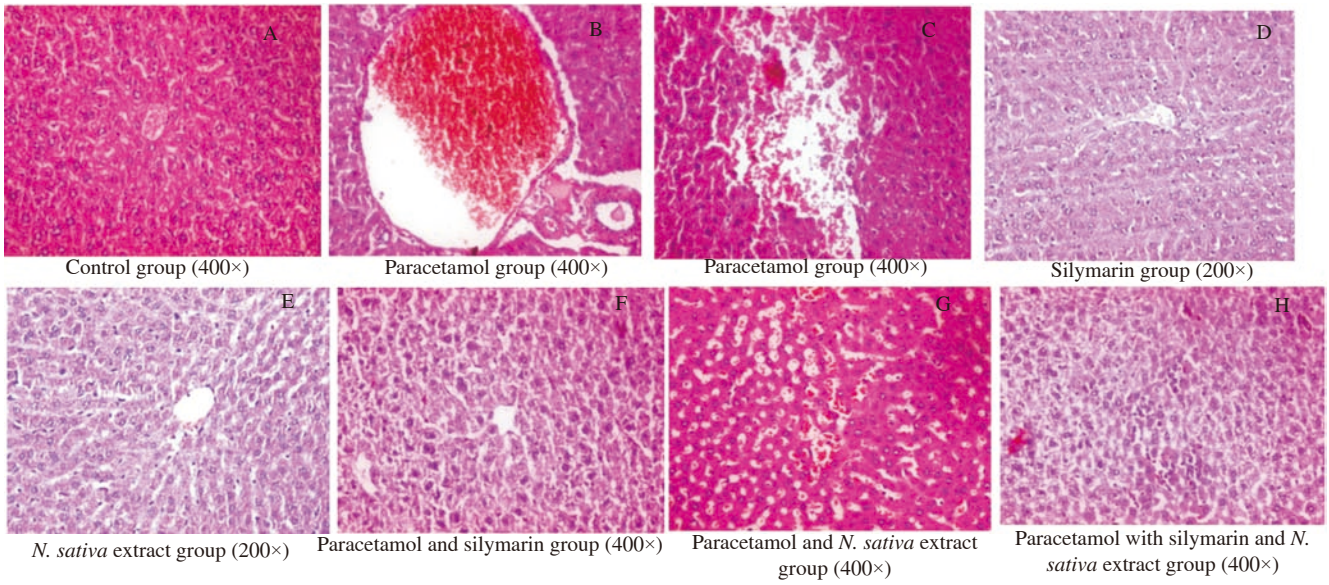

Figure 1. Photomicrographs of liver sections of control and experimental mice stained with Hematoxylin-Eosin under light microscopy.

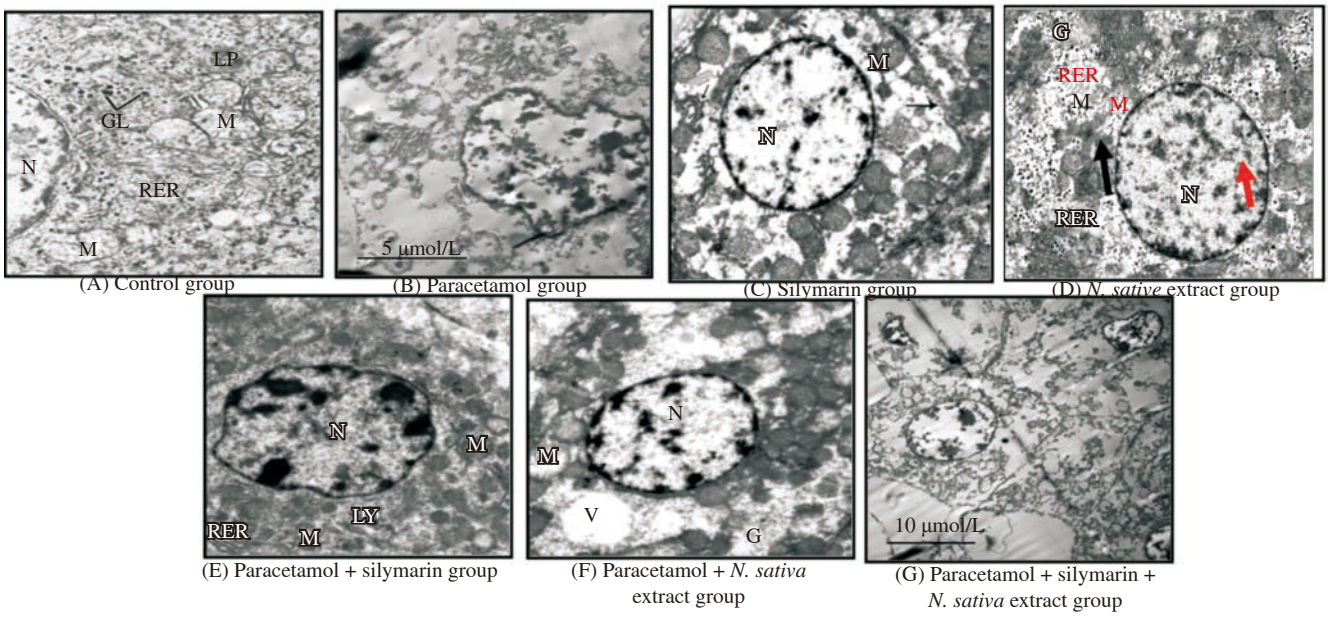

Figure 2. Electron micrographs of liver sections of control and experimental mice.

N: Nucleus; M: Mitochondria; GL: glycogen inclusions; LP: Lipid droplet; LY: lysosomes; V: vacuoles.

mitochondria, lysosomes and RER (scale bar 2 μm). Figure 2F of group treated with paracetamol and *N. sativa* extract shows little depris mitochondria, vacuoles, normal glycogen rosettes and regular nucleus (scale bar 2 μm). Treatment of the mice with paracetamol with silymarin and *N. sativa* extract showed nearly normal appearance of binuclei with smaller size (black arrow), one pyknotic nuclei (red arrow), nearly small mitochondria and nearly restoration of some distingerated cell contents (scale bar 10 μm) (Figure 2G).

4. Discussion

The major goal of this work was to evaluate the potential benefit of silymarin and *N. sativa* extract administration on APAP tissue injury, compared to silymarin or *N. sativa* extract treatment alone. To our knowledge, no study has been conducted on the co-effect of silymarin and *N. sativa* extract on APAP toxicity at the biochemical, histopathological and ultrastructural levels for the liver as well as antioxidant parameters of the mice. This study investigated the ability of silymarin and *N. sativa* extract to counteract APAP-induced toxicity in mice. Exposure to APAP at the tested dose to mice led to an alteration of liver functions and antioxidant capacities, decreased the total protein, increased ALT, AST and LDH levels as well as increased MDA and SOD while decreased catalase, GPX and GR. The biochemical and oxidative changes in APAP-treated groups were dose dependent. Concomitant administration of silymarin or/and *N. sativa* extract with APAP significantly protected most of the altered biochemical and antioxidant variables induced by APAP suggesting their protective efficacy. These two antioxidant compounds also improved the structure of vital organ (liver) that was evaluated on the basis of histopathological and ultrastructure findings. The biochemical markers used to evaluate liver function were ALT, AST, total protein and LDH. ALT and AST are the most sensitive biomarkers directly implicated in the extent of hepatic damage and toxicity[27,28].

Our results showed that levels of serum transferases (AST and ALT) were markedly elevated by acetaminophen (paracetamol) administration to mice when compared with control group. Acetaminophen (paracetamol) in its recommended doses elicited a marked elevation in serum LDH activity after the end of the study when compared with control group. ALT, AST and LDH are important indicators of liver damage in clinical findings. These enzymes were secreted into the blood in hepatocellular injury and their levels increase. Changes in these enzyme levels might differ depending on exposure time and dose.

Our results are reinforced by Mossa *et al*. as they noticed the increase in the levels of aminotransferase (ALT and AST) and the level of ALP as well as the decrease in the levels of total protein and albumin (are the major diagnostic symptoms of liver diseases) in the serum of paracetamol treated group[29].

Our results are reinforced by Yousef *et al*. who reported that paracetamol-induced hepatocellular necrosis marked by increased plasma AST, ALT, ALP and LDH activities[30]. Paracetamol also decreased plasma total protein as reported in our results.

The liver is the major source of most of the serum proteins, in which the parenchymal cells are responsible for synthesis of albumin, fibrinogen and other coagulation factors and most of the α and β globulins[31]. Albumin, being the most abundant plasma protein, accounts for 60% of the total serum protein and is incorporated in many physiological processes. The observed decrease in albumin by paracetamol, which is consequently reflected the decrease in total protein, could be a result of a decline in the number of cells responsible for albumin synthesis in the liver through necrosis on the same grounds. This explains the decline in total protein in our study after treatment of mice with paracetamol for 30 successive days[32].

Paracetamol overdose can cause liver function failure and death in human as well as experimental animals which is in agreement with our results and this could be due to the formation of highly reactive radicals because of oxidative threat caused by paracetamol[33].

Our results are also supported by Hawkins *et al*. who showed that paracetamol could in severe cases lead to liver failure in experimental animals and humans when taken in overdose[34]. The mechanisms of polyarylamide hepatotoxicity have been extensively studied[35].The initial step in toxicity is cytochrome P450 metabolism of acetaminophen to the reactive metabolite NAPQI. NAPQI is an electrophilic intermediate, which is oxidized by cytochrome P450 and converted to a highly reactive and toxic metabolite as in cases of APAP overdose[36].

NAPQI can rapidly react with glutathione and lead to 90% of total hepatic glutathione depletion in cells and mitochondria, which can result in hepatocellular death and mitochondrial dysfunction. NAPQI can also induce DNA strand breaks and promote apoptosis and hepatic necrosis[37].

LDH is localized in the cytoplasm of cells and thus is extruded into the serum when cells are damaged or necrotic. The measurement of total LDH can be useful when only a specific organ, such as the liver, is known to be involved. LDH is increased in acute necrosis of the liver. LDH is a sensitive intracellular enzyme which increases in serum and is an indication of liver cell damage and this supports our obtained finding that recorded increment in LDH level in paracetamol treated group[38].

Our results showed that acetaminophen (paracetamol) when given daily for successive 30 days afforded a significant increase in serum ALT activity post drug administration along the entire period of the experiment when compared with control group.

Our results seem to be conceivable with that obtained by Greenlee *et al*.[39] and Tamayo and Diamond[40]. They reported that milk thistle (silymarin) have protective effects on the liver and greatly improve its function since it is typically used to treat liver cirrhosis and chronic hepatitis (liver inflammation).

In addition, milk thistle extract prevent and repair damage from toxic chemicals and medications. Workers who had been exposed to vapors from toxic chemicals for 5-20 years which were given silymarin for 30 days. They showed significant improvement in liver function tests (ALT and AST) and platelet counts[41]. The liver regenerating effect induced by silymarin results from stimulation of RNA polymerase enzyme in the nucleus of liver cells. This results in an increase of ribosomal protein synthesis which helps to regenerate hepatocytes[42]. On similar grounds, silymarin might also affect bone marrow.

The combination of acetaminophen (paracetamol) with either *N. sativa* extract or silymarin afforded also a significant elevation in serum ALT level after 4th weeks post-treatment but less than increment recorded in paracetamol treated group indicating that both *N. sativa* and silymarin succeed to some extent in correcting the effect produced by acetaminophen (paracetamol).

Either silymarin or *N. sativa* extract caused a significant reduction in the elevated serum transaminases activity along the entire course of the study when compared with acetaminophen group alone. This plant drug and plant extract succeeded in improving the status of injured liver caused by acetaminophen. Chopra *et al*.[43],

and Rooney and Ryan[44] reported that thymoquinone, one of the active constituents of *N. sativa* has a hepatoprotective activity. An *in vitro* study showed the protective effect of *N. sativa* against tert-butyl hydroperoxide induced oxidative damage to hepatocytes. The activity was demonstrated by a decreased leakage of ALT and AST.

The hepatoprotective effect of *N. sativa* was supported also by the results of Mahmoud *et al*.[45]. The same previous effects were observed on serum AST level with the exception of effects of *N. sativa*, silymarin and their combination with acetaminophen (paracetamol) which showed some elevation when compared with control group. This may be attributed to the fact that AST is not a liver specific enzyme.

Our results coincide also with Al-Anati *et al*.[41]. They reported that milk thistle (silymarin) repair damage caused by toxic chemicals and medications. They exposed workers to vapors from toxic chemicals (toluene and/or xylene) for 5-20 years who were given either a standardized milk thistle (80% silymarin) for 30 days. The workers showed significant improvement in liver function tests (ALT and AST) when compared with placebo.

On the same basis, milk thistle (silymarin) is used primarily to treat various liver diseases and dysfunctions including alcoholic cirrhosis, hepatitis (due to viral infection or drug–induced) as well as hepatic problems related to diabetes[43]. Silymarin has liver regenerative effects by stimulating the enzyme known as RNA polymerase in the nucleus of liver cells. This results in an increase of ribosomal protein synthesis which helps to regenerate hepatocytes[43].

Our result was in accordance with Floyd *et al*. as previously mentioned with serum ALT and AST[46]. At the meantime, it was obvious that acetaminophen afforded a significant elevation in serum LDH activity when compared with control group along the entire period of the experiment. The administration of acetaminophen (paracetamol) for 30 successive days afforded also a more profound elevation in serum LDH level when compared with both silymarin and *N. sativa* extract group. The combination of acetaminophen (paracetamol) with either *N. sativa* or silymarin afforded slight significant increase in the enzyme activity.

Concerning the effect on serum total proteins, our results revealed that acetaminophen (paracetamol) induced significant decrease along the course of the study post drug administration when compared with normal control group. Whereas, silymarin induced nonsignificant elevation in serum total proteins after the end of the 4th week post-drug administration as well as *N. sativa* induced when compared with normal control group. Meanwhile, the combination of acetaminophen (paracetamol) and *N. sativa* afforded a marked decrease in serum total proteins of the normal group along the entire course of the experiment. The significant decrease in serum total proteins induced after the 4th week by acetaminophen (paracetamol) post-drug administration coincides with Forbes *et al*.[47]. They stated that a number of factors had been shown to regulate protein synthesis; the most important factors are insulin excretion and plasma amino acids concentration. Insulin has been shown to be a powerful inhibitor of protein degradation *in vivo*. In addition it is particularly noteworthy that those cirrhotic patients are characterized by severe resistance to action of insulin on glucose metabolism. As a result, there is higher probability to protein degradation because of hindering insulin action. Our result showed that acetaminophen (paracetamol) decreased significantly insulin level along the entire period of the study giving further support to our findings.

About 90% of total hepatocyte enzymes represented by these transaminases and among them high level of ALT is a better index of liver injury as it resides only in hepatocytes rather than AST which is inherent in liver parenchymal cells, blood and cardiac/muscle cells. ALP resides in cells lining biliary duct of livers, bone and placental tissue is elevated due to cholestasis and increased biliary pressure and this coincides with our results and refer to liver damage caused by paracetamol by high level of ALT in paracetamol treated group[48].

The nonsignificant changes in serum total proteins induced by silymarin after the end of the 4th week post-treatment coincide with Balandrin *et al*.[49]. They reported that silymarin can enter into the nucleus and act on RNA polymerase I enzyme and the transcription of rRNA result in an increase of ribosomal formation. This in turn hastens protein and DNA synthesis which enhances the biosynthetic apparatus in the cytoplasm, thus leading to an increase in the synthesis rate of both structural and functional proteins. This action has important therapeutic implication in the repair of damaged hepatocytes and restoration of normal functions of the liver. Moreover, Féher and Lengyel found that silymarin protects liver in 3 ways: by enhancing DNA polymerase, stabilizing cell membrane and scavenging free radicals[50]. Whereas, silymarin stimulates DNA polymerase, increase synthesis of ribosomal RNA and stimulates liver cell regeneration and it stabilizes cellular membranes and increases the glutathione content of liver[51].

The increased total protein content obtained in our study as a result of administration of *N. sativa* or its combination with acetaminophen (paracetamol) for 30 days into normal mice was in full agreement with Haq *et al*.[52].

Our results showed that cross section of liver tissues from group treated with acetaminophen (paracetamol) (1 000 mg/kg) markedly dilated congested central vein filled by large number of red blood cells and surrounded by hepatic cords with congested dilated central parts of sinusoids and markedly pyknotic nuclei, focal hepatocytes necrosis with drop off hepatocytes and markedly pyknotic nuclei.

Lipid droplet, the integrity of cellular membranes, leads to the leakage of cytoplasmic enzymes[53]. Therefore, the increased activities of liver enzymes in the present study could be due to severe histopathological damages and hepatotoxicity of paracetamol.

Our results are in full agreement with Mossa *et al*. as their observations indicated marked changes in the overall histoarchitecture of liver in response to paracetamol which could be due to its toxic effects primarily by the generation of reactive oxygen species (ROS), causing damage to the various membrane components of the cell[29]. One consequence of oxidative stress and lipid peroxidation is the formation of DNA adducts. Since DNA is believed to be the target molecule for carcinogens, endogenous DNA adducts are derived from oxidative stress, lipid peroxidation, and other sources have been proposed to contribute to the etiology of human cancers[54].

In accordance with our results, Mossa *et al*. observed that the necrotic conditions observed in the liver of paracetamol treated animals are in corroboration with the observed biochemical changes, wherein an increased level of lipid peroxidation was noticed[29]. Previous studies showed that paracetamol cause mild focal hepatitis in the lobules and portal areas necrosis in rats and hemorrhagic necrosis in humans mostly characterized by pyknosis and eosinophilic cytoplasm which are greatly in agreement with our results[55].

In accordance with our results, Ratnasooriya and Jayakody showed that there was a significant serum glutamic-oxaloacetic

transaminase elevation and moderate leucocyte infiltration of the hepatic vessels, suggesting a damaging effect on the liver[56]. It has been indicated that paracetamol overdose causes liver necrosis and failure.

In agreement with our results, administration of an overdose of APAP (1.5 g/kg) to rats developed a significant liver damage, after 12 h, evident by marked increase in serum ALT, concurrently with the development of severe histopathological changes in liver tissues. Our results are in the same line with Fujimura *et al*. who showed that early histological alterations result from a single large dose of acetaminophen in liver of rats showed cytoplasmic swelling and followed by hydropic vacuolation and necrosis[57].

Similar results were obtained by Wu *et al*. as they showed that silymarin has therapeutic effects on the early stages of liver damage, involving the reversal of fatty changes and a regression of the liver morbid histopathology[58].

Similar findings were obtained by Gani and John as they found absence of any adverse toxic effects of *N. sativa* extract in the liver[59]. These findings suggested the possibility of *N. sativa* extract being able to protect liver tissues and thus decrease the leakage of the enzymes (AST, ALT and ALP) in to the circulation.

In accordance with our results, El-kott *et al*. indicated that the *N. sativa* treated group showed the liver sections nearly with normal architectures and normal hepatocytes[60].

Our obtained results indicated that ultrastructure of liver sections from paracetamol treated group shows irregular nuclei with some pyknosis in nuclei with appeared nucleolus, appeared mitochondria, fragmented RER and nearly disintegration of most of cellular contents except few number of mitochondrias and these findings are in harmony with Fujimura *et al*. as they reported ultrastructurally, depletion of glycogen, degranulation of RER and swelling or disruption of mitochondria were observed with enlargement of mitochondria, loss of glycogen granules and degranulation of RER in acetaminophen (paracetamol) treated group[57].

In agreement with Fujimura *et al*., they showed that the main alteration in acetaminophen (paracetamol) treated rats was prominent enlargement of mitochondria associated with other various mitochondrial alterations including irregular configurations and deposition of some materials[57].

It has been demonstrated that acetaminophen is oxidized by cytochrome P-450, producing an acetaminophen free radical and hydrogen peroxide. In the presence of oxygen, the free radical generates superoxide. On the other hand, mitochondrion as well as endoplasmic reticulum and the plasma membrane are considered to be acetaminophen hepatotoxicity target sites and thus mitochondrial dysfunction is considered to result to acetaminophen hepatotoxicity[57].

Our results are in accordance with Wu *et al*. as they showed significant recovery of hepatocyte ultrastructure of the 6-week-old HBx transgenic mice given with silymarin (300 mg/kg/d) for 2 weeks[58].

Previous studies by El-kott *et al*. reported that *N. sativa* treated animals showed that some degree of mitochondrial swelling with some variation and size was noticed and this disagrees with our obtained results[60]. However, nuclear structures along with bile canaliculus and other organelles are normal in these animals and this agrees with our obtained results. The protective potential of *N. sativa* in restoring the altered hepatic histoarchitecture was close to the histoarchitecture of normal animals.

Our results are reinforced by Yousef *et al*.[30]. They reported that paracetamol treatment caused a significant elevation in thiobarbituric acid reactive substance levels with simultaneous inhibition in the activities of antioxidant enzymes and glutathione transferase (GST), GPX, SOD and catalase in rat plasma, liver, kidney, brain, lung, heart and testis. Furthermore, it decreased glutathione content significantly in rat liver, kidney and lung. These features might be attributed to the metabolic activation of paracetamol, which is considered a major mechanism of its toxicity.

In accordance with our results, Jaeschke *et al*.[61] and Newton *et al*.[62] were found that paracetamol trigger a rapid loss of glutathione and lipid peroxidation in both liver and kidney. The basic mechanism of paracetamol toxicity in the liver is the covalent binding of NAPQI, the reactive metabolite of paracetamol, to sulfhydryl groups of glutathione and various proteins and their subsequent oxidation[63].

On the other hand, several mechanisms are suggested as probable pathways for paracetamol-mediated nephrotoxicity.These include the oxidative metabolism to NAPQI similar to that in the liver, deacetylation to p-aminophenol and further oxidation to an aminophenoxy radical and benzoquinone imine, and also are the hepatically-derived metabolites from paracetamol-glutathione conjugates[64].

The elevation in thiobarbituric acid reactive substance level is an indicator of lipid peroxidation, which has been suggested to be closely related to paracetamol-induced tissue damage and this finding goes hand in hand with our obtained results[65]. It has been proven that hydrogen peroxide and superoxide anion are produced during metabolic activation of paracetamol in the CYP450 system[66] and from mitochondria during paracetamol intoxication[67]. It has been further suggested that the generation of ROS appears as an early event which precedes intracellular glutathione depletion and cell damage in paracetamol hepatotoxicity.

The superoxide formation may promote peroxynitrite generation and protein nitration that may further result into oxidative damage to proteins, DNA and lipids[68]. In addition, both paracetamol and NAPQI can interact with mitochondria, thereby inducing depletion of mitochondrial glutathione content, decline in ATP content, and uncoupling of the mitochondrial respiratory chain combined with electron leakage and this explanation is greatly supported by our findings[69].

Glutathione is a ubiquitous tripeptide present in all cell types in millimolar concentrations. The major roles of glutathione maintain the intracellular redox balance and eliminate xenobiotics and ROS[70].

Our results are completely in agreement with Halliwell who showed that they had chosen the liver, kidney and lung to estimate the changes in glutathione because they possess a high content of this protein[71]. According to the decline in hepatic, renal and lung glutathione content, it was evident that paracetamol-induced toxicity involved a change in cellular redox status toward a state of oxidative stress. A wide variety of oxidizing molecules such as ROS and/or depleting agents can alter glutathione redox state, which is normally maintained by the activity of glutathione-depleting (GPX, GST) and glutathione-replenishing enzymes. Therefore, it can be assumed that the decrease in glutathione concentration might cause the effectiveness of GST and GPX activity to be restricted, as evident by the intensification of lipid peroxidation.

The fall in catalase activity which proven in our results indicated a decrease in the antioxidative capacity as well. It has been shown that the decreased activity of SODs may be attributed to the consumption of these enzymes in ROS detoxification and also it's increasing is due to increased lipid peroxidation. It is also known

that antioxidant enzymes can be inactivated by lipid peroxides and ROS[72]. SOD is inhibited by hydrogen peroxide, while GPX and catalase are inhibited by an excess of superoxide radical on the same grounds as indicated in our study[73].

Lipid peroxidation is supposed to cause the destruction and damage to cell membranes, lead to changes in membrane permeability and fluidity and enhance the protein degradation in mice[74]. In the present study, the levels of lipid peroxidation were increased, indicating an increase in the generation of free radicals in the paracetamol treated group and this level was decreased in other groups treated with silymarin and *N. sativa* extract.

Conflict of interest statement

We declare that we have no conflict of interest.

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