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Potential protective effects of white mulberry (*Morus alba* L.) leaves on rat liver injuries induced by carbon tetrachloride

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Abstract: This study aimed to investigate the potential protective effects of white mulberry leaves on rat liver injuries induced by carbon tetrachloride. Mulberry leaves powder (MLP) was prepared and its chemical composition, bioactive compounds content and antioxidant activity were determined. The data indicated that MLP contain high levels of protein, ash, crude fiber, carbohydrates, antioxidant vitamins (A, C and E) and non-nutrient antioxidants (phenolics and carotenoids). Also, MLP exhibited high antioxidant properties (antioxidant activity, 63.04% and DPPH•, 129.52 µMol Trolox /g DW). For biological experiments, MLP has been fed at concentrations ranged 0.75 to 6 % in basal diet of rats for 2 weeks then injected withCCl4 to induce liver damage. Treatment of animals with CCl4 caused a significant increased ($p \leq 0.05$) in AST (90.76%), ALT (62.67%) and ALP (183.15%) compared to normal controls. Supplementation of the rat diets with MLP prevented the rise of mean serum AST, ALT and ALP activities. The same behavior was recorded for thiobarpituric acid reactive substances (TBARS) level in serum, the biomarkers of oxidative stress in cells. The opposite directions were observed for some immunological parameters including albumin levels and protease activity in serum and reduced glutathione (GSH) fractions (non-enzymatic antioxidant) in serum. The results were confirmed by microscopical liver examination. These results supported our hypothesis that MLP could be used successfully as functional food for containing several classes of bioactive compounds and exhibited antioxidant activity that are able to prevent or inhibit liver injuries induced by chemical toxin (CCl4) . Therefore, we recommended MLP by a concentration about 6% (w/w) to be included in our daily dishes and drinks as well as in different food products as a natural food additive.

Keywords: Mulberry leaves powder, liver functions, glutathione, immunological parameters, TBARS, histopathological examination

Introduction

Liver plays a critical role in all vertebrates. It performs several vital functions including: 1) processes many of the products that are released into the blood stream (e.g. glucose derived from glycogenesis, plasma proteins and urea), 2) secretes bile into the intestine to help absorb nutrient, 3) makes some of the clotting factors needed to stop bleeding from acute or injury, 4) stored several products in the parenchymal cells (e.g. glycogen, fat and fat soluble vitamins), and 5) plays a very important part in biotransformation and removing the xenobiotics from the body, of which alcohol and dietary toxins are particularly noteworthy (Crawford, 1999; Elhassaneen, 1996 and Kebamo et al., 2015). The enormous functional reserve of the liver often masks the clinical impact of early liver damage. With progression of diffuse disease or disruption of bile flow, however, the consequences of liver damage can easily become life-threatening. Therefore, liver diseases are a major problem throughout the world (Lawrence and Emmet, 2012).

The burden of liver diseases has been increasing in Egypt with a doubling of the incidence rate in the past 10 years. This has been attributed to several biological (e.g. virus infection) and environmental/dietary factors (e.g. Aflatoxin, polycyclic aromatic hydrocarbons) (Elhassaneen, 2004 and Elhassaneen *et al.,* 2016). Other factors such as cigarette smoking, occupational exposure to chemicals such as pesticides and heavy metals, and endemic infections in the community, as schistosomiasis, may have additional roles in the etiology or progression of the disease (Anwar *et al.,* 2008). The World Health Organization (WHO, 2010) reported that Egypt has one of the highest incidences of hepatitis C, one of the main causes of liver cancer, in the world. The number of deaths resulting from liver cancer in Egypt had risen from 4% in 1993 to 11% in 2009.

The modern pharmacological therapy is costly and associated with multiple side effects resulting in patient non-compliance. Thus there is a need to explore alternative therapies particularly from herbal/plant sources as these are cost effective and possess minimal side effects. Also, it was reported that some plants exercise various bioactivities, including antioxidant, anti-inflammatory, antidiabetic, anticarcinogenic, antimutagenic etc (El-Safty, 2012 and Elhassaneen and Sayed, 2015 and Elmaadawy, 2016).

One of the less studied plants is the white mulberry (*Morus alba* L.). It is a deciduous tree originating from Asia but currently cultivated in subtropical, tropical, and moderate environments (Amarowicz *et al.,* 2000). Different parts of the mulberry plant (fruit, bark, leaf and root) have been used over the centuries in traditional Chinese medicine as a common agent to treat a variety of conditions including diabetes, atherosclerosis, cancer as well as for boosting the immune system through potent antioxidant activity (Butt *et al.,* 2008). The mulberry leaves are nutritious, palatable and nontoxic (Srivastava et al., 2003). Several studies indicated that mulberry leaves contain many nutrients (e.g. proteins, dietary fiber and carbohydrates), minerals (e.g. iron, zinc, calcium, magnesium and phosphorous) and vitamins (e.g. ascorbic acid, β-carotene, B_1, D, and folic acid) (Ewa *et al.,* 2013). Also, many bioactive compounds (e.g. flavonoids, phenolics acids, quercetin, isoquercetin and alkaloids) have been found in mulberry leaves (Doi *et al.,* 2001). Such bioactive compounds found in mulberry leaves possesses medical benefits, including diuretic, hypoglycemic, antibacterial, antiviral, hypotensive properties and neuroprotective functions [Harauma *et al.,* 2007]. Unfortunately, there are a dearth of information regarding the effect of such natural compounds found in mulberry leaves on liver diseases. Therefore, in the present study we investigated the potential protective effects of MLP on rat liver injuries induced by carbon tetrachloride.

Materials and Methods

Materials

White mulberry (*Morus alba* L.) leaves were obtained by special arrangements with some farmers in Zagazig City, Sharkia Governorate, Egypt. Carbon tetrachloride (CCl_4) was purchased from Sigma Chemical Co. (St. Louis, MO, Company agent, Cairo, Egypt).). Casein was obtained from Morgan Chemical Co., Cairo, Egypt. All organic solvents, buffers and other chemicals of analytical grade were purchased from El-Ghomhorya Company for Trading Drugs, Chemicals and Medical Instruments, Cairo, Egypt.

Throughout this study a SP Thermo Separation Products Liquid Chromatograph (Thermo Separation products, San Jose, CA) was used with a Consta Metvic 4100 pump, a Spectra Series AS100, Spectra System UV 1000 UV/Visible Spectrophotometer Detector, Spectra

System FL 3000 and a PC 1000 system software. The columns used (Alltech, Deerfield, IL, USA) were a Spherosorb ODC-2 (5 µm, 150 x 4.6 mm I.d.) for glutathione fractions; a reversed phase water Adsorbosil C18 (5 µmol/L, 100 mm x4.6–mm internal diameter) for vitamin C; and normal Ultrasphere Si (5 µmol/L, 250 mm x4.6–mm internal diameter) for analysis of vitamins A and E.

Mulberry leaves powder (MLP) preparation

Mulberry leaves were washed and then dried in a hot air oven (Horizontal Forced Air Drier, Proctor and Schwartz Inc., Philadelphia, PA) at two stages 50°C for 6 hrs followed by 40°C for 10 hrs. The dried peels were ground into a fine powder in high mixer speed (Moulinex Egypt, Al-Araby Co., Egypt). The material that passed through an 80 mesh sieve was retained for use.

Chemical analysis of MLP

MLP samples were analyzed for moisture, protein (T.N. × 6.25, micro - kjeldahl method using semiautomatic apparatus, Velp company, Italy) , fat (soxhelt miautomatic apparatus Velp company, Italy , petroleum ether solvent), ash, fiber and essential oil (using rotary evaporator apparatus, Velp company, Italy) contents were determined using the methods described in the A.O.A.C. (1995). Carbohydrates calculated by differences:

Carbohydrates (%) = 100 - (% moisture + % protein + % fat + % Ash + % fiber).

Total energy calculated by following equation:

Total energy (Kcal/100g) = (% protein + % carbohydrates) x4 + % fat x9.

Total phenolics, carotenoids and total dietary fiber in MLP samples were analyzed as follow: MLP was extracted with 80% acetone and centrifuged at 10,000g for 15 min at room temperature. The supernatant obtained from the samples were used for the analysis of total phenolics, carotenoids and antioxidant activity.

Total phenolics were determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Two hundred milligrams of sample was extracted for 2 h with 2 mL of 80% MeOH containing 1% hydrochloric acid at room temperature on an orbital shaker set at 200 rpm. The mixture was centrifuged at 1000g for 15 min and the supernatant decanted into 4 mL vials. The pellets were combined and used for total phenolics assay. One hundred microliters of extract was mixed with 0.75 mL of Folin-

Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22 0C for 5 min; 0.75 ml of sodium bicarbonate (60g/L) solution was added to the mixture after 90 min at 22 0C, absorbance was measured at 725 nm. Results are expressed as ferulic and equivalents. The total carotenoids in 80% acetone extract were determined by using the method reported by Litchenthaler (1987). Total dietary fiber content in the TRP was estimated according to the method described by Asp *et al.* (1983).

All vitamins (A, C, and E) were extracted according to methods previously detailed (Epler *et al.,* 1993; Moeslinger *et al.,* 1994 and Hung *et al.,* 1980) and were analyzed by HPLC techniques. For vitamins A and E, the chromatographic conditions were as follows: flow rate, 1.5 mL/min; detection, UV absorption at 265 nm, volume of injection, 20 µL; temperature, room temperature; and the mobile phase composition was an isocratic system of isopropanol:hexane (1:99). For vitamin C, the conditions were: flow rate, 1 mL/min; detection, UV absorption at 254 nm, volume of injection, 20 µL; temperature, room temperature, and mobile phase composition was an isocratic system of 100% methanol. Retention times and absorbance ratio against those of standards were used to identify the separated vitamins. Quantitative determination of each vitamin was determined from its respective peak area and corresponding response factor. The percent recoveries of vitamins were also studied by adding each vitamin to plasma after sample preparation and HPLC determination. Under such chromatographic conditions, mean values (±SD) of vitamins A, C and E, and curcumin recoveries were 86.04 ± 3.55, 88.90 ±2.21 and 82.98±5.17 %, respectively.

Antioxidant activity

Antioxidant activity of MLP extracts and standards (α-tocopherol, BHA, ans BHT; Sigma Chemical Co., St. Louis, Mo) was determined according to the *β*-carotene bleaching method following a modification of the procedure described by Marco (1968). For a typical assay, 1mL of *β*-carotene (Sigma) solution, 0.2 mg/mL in chloroform, was added to round-bottom flasks (50 mL) containing 0.02 mL of linoleic acid (J.T. Baker Chemical Co., Phillipsburg, NJ) and 0.2 mL of Tween 20 (BDH Chemical Co., Toronto, On). Each mixture was then dosed with 0.2 mL of 80% MeOH (as control) or corresponding plant extract or standard. After evaporation to dryness under vacuum at room temperature, oxygenated

distilled water (50 ml) was added and the mixture was shaken to form a liposome solution. The samples were then subjected to thermal autooxidation at 50 ^{0}C for 2 h. The absorbance of the solution at 470 nm was monitored on a spectrophotometer (beckman DU-50) by taking measurements at 10 min intervals, and the rate of bleaching of β-carotene was calculated by fitting linear regression to data over time. All samples were assayed in triplicate. Various concentrations of BHT, BHA, and α-tocopherol in 80% methanol was used as the control. Antioxidant activity (AA) was all calculated as percent inhibition ralative to control using the following equation (Al-Saikhan *et al*., 1995).

$$AA= (R_{control} - R_{sample}) / R_{control} \times 100$$

Where: $R_{control}$ and R_{sample} were the bleaching rates of beta-carotene in reactant mixture without antioxidant and with plant extract, respectively.

Radical scavenging capacity against DPPH

The free-radical scavenging potentials of crude ex- tracts were tested in a methanolic solution of DPPH• as mentioned in Kim *et al.,* (1999). The extent of discoloration of the solution indicates the scavenging efficacy of the added substance. A 1mL aliquot of extract solution was combined with 2 mL of CH3OH and then 0.25 mL of a 1 mM ethanolic solution of DPPH•. The mixture was vortexed for ~60 s and incubated at room temperature for 20 min followed by measurements of absorbance at λ = 517 nm (beckman DU-50). A reference sample was prepared with methanol instead of DPPH• and the control instead of the extract sample. To construct a calibration curve, absorbance of samples containing 0.5, 1.0, 1.5, and 2.0 mg/mL Trolox were measured simultaneously. Results were expressed as μMol Trolox equivalents/g extract dry weight. Antioxidant activity was calculated as a percentage of DPPH• change in absorbance using the following equation:

$$\text{Radical scavenging activity} = 100 - [(Abs_{sample} - Abs_{reference})/Abs_{control})] \times 100$$

Biological Experiments

Animals

Animals used in this study, adult male albino rats (160±9.1 g per each) were obtained from National Research Center (NRC), Cairo, Egypt.

Basal Diet:

The basic diet prepared according to the following formula as mentioned by (AIN, 1993) as follow: protein (10%), corn oil (10%), vitamin mixture (1%), mineral mixture (4%), choline chloride (0.2%), methionine (0.3%), cellulose (5%), and the remained is corn starch (69.5%). The used vitamins mixture component was that recommended by Campbell, (1963) while the salts mixture used was formulated according to Hegsted, (1941).

Experimental design

All biological experiments performed a complied with the rulings of the Institute of Laboratory Animal Resources, Commission on life Sciences, National Research Council (NRC, 1996). Rats (n=36 rats) were housed individually in wire cages in a room maintained at 23 ± 5 ^{0}C, relative humidity (50±6%), a 12-hr lighting cycle and kept under normal healthy conditions. All rats were fed on basal diet for one-week before starting the experiment for acclimatization. After one week period, the rats were divided into six groups (6 rats per each) as follow:

- Group (1): as a negative control group, still fed on basal/standard diet and injected with paraffin oil (5 ml/kg body weight) which was used as a vehicle for the treatment of animals in CCl4 group.
- Group (2) as a positive control group, was challenged with an subcutaneous injection of CCl4 in paraffin oil (50% v/v, 2ml/kg) twice a week to induce chronic damage in the liver according to the method of Jasekhar et al., (1977).
- Group (3): fed on standard diet containing 0.75 % (w/w) MLP.
- Group (4): fed on standard diet containing 1.50 % (w/w) MLP.
- Group (5): fed on standard diet containing 3.00 % (w/w) MLP.
- Group (6): fed on standard diet containing 6.00 % (w/w) MLP**.**

The treatment with MLP to the animal belonging to groups (3-6) was started 14 days prior to CCl_4 injection. All the rats had free access to the diet and water and the treatments continued for a total duration of 6 weeks.

Blood sampling

At the end of experiment period, 4 weeks, blood samples were collected after 12 hours fasting using the abdominal aorta and rats were scarified under ether anesthetized. Blood samples were received into

clean dry centrifuge tubes and left to clot at room temperature, then centrifuged for 10 minutes at 3000 rpm to separate the serum according to Drury and Wallington, (1980). Serum was carefully aspirate, transferred into clean covet tubes and stored frozen at -20°C until analysis. The liver was excised for histological examination.

Hematological analysis

Liver functions

Serum aspartate aminotransferase (AST) and Serum alanine aminotransferase (ALT), and Serum alkaline phosphatase (ALP) activities were measured in serum using the modified kinetic method of Tietz *et al.*, (1976) and Vassault *et al.,* (1999), respectively.

Albumin

Albumin was determined in plasma using kits purchased from El-Nasr Pharmaceutical Chemicals Company, Cairo, Egypt.

Protease activity assay

The protease activity was determined by adaptation the method of Rinderrnecitt *et al*., (1968). Briefly, 100 µl of plasma were added to 40 µl of buffer (150 mM Tris base, 30 mM $CaCl_2$, 0.05% Brij 35) and 50 µl of protease substrate (20% Hide powder azure, HPA, 20 % sucrose, 0.05% Brij). The tubes contents were incubated at 37^{0}C with continuous shaking for 2 hours. The reaction was stopped by the addition of 50 µl of 10% TCA and the tubes were stored at 4°C for about 15 min. After spined the tubes at 8500 rpm for 5 min, the supernatants were transferred to new tubes and the absorbencies were measured at 540 nm. Blank tubes were prepared by the same previous steps without samples addition.

Glutathione (GSH) fractions

GSH was determined by HPLC according to the method of McFarris and Reed (1987). In brief, 100 µl of aliquot were placed in 2 ml of 10% perchloric acid containing 1 mM bathophenanthroline disulfonic acid and homogenized. The homogenate was cold centrifuged at 10000 rpm for 5 min and the internal standard (γ-glutamyl glutamate) was added to the supernatant. A 250 µl aliquot of acidic extract was mixed with 100 µl of 100 mM iodoacetic acid in 0.2 mM cresol purple solution. The acid solution was brought to pH 8.9 by the addition of 0.4 ml of KOH (2 M) –

$KHCO_3$ (2.4 M) and allowed to incubate in the dark at room temperature for 1 hr to obtain S-carbooxymethyl derivatives. The N-nitrophenol derivatization of the samples were taken overnight at 4 ^{0}C in the presence of 0.2 ml of 1% 1-fluoro-2,4-dinitrobenzene and injected onto the HPLC system.

Thiobarpituric acid reactive substances (TBARS) content

TBARS were measured as described by **Buege and Aust, (1978).** Half milliliter of plasma were added to 1.0 ml of thiobarbituric acid reagent, consisting of 15% TCA, 0.375% thiobarbituric acid (TBA) and 0.01% butylated hydroxytoluene in 0.25 N HCl. Twenty-five microliters of 0.1 M FeSO4.7H2O was added and the mixture was heated for 20 min in boiling water. The samples were centrifuged at 1000 xg for 10 min and the absorbance was read at 535 nm using Labo-med. Inc., spectrophotometer against a reagent blank. The absorbance of the samples was compared to a standard curve of known concentrations of malonaldialdehyde.

Histological examination

Liver was prepared for histological examination according to Ji-Young *et al.,* (2015). The liver was isolated and fixed in 10 % neutral buffered formalin. The liver tissues were dehydrated and then embedded in paraffin wax. Histological sections (4 lm) of
these tissues were cut and stained with hematoxylin and eosin (H&E). Liver sections were observed using an optical microscope (HS-100, OPTICAL, China).

Statistical Analysis

All measurements were done in triplicate and recorded as mean±SD. Statistical analysis was performed with the Student *t*-test and MINITAB 12 computer program (Minitab Inc., State College, PA). Data were expressed as mean ± SD. The significance of differences was determined by one-way ANOVA followed by Duncan's test for multiple comparisons using an MINITAB 12 computer program (Minitab Inc., State College, PA). A probability level of $P<0.05$ was considered statistically significant.

Results and Discussion

Chemical analyses of mulberry leaves powder (MLP)

The proximate chemical composition of MLP is shown in Table (1). The results showed that the moisture content was 8.29%, total protein was 19.54%, crude fat was 2.65%, crude fiber was 10.76%, ash content was 9.94% and total carbohydrate content was 48.82%. Also, the total energy was recorded 297.29 Kcal/100g. The proximate composition reported was accordance with that observed by Butt *et al.,* (2008) and reviewed by Małgorzata, (2015). All of these components in MLP might be important from the nutrition point of view. Therefore, enrichment of different food products with MLP would enhance the nutritional quality of the product better than many food sources.

Table 1. Chemical analysis of mulberry leaves powder (MLP)

Component	Value*
Water (g/100g)	8.29 ± 0.76
Total protein (g/100g)	19.54 ± 3.45
Crude fat (g/100g)	2.65 ± 1.03
Ash (g/100g)	9.94 ± 1.22
Crude fiber (g/100g)	10.76 ± 1.17
Carbohydrate (g/100g)	48.82 ± 4.02
Total energy (Kcal/100g)	297.29 ±12.67

* Each value represents the mean of three replicates ±SD.

Antioxidant activity, antioxidant vitamins and bioactive compounds of MLP aqueous extracts

Antioxidant activity, antioxidant vitamins and bioactive compounds of MLP aqueous extracts were illustrated in Table (2). From such data it could be noticed that, MLP exhibited antioxidant activity (AA, 63.04% and DPPH•, 129.52 µMol Trolox /g DW). In parallel, many antioxidant nutrients such as vitamins C, E and β-carotene for which there are Dietary Reference Values (DRVs) were recorded in MLP. However, there are thousands of other bioactive compounds in foods that have antioxidant activity but are not classified as "nutrients." These "non-nutrient antioxidants" include phenolics and carotenoids (found MLP) (Ajila *et al.,* 2008). The present data are in accordance with that observed by Ewa *et al.,* (2013). Also, numerous studies indicate significance of the antioxidative properties of the white mulberry in preventing and treating lifestyles diseases (Małgorzata, 2015 and Sánchez-Salcedo *et al.,* 2015).

In this direction, many studies indicated that there was a positive and significant ($p < 0.01$) relationship between all of the previous bioactive compounds recorded in MLP and the antioxidant activity in different plant parts (Khoneem, 2009; Jaggi, 2012 and Elhassaneen *et al.,* 2013). Plant-based foods generally are considered important sources of antioxidants in the diet. Antioxidants help protect cells from the potentially damaging physiological process known as "oxidative stress" (damage to healthy cells or DNA by unpaired electrons known as free radicals). Oxidative stress is thought to be associated with the development of chronic diseases including cancer, heart disease, diabetes, rheumatoid arthritis, obesity, conditions of ageing including neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Halliwell, 1991, Chaitanya *et al.,* 2010 and Elmaadawy *et al.,* 2016; Sello and Eldemery, 2016). Such phytochemical composition and antioxidant properties of the MLP are giving such food high significant as an important functional food.

Table 2. Antioxidant activity, antioxidant vitamins and bioactive compounds of MLP aqueous extracts

Assay	Value*
Antioxidant activity (AA, %)	63.04 ± 5.17
DPPH• (µMol Trolox /g DW)	129.52 ± 20.65
Ascorbic acid (mg/100g)	125.76 ± 10.7
Vitamin A (mg/100g)	7.51 ± 1.12
Vitamin E (mg/100g)	3.16 ± 0.85
Total phenolics content (mg GAE.g-1)	82.10 ± 1.18
Total carotenoids (mg.100g)	69.81 ± 7.90

*Each value represents the mean of three replicates ±SD.

Effects of MLP on hepatic function disorders of rats induced by CCl_4

Liver functions of rats injected CCl_4 and consumed MLP powder were shown in Table (3). From such data it could be noticed that treatment of animals with CCl_4 caused a significant increased ($p \leq 0.05$) in AST (90.76%), ALT (62.67%) and ALP (138.15%) compared to normal controls. Supplementation of the rat diets with MLP (0.75 to 6.0% w/w) prevented the rise of mean serum AST, ALT and ALP activities. The rate of preventative was increased with the increasing of the MLP concentration.

Table 3. Effects of MLP on hepatic function disorders of rats induced by CCl_4

Value	Control (-)	Control (+)	MLP (%, w/w)			
			0.75	1.50	3.00	6.00
Serum aspartate aminotransferase (AST,U/L)						
Mean	42.08	80.27	67.27	62.35	51.55	48.89
SD	3.78	6.98	9.09	6.72	4.57	4.45
% of Change	0.00	90.76	59.87	48.19	22.52	16.20
Serum alanine aminotransferase (ALT,U/L)						
Mean	70.25	114.28	99.82	90.21	83.74	79.61
SD	4.12	5.79	10.51	6.12	8.23	6.70
% of Change	0.00	62.67	42.09	28.40	19.20	13.33
Serum alkaline phosphatase (ALP,U/L)						
Mean	151.42	360.59	282.93	260.24	208.52	199.80
SD	10.92	30.56	10.84	19.18	9.96	20.94
% of Change	0.00	138.15	86.86	71.87	37.71	31.95

[*] Means in the same row with different litters are significantly different at $p \leq 0.05$

Long time ago, CCl_4 is a well known hepatodestructive agent that is widely used to induce acute-toxic liver injury in a large range laboratory animals (Dandiya and Collumbine, 1959). In related studies, Elhassaneen et al., (2012) and El-Sayed *et al.,* (2012) reported that elevations in liver functions enzymatic activities including AST, ALT and ALP in rats as the result of CCl_4 injection. Data of the present study indicated that MLP is a rich source of different classes of phytochemicals (e.g. carotenoids and phenolics) and antioxidant vitamins (e.g. vitamins A, C and E). Also, organosulfur compounds, flavonoids and alkaloids have been found in MLP (Doi *et al.,* 2001). Many studies reported that the effect of many plant parts on decreasing the serum liver function enzymes activity could be attributed to their high level content of such bioactive compounds (Hassan, 2014; El-Sayed *et al.,* 2012 and Sayed Ahmed, 2016). The possible mode of action of liver serum enzymes-lowering activity of the MLP could be explained by one or more of the following process: 1) block the hepatocellular uptake of bile acids (Dawson, 1998), 2) improve the antioxidant capacity of the liver (Beattic

et al., 2005) and 3) improve the of antioxidant defense systems in both serum and red blood cells. The obtained result are confirmed by the data of chan *et al.,* (2013) who found that mullberry leaves have not only anticholestermic effects but also cardiovascular and hepatoprotective properties.

Effects of MLP on serum immunological parameters levels of rats induced by CCl_4

Data presented in Table (4) showed effect of feeding MLP on some immunological parameters (albumin level and protease activity) in serum of rats treated with CCl_4. From such data it could be noticed that treatment of animals with CCl_4caused a significant decreased ($p \leq 0.05$) in albumin level (-23.45%) and protease activity (-29.22%) compared to normal controls. Supplementation of the rat diets with MLP (0.75 to 6.00 % w/w) prevented the decreasing in serum albumin level and protease activity. The rate of preventative was increased with the increasing of the MLP concentration.

Table 4. Effects of MLP on serum immunological parameters levels of rats induced by CCl_4

Value	Control (-)	Control (+)	MLP (%, w/w)			
			0.75	1.50	3.00	6.00
Serum albumin concentration (Alb, g/dl)						
Mean	3.66	2.80	2.98	3.11	3.30	3.34
SD	0.27	0.22	0.35	0.58	0.49	0.39
% of Change	0.00	-23.45	-18.55	-15.10	-9.73	-8.66
Serum protease activity (PA, U/L)						
Mean	3.40	2.41	2.60	2.74	2.99	3.16
SD	0.21	0.18	0.18	0.45	0.45	0.60
% of Change	0.00	-29.22	-23.43	-19.41	-11.95	-7.00

* Means in the same row with different litters are significantly different at p≤0.05

In according with the study of Wang *et al.* (2007), the present study showed that CCl_4 induced significant ($p \leq 0.05$) decreasing in the serum albumin content. It was reported that hypoalbuminaemia is most frequent in the presence of advanced chronic liver diseases, hence decline

in albumin can be deemed as a useful index of the severity of cellular dysfunction in chronic liver diseases (El-Sayed *et al.,* 2012)

Regarding the proteases activity, numerous studies have revealed that proteases could play an important role in immunological functions and humoral host defense (reviewed in Neurath,1989). Other studies outlining the direct relationship between protease expression and protozoal virulence have implicated proteases as being involved in pathogenicity (Wilson *et al*., 1989; Keene *et al*., 1989). In several studies, protease activity was found decreased as found in the present study. For example, Elhassaneen *et al*., (1997) and Elhassaneen, (2001) reported that a significant decreasing in the protease activity of fish isolated liver cells as a consequence of exposure to hepatotoxins (e.g. pesticides, heavy metals, polycyclic aromatic hydrocarbons etc). Data of the present study confirmed that MLP exhibited therapeutic effects against CCl4 (i.e. hepatotoxin) through different mode of actions including the elimination of some immunotoxic effects (e.g. albumin levels and protease activity).

Effects of MLP on serum reduced glutathione (GSH) concentration of rats induced by CCl_4

Data presented in Table (5) showed the effect of feeding MLP on serum GSH content of rats treated with CCl_4. From such data it could be noticed that treatment of animals with CCl_4 caused a significant decreased ($p \leq 0.05$) in serum GSH (-34.65%) compared to normal controls. Supplementation of the rat diets with MLP (0.25 to 1.0 g/100g w/w) prevented the decreasing of mean serum GSH levels. The rate of preventative was increased with the increasing of the MLP concentration.

Several years ago, GSH has received considerable attention in terms of its biosynthesis, regulation, and various intracellular functions (Larsson *et al*., 1983). Among of these functions, its role in detoxifications process represent the central role through as a key conjugate of xenobiotics electrophilic intermediates and as an important antioxidant. The antioxidant functions of GSH includes its role in the activities of the antioxidant enzymes system (GSH-Px and GSH-Rd). In addition, GSH can apparently serve as a nonenzymatic scavenger of oxyradicals/reactive oxygen species (Halliwell and Gutteridge, 1985 and Almaadawy *et al.,* 2016).

Table 5. Effects of MLP on serum GSH concentration (μmol/L) of rats induced by CCl_4

Value	Control (-)	Control (+)	MLP (%, w/w)			
			0.75	1.50	3.00	6.00
Mean	7.98	5.22	5.68	6.22	6.73	7.11
SD	1.03	1.33	1.17	0.55	1.39	1.12
% of Change	0.00	-34.65	-28.88	-22.11	-15.71	-10.99

* Means in the same row with different litters are significantly different at p≤0.05

Several studies have reported the potent antioxidant capacity of MLP where by mitigation of lipid peroxidation and oxidative stress in several organs tissues including liver were demonstrated (Naowaratwattana *et al.,* 2010 and Priya, 2012). Such data with the others suggested that secretion of GSH from liver to blood might be blocked because of intracellular structural failure and elevation of the lipid peroxide content (Hasegawa *et al.,* 1995). MLP was described by its higher content of different classes of phytochemicals including carotenoids, quercetin, flavonoids, phenolics which exhibited high antioxidative activities (Doi *et al.,* 2001) and anticarcinogenic effects (Fathy *et al.,* 2013).

Effects of MLP on serum TBARS concentration of rats induced by CCl_4

Serum lipid peroxidation of rats injected CCl_4 and consumed MLP was shown in Table (4). From such data it could be noticed that the serum lipid peroxidation level was increased 65.13% by CCl_4 injection which was significantly (p≤0.05) reduced in CCl_4+MLP treatment. The rate of TBARS (malonaldialdehyde level, MDA) reducing was increased with the increasing of MLP concentration.

Table 4. Effects of MLP on serum TBARS concentration (nmol/mL) of rats induced by CCl_4

Value	Control (-)	Control (+)	MLP (%, w/w)			
			0.75	1.50	3.00	6.00
Mean	0.161	0.266	0.239	0.212	0.198	0.195
SD	0.044	0.051	0.043	0.037	0.029	0.031
% of Change	0.00	65.13	48.34	31.79	23.09	20.98

* Means in the same row with different litters are significantly different at p≤0.05

Accompanied by a concomitant reduce in nonenzymatic antioxidants (GSH), high concentrations of oxidant i.e. TBARS as established in the present study in rats feeding MLP. So, if there were no change in the antioxidant defense system of rats feeding ingested MLP, it would be difficult to observe high concentrations of TBARS. High levels of malonaldialdehyde (MDA), one of the most important compounds in TBARS, in the plasma of patients were associated with rather low levels of nonenzymatic antioidants (Elhassaneen, 2004). Our present data with the others interpreted the effect of MLP on reducing the serum lipid perioxidation which could be attributed to several effects such as scavenging free radicals, inhibiting oxidation, and reducing atherogenic risk (Liu *et al.,* 2008).

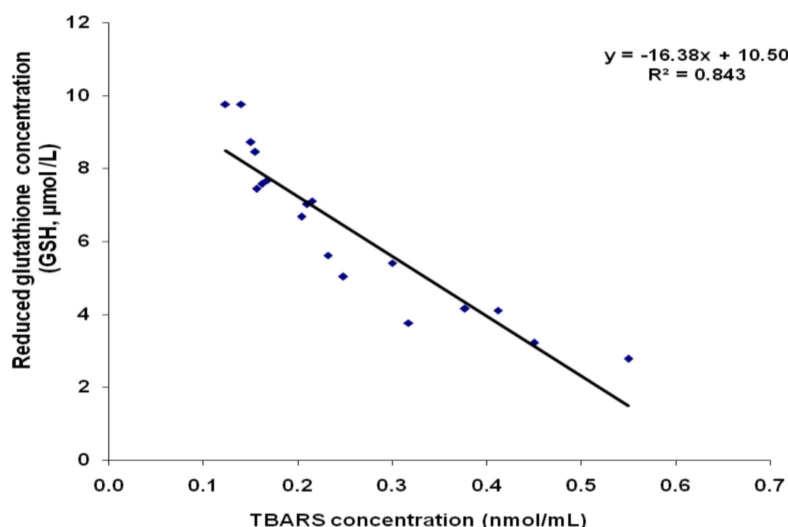


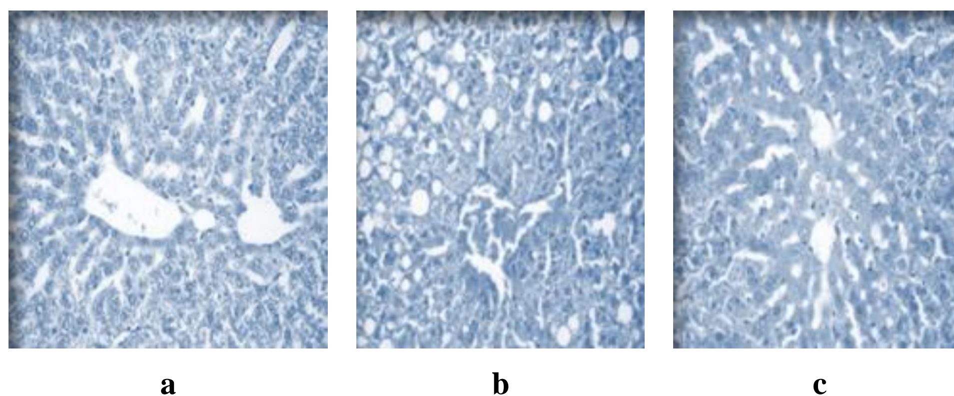
Figure 1. Correlation between lipid peroxidation (TBARS) and non-enzymatic antioxidant (GSH) in rats treated with CCl4 and feeding MLP

On the other side, correlation analysis indicated that important differences were found between lipid peroxidation (TBARS) and non-enzymatic antioxidant (GSH) in rats subjected to CCl4 and the same rats feeding MLP (Figure 1). From such data it could be noticed that there was a significant ($p \leq 0.05$) negative relationship between GSH and TBARS concentrations in plasma (r^2 = -0.843). By other meaning, a fall in GSH

level observed in rats of all groups are generally accompanied by a concomitant increased in the serum lipid peroxidation (MDA content). In similar study, Fayez, (2016) reported that high levels of TBARS/MDA in the plasma of rats treated with benzo(*a*)pyrene were associated with rather low levels of different oxidative parameters including GSH. Also, Bohm *et al*., (1997) found that a combination of different bioactive compounds (α-tocopherol and β-carotene) i.e. such as found in MLP interact synergistically to inhibit lipid peroxidation subsequently increased TBARS in some model systems.

The effect MLP on liver histopathological changes induced by CCl4 in rats.

Liver of rats fed the control diet (negative control) showed the normal histological structure of the central veins and surrounding hepatocytes (Figure 2a). Rats treated with CCl4 (positive control) for 6 weeks showed congestion of central vein and cytoplasm vacuolization of hepatocytes (Figure 2b). Feeding on diet containing 6% w/w MLP for 4 weeks and administrated CCl4 for 2 weeks showed Kupffer cells activation and slight hydropic degeneration of some hepatocytes (Figure 2c). Such histopathological changes were confirmed by the biochemical results.



a **b** **c**

Figure (2). The effect MLP on liver histopathological changes induced by CCl4 in rats. (a) normal (control diet), (b) fed the control dies and administrated CCl4 for 6 weeks, (c) fed diet containing 6% w/w MLP for 4 weeks and administrated CCl4 for 2 weeks.(H and E, X40)

Conclusion

The burden of liver diseases has been increasing in Egypt with a doubling of the incidence rate in the past 10 years. This has been attributed to several biological and environmental/dietary and occupational exposure factors. Oxidative stress and immunedeficiency parameters appear as major contributors in the development of many metabolic complications associated with the liver toxicities/injuries by chemical toxins. Lowering oxidative stress and increasing the immunological parameters prevent such metabolic disorders and complications therefore constitutes an interesting target. Feeding MLP successfully applied as functional food for containing several classes of bioactive compounds and exhibited antioxidant activity that are able to prevent or inhibit liver injuries induced by chemical toxin (CCl_4) . We recommended MLP by a concentration about 6% (w/w) to be included in our daily dishes and drinks as well as in different food products as a natural food additive.

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التأثيرات الوقائية المحتملة لأوراق التوت الأبيض على إعتلالات كبد الفئران المستحث بواسطة رابع كلوريد الكربون

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الملخص العربى

أجريت الدراسة الحالية بهدف إستيضاح التأثيرات الوقائية المحتملة لأوراق التوت الأبيض على إعتلالات كبد الفئران المستحث بواسطة رابع كلوريد الكربون. لذلك تم تجهيز مسحوق من أوراق التوت الأبيض وتقدير التركيب الكيميائى والمركبات النشطة حيويا والنشاط المضاد للأكسدة. ولقد أوضحت النتائج أن مسحوق أوراق التوت الأبيض يحتوى على مستويات عالية من البروتين والرماد والألياف الخام والكربوهيدرات والفيتامينات المضادة للأكسدة (أ، جـ، هـ) والمؤكسدات غير الغذائية (الفينولات والكاروتينات). أيضا أظهر مسحوق أوراق التوت الأبيض خواص عالية مضادة للأكسدة (النشاط المضاد للأكسدة، ٦٣,٠٤%، •DPPH ، ١٢٩,٥٢ ميكرومول ترولكس/جرام وزن جاف). أما ما يتعلق بالتجارب البيولوجية، فقد تم تغذية الفئران على مسحوق أوراق التوت الأبيض بتركيزات ٠,٧٥-٦% فى الوجبات الأساسية للفئران لمدة ٢ أسبوع ثم تم الحقن بواسطة رابع كلوريد الكربون لإحداث إعتلالات بالكبد. وقد أحدث رابع كلريد الكربون زيادات معنوية فى درجات نشاط إنزيمات الكبد AST (90.76%), ALT (62.67%) ALP (183.15%) وذلك عند المقارنة بالمجموعة الضابطة. كما أدى تدعيم الوجبات الخاصة بالفئران بمسحوق أوراق التوت الأبيض الى تحسن واضح فى درجة نشاط تلك الإنزيمات. كما سجل نفس السلوك فيما يتعلق بالمواد النشطة لحامض الثيوباربتيويك والذى يشكل مؤشرا حيويا على الإجهاد التأكسدى فى الخلايا. كما سجل سلوكا معاكسا فيما يتعلق بالمقاييس المناعية التى تشمل مستويات الألبيومين ونشاط انزيم البروتيز وجزيئات الجلوتاثيون فى السيرم. كما تم تأكيد تلك النتائج بالفحص الميكروسكوبى للكبد. ولعل نتائج تلك الدراسة تدعم الفرضية أن مسحوق أوراق التوت الأبيض يمكن أن يستخدم بنجاح كأغذية وظيفية لإحتوائها على المجموعات العديدة من المركبات النشطة حيويا والتى لها القدرة على الوقاية من أو تثبيط إعتلالات كبد الفئران المستحث بواسطة رابع كلوريد الكربون. لذلك،توصى الدراسة بخلط مسحوق أوراق التوت الأبيض بتركيزات ٦% (وزن/وزن) فى أطباقنا ومشروباتنا اليومية وكذلك مختلف المنتجات الغذائية كإضافات غذائية طبيعية.

الكلمات المفتاحية: مسحوق من أوراق التوت الأبيض، وظائف الكبد، الجلوتاثيون، المقاييس المناعية، حامض الثيوباربتيوريك، الفحص الهستوباثولوجى.