Hepatoprotective Effects of White, Red and Black Mulberry Against CCl4-Induced Hepatotoxicity in Rats

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Abstract

Berries (black, blue, red and rasp) are among the better plants with medicinal value due to its higher content of bioactive phenolic compounds. The present study was performed to find out the effectiveness of white, red, and black mulberry in protection against CCl_4-induced-hepatotoxicity in rats. The total phenolics, flavonoids, anthocyanin, and carotenoids as well as the total antioxidant capacity of white, red, black mulberry were identified. To accomplish the current study forty-two male rats were divided into six groups, of 7 rats each. Group 1, kept as the negative control group fed on the basal diet alone. Groups 2, 3, 4, 5 and 6 were intraperitoneal injected (IP) once a week for six weeks with 1 ml/kg b.wt of CCl_4 to induce liver toxicity. Group 2, kept as a positive control group and fed on the basal diet only, while hepatotoxic groups 3, 4, 5 and 6 were nourished on a supplemented basal diet with 5% of dried white, black, red mulberries and their mixture in proportion to 1:1:1, respectively. In comparison to untreated hepatotoxic-rats (positive control group) fed on the basal diet alone, feeding hepatotoxic-groups on an enriched diet with the three different berry colors caused a significant increased ($p<0.05$) in body weight gain, serum levels of HDL-c, total protein and albumin, activities of antioxidant enzymes (CAT, SOD, GSH and GPx), and decreased in the activities of AST, ALT and ALP enzymes, and serum levels of total, direct and indirect bilirubin, total lipids, cholesterol, triglycerides, LDL-c, VLDL-c and MDA. In addition, histopathological examination confirmed the results of biochemical analysis and showed an obvious improvement in the liver sections of hepatotoxic-rats fed on the enriched basal diet with three different berry colors. Finally, the existing study demonstrated that different colors of berries could improve the liver functions and activity of antioxidant enzymes by eliminating the deleterious toxic effect of CCl_4, especially with their mixtures.

Keywords: Berries; Antioxidant Enzymes; Carbon Tetrachloride; Liver Injuries.

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Introduction

Berries, in particular the nation ones such as black, blue, red and rasp are among the better plants with medicinal value due to the presence of a wide range of bioactive phenolic compounds (**Baby *et al.*, 2018).** Mulberry (*Morus*) is a perennial, woody, economic, fast-growing deciduous tree, distributed worldwide and used for centuries in agriculture, food, cosmetics and medicament due to its chemical composition and pharmacological effectiveness **(Gr *et al.*, 2017 and Khalifa *et al.*, 2018)**. There are about 11 main species of mulberry in the world, however the most common sorts consisting of black (*Morus nigra L.*), white (*Morus alba L.*) and red (*Morus rubra L.*) **(Ma *et al.*, 2022)**, also called America mulberry and native to the eastern United States, followed by other species: *Morus australis*, *Morus bomycis*, *Morus laevigata*, *Morus serrata*, *Morus macroura*, *Morus cathayana*, *Morus multicaulis* and *Morus insignis* **(Turan *et al.*, 2017 and Wei *et al.*, 2018)**. Mulberry exhibited a wide spectrum biological effects including hepatoprotective **(Deniz *et al.*, 2018)** and neuroprotective, anticancer, antidiabetic, lipid metabolism regulatory activities and antiaging **(Forbes *et al.*, 2017)**, anti-inflammatory, antiviral, cytotoxic and anti-hypertensive **(Wen *et al.*, 2019 and Chen *et al.*, 2021).**

The liver is the body's largest gland, and a vital immunity organ. It has an important vital function in removing poisons, secretory and excretory functions and secretion of bile, as well as carbohydrate, protein and fat metabolism and storing energy. **(Ahmed *et al.*, 2019)**. The liver being an important organ and is often exposed to array of threats **(Zhang *et al.*, 2020)**.

Liver may face with several kinds of fatal diseases caused by viruses such as hepatitis (A, B, C, and delta virus), hepatotoxic chemicals or drugs, fatty liver, cirrhosis, inherited disorders (e.g.,

hemochromatosis and Wilson diseases), obesity, malnutrition, alcohol, autoimmune disorders, type-2 diabetes, and genetic factors (**Zhang *et al.*, 2020**). Notwithstanding, there are some symptoms like fatigue, vomiting, nausea, yellowing of the eyes and skin, as well as weight loss, back pain, swelling of the abdomen and legs, and in some cases no symptoms are observed in the patients. **(Nahar and Ara, 2018)**. Liver injury can lead to violation of its functions and may sustain failure (**Wang *et al.*, 2019**). Liver failure is complemented by chronic fibrosis due to cirrhosis, hematoma and hepatocellular carcinoma. **(Koyama *et al.*, 2016)**.

The liver diseases are of public health interest because the traditional medical solution for liver diseases produce restricted results with related side effects. So, the utilization of complementary and alternative herbal medicine has attracted research concern for novel plausible hepatoprotective agents, efficient medicines at ameliorating or reversing liver damage with few side effects (**Zhang *et al.*, 2012**). In the last decade, considerable progress has been made in understanding of the pathophysiology of liver disorders. It is the main target tissue complicated in responding to different classes of oxidative stresses **(Baradaran *et al.*, 2019)**. Oxidative stress and inflammation are the quintessence of mechanisms of liver injury induced by agents of chemicals and/or viruses (**Ramachandran and Jaeschke, 2018**). Natural antioxidant and anti-inflammatory effects of plants and their phytochemicals have been well documented (**Kazemi *et al.*, 2018**). Finding effective and safe natural hepatoprotective agents is one of the future directions. Therefore, the present study was performed to find out the effectiveness of white, red, and black mulberry in protection against CCl_4-induced-hepatotoxicity in rats. In addition to, the total phenolics, flavonoids, anthocyanin, and carotenoids as well as the total antioxidant capacity of white, red, black mulberry were identified.

Materials and Methods:

Materials:

Fresh white, red, black mulberry: Fresh white, red and black mulberry fruits (MFs) were purchased from the local market, Cairo, Egypt.

Rats: Forty-two of male adult rats (Sprague Dawley Strain), weighing (180±7g) were obtained from the Laboratory Animal Colony, Helwan, Egypt.

Basal Diet Constituents: Basal diet constituents (AIN-93M) were purchased from the El-Gomhorya Company for Trading Drugs and Chemicals, Cairo, Egypt. Starch, soybean oil, and sucrose were obtained from the local market, Cairo, Egypt.

Chemicals and Kits for Biochemical Analysis: Carbon tetrachloride (CCl_4), diethyl ether and other chemicals used in this study were acquired from the El-Gomhoriya Company for Trading Drugs and Chemicals, Cairo, Egypt. Kits for biochemical assay were purchased from the Gamma Trade Co., for Pharmaceutical and Chemical, Dokki, Egypt.

Methods:

Preparation of Dried White, Red, Black Mulberry: The fresh white, red and black mulberry fruits were cleaned, sorted and washed from dust and removed all invalid parts and dried in an oven-under vacuum at 50 C°. A grinder mill and sieves were used to obtain a powder particle size of less than 0.4mm of fruits. Then, the final powder was packaged in an enclosed bags and stored in the refrigerator at 5 ºC till use.

Proximate analysis of White, Red and Black Mulberry: Proximate analysis of white, red and black mulberry was performed according to official methods procedures (**AOAC., 2000**) including crude proteins, carbohydrates, fats, fibers and PH.

Nitrogen content was determined by using the Kjeldahl method and multiplied by a factor of 6.25 to determine the crude protein content. The amount of total carbohydrates was obtained by the difference between weight of the sample taken and sum of its moisture, ash, total lipid, protein, and fiber contents. Crude fat was obtained by exhaustively extracting 5.0 g sample power in a Soxhlet apparatus using petroleum ether (boiling point range 60-90°C) as extractant. The pH was determined at 20 °C with a digital pH meter calibrated with pH 4.00 and pH 6.86 buffer solutions

Determine of Total Phenolics, flavonoids, Anthocyanin and Carotenoids in White, Red and Black Mulberry: The total content of phenolics, flavonoids, anthocyanins and carotenoids in white, red and black berries was estimated according to the described methods by **Singleton *et al.*, (1999), Djeridane *et al.*, (2006), Giusti and Wrolstad., (2005) and Aljane and Sdiri (2014),** respectively.

Determination of Total Antioxidant Capacity: The DPPH scavenging activity of each sample was determined as described by **Bey *et al.*, (2013)** with some modification. Extracts of mulberry fruits were tested at a polyphenols concentration of 0.1

mmol·l-1(GAE). Briefly, 100 µmol/L of DPPH in ethanol was prepared and 2.0 ml of this solution was added to a test sample (2.0 ml). The reaction mixture was shaken well and incubated for 30 min at room temperature in the dark. The absorbance of the resulting solution was measured at 517 nm against a blank. Ascorbic acid was used as a positive antioxidant control.

Formularization of The Basal Rat-Diet (AIN-93M): Dietary components of basal diet for rats were formulated collectively to meet the desirable adequate dietary intake of rats as revealed by **Reeves *et al.*, (1993)** in Table **1**.

Table 1: **Components of Rat-diet (AIN-93M) per 1 kg. Diet**

Components	Amounts (g)
Casein (85% protein)	140
Cornstarch	465. 692
Dextrinized cornstarch	155
Sucrose	100
Fiber	50
Soybean oil	40
Mineral mixture	35
Vitamin mixture	10
Choline chloride	2.5
L-cysteine	1.80
Tert-butylhydroquinone	0.008

Induction of Hepatotoxicity in Rats: In this study, hepatotoxicity in all rats with the exception of negative control rats (7 rats) was induced by intraperitoneal injection (IP) once a week for six weeks with 1 ml/kg b.wt of CCl_4 dissolved in paraffin oil in a 1:1 portion (v:v) as demonstrated by **Karthikeyan and Deepa, (2010).**

Experimental Design and Grouping of Rats: All rats were housed at the animal house of the Faculty of Home Economics, Helwan University in wire cages under controlled environmental conditions of the light/dark cycle (12/12 hr), temperature (22±4°C) and relative humidity (45% to 50%). The food and water supplies was uninterrupted during the experimental period. Prior to the trial study, rats were kept for a week to acclimatize. Subsequently, rats were randomized into six groups, each with seven rats as follows:

- Group (1), rats were kept as a negative control group (healthy rats) and fed on the normal basal diet.
- Group (2), rats were maintained as a positive control group, IP injected with CCl_4 and fed on the normal basal diet.
- Group (3), rats with hepatotoxicity were fed on the supplemented diet with 5% of dried white berries.
- Group (4), rats with hepatotoxicity were fed on the supplemented diet with 5% of dried black berries.
- Group (5), rats with hepatotoxicity were fed on the supplemented diet with 5% of dried red berries.
- Group (3), rats with hepatotoxicity were fed on the supplemented diet with 5% of the dried mixture of white, red and black berries at 1:1:1 ratio.

Determination of FI (g) and BWG (g) and RWG (%): Food intake (FI) was calculated every day during the experimental period (6 weeks). The changes in body weight were determined by weighing the animals on a balance scale prior the experiment (initial body weight) and at the end of the experimental period (final body weight). The biological value of the diet was assessed by the determination of its effect on body weight gain (BWG) and the relative body weight gain (RWG%) was calculated using the following formula as described by **Kratochvílova *et al.*, (2002).**

BWG = Final Body Weight (FBW)- Initial Body Weight **(IBW)**

Change of body weight gain %= BWG/IBW X 100

Collection of Blood Samples: At the end of the experimental period (6 weeks), rats in all groups were fasted for 12 hours, anesthetized with diethyl ether. Portal vein blood samples were collected in clean, dry centrifuge tubes and left to coagulate at room temperature. The clotted blood was centrifuged at 3000 rpm for 15 minutes to obtain serum. Then, clear serum samples were taken into the Eppendorf's tubes (1.5 mL) and stored at –20ºC until they were used in biochemical analysis.

Biochemical Assay:

Estimation of Liver Functions: The activity of Alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) enzymes was measured colorimetric by utilizing (Diamond Co, Hanover, Germany) Kits according to instruction of **Young (1990)** and

Sherwin (1984), respectively. The biometrics were measured using a spectrophotometer (Hum star 200, automatic biochemistry analyzer, Germany) adjusted at 505 and 510 nm, respectively.

Serum concentrations of total protein (TP), albumin (Alb), total bilirubin (TBL) and direct bilirubin (DBL) were measured colorimetrically using a spectrophotometer (Hum star 200, automatic biochemistry analyzer, Germany) as described by **Tietz (1994), Young (2000), Henry (1991)** and **Burtis and Ashwood (1999)**, respectively. The device was regulated at 545, 628, 520 and 548 nm, respectively for measuring the color intensity that reflect the serum concentration of the tested parameters. While indirect bilirubin (IDBL) was estimated by calculating the difference between total and direct bilirubin using the present formula:

Indirect bilirubin (mg/dl) = Total bilirubin-Direct bilirubin

Estimation of Lipid Profile: Serum levels of total lipid (TL), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-c), and low density lipoprotein cholesterol (LDL-c) were estimated using commercial reagent kits (Biomed diagnostic, Egypt) as described by **Zollner and kirsch (1962), Vassault *et al.*, (1986), Hostmark *et al.*, (1991), Friadwald *et al.*, (1972) and Young, (2001),** respectively. While very low-density lipoprotein cholesterol (VLDL-C) was calculated using Friedewald's formula.

VLDL-c (mg/ dL) = TG/5

Estimation of Malondialdehyde and Activities of Antioxidant Enzymes: The serum concentration of MDA and the activity of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxides (GPx) enzymes were determined using commercial assaying kits (Cayman Practice ELISA Kits). The principal method for the determination of oxidative stress depends on colorimetric by quantifying thiobarbituric acid (TBA) reactivity as malondialdehyde (MDA) in a spectrophotometer adjusted at 534 nm according to the described method by **Ohkawa *et al.*, (1979)**. The procedure that is used for the evaluation of CAT activity depends on the reaction of the enzyme with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced is measured spectrophotometrically at 510 nm as described by **Aebi., (1984).**

The standard technique to assay the activity of SOD is that the kits used use an enzyme linked immunosorbent assay double antibody principle. The color change is measured spectrophotometrically at 560 nm as described by **Nishikimi *et al.*, (1972)**. The serum activity of GSH and GPx was assayed according to the kit's instructions as described by **Beutler *et al.*, (1963)** and **Paglia and Valentine., (1967)** using spectrophotometrically at 405 nm and 340 nm.

Histopathological Examination: The liver of all the scarified rats were immersed in 10% formalin solution. The fixed specimens were then trimmed, washed and dehydrated in ascending grades of alcohol. Specimens were then cleared in xylol, embedded in paraffin, sectioned at 4-6 microns thickness, and stained with Heamtoxylin and Eosin stain for examination of the liver as described by **Carleton, (1979).** The Histopathological examination were conducted at the Faculty of Veterinary Medicine, Cairo University.

Statistical Analysis: Data was evaluated statistically using computerized SPSS package program (SPSS 22.00 software for Windows) by one-way analysis of variance (ANOVA). The obtained data was expressed as Mean ± SE and the significant difference among means was estimated at $p<0.05$ (**Snedecor and Cochran 1980).**

Results

Proximate Composition of White, Black, and Red Mulberry Fruits:

The Quantitative content of proximate composition of the white, black, and red mulberry fruits is recorded in Table **(2).** Recorded results showed that the total protein and total carbohydrate in black mulberry fruit were higher than that of white and red mulberry fruits. Content of total fat of red Mulberry fruit was higher than that of white and black Mulberry fruits. Total fiber content in white mulberry fruit was higher than that of both black and red Mulberry fruits. Regarding to PH value of white, black, and red were 5.88, 6.13 and 5.98, respectively.

Table (2): Proximate Composition of White, Black, and Red Mulberry Fruits.

Mulberry Fruits / Major Constituents	White	Black	Red
Total Protein (%)	14.40±1.10	16.23±0.60	15.16±0.55
Total Fat (%)	0.39±0.04	0.35±0.03	0.41±0.02
Total Carbohydrate (%)	23.80±1.22	24.31±0.51	23.11±0.48

Total Fiber (%)	14.20±0.34	13.90±0.57	12.95±0.52

Total Phenolics, Flavonoids, Anthocyanin and Carotenoids in White, Black, and Red Mulberry Fruits

The total content of phenolics, flavonoids, anthocyanins and carotenoids in white, red and black mulberry fruits is presented in Table **(3).** The tabulated results revealed that black mulberry have the highest content of phenolic compounds, followed by white (1352±2.36) and the red mulberry. The highest content of flavonoid compounds and TAC was found in black mulberry followed by red and the white mulberry. Also, the highest content of total anthocyanin and carotenoids was in founded in red mulberry followed by black and white mulberry.

Table (3): Total Phenolics, Flavonoids, Anthocyanin and Carotenoids in White, Black, and Red Mulberry Fruits

Mulberry Fruits / Phytochemical compound	White	Black	Red
Total Phenolics (mg/100g)	1352±2.36	1375±2.53	1035±2.01
Total Flavonoids (mg/100g)	188.99±0.38	265±1.05	241±1.03
Total Anthocyanin (mg/100g)	25.21±0.25	28.10±0.34	31.28±0.41
Total Carotenoids (mg/100g)	0.57±0.16	0.85±0.03	1.28±0.05
TAC (mg/100g)	125.53±0.29	175±0.96	133±0.85

Effect of Supplemented Diet with White, Black, and Red Mulberry Fruits on FI, BWG and RBWG (%) in Rats with Hepatotoxicity:

The effect of feeding on the supplemented diet with white, black, and red mulberry fruits and their mixture (1:1:1) at a level of 5% on FI, BWG and RBWG (%) in rats with hepatotoxicity is recorded in Table (4). Recorded results interpreted that the injected-rats with CCl4 and fed on the basal diet alone (positive control group) have a significant ($P<0.05$) decrease in FI, FBW, BWG and RWG (%), compared to that of normal rats (negative control group). Whereas, feeding rats on a supplemented diet with white, black, and red mulberry fruits and their mixture and co-combined with IP injection by CCl_4 have significant ($P<0.05$) increase in FI, FBW, BWG and RWG (%), comprised with the positive rats feed on the basal diet alone. The superior results in FI, FBW, BWG and RWG (%) were established in treated groups by red mulberry and the combination of the three different colors mulberry fruits, compared to the other treated groups with white and black mulberry fruits

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Table (4): Effect of supplemented diet with White, Black, and Red Mulberry Fruits on FI, BWG and RBWG (%) in Rats with Hepatotoxicity

Groups / Parameters	− ve	+ ve	Tread group with			
			White	Black	Red	Combination
FI (g)	15.43±0.80^{a}	12.90±0.70^{c}	13.90±0.95^{b}	14.90±0.97^{a}	15.90±0.96^{a}	15.70±0.86^{a}
IBW(g)	180.75±0.99^{a}	180.75±0.99^{a}	181.43±0.80^{a}	181.50±0.80^{a}	181.00±0.82^{a}	180.80±0.88^{a}
FBW (g)	220.86±1.05^{a}	209.01±1.30^{c}	216.44±1.50^{d}	219.90±0.70^{a}	220.70±1.70^{a}	221.50±1.30^{a}
BWG (g)	40.11±1.25^{a}	28.26±1.11^{c}	35.01±1.04^{b}	38.4±0.78^{a}	39.7±1.33^{a}	40.70±1.23^{a}
RBWG (%)	22.19±1.44^{a}	15.63±1.43^{c}	19.30±1.02^{b}	21.16±0.48^{a}	21.93±0.69^{a}	22.51±0.39^{a}

Data are expressed as the mean ± SE; Mean values with different superscript letters at the same row are significantly different at $P < 0.05$

Effect of Supplemented Diet with White, Black, and Red Mulberry Fruits on the Activity of ALT, AST and ALP Enzymes in Rats with Hepatotoxicity:

The attained results in Table (**5**) exhibit the effect of CCl4-induced liver toxicity without or with feeding on the supplemented diet with white, black, and red mulberry fruits and their

combination on the activity of liver enzymes in rat groups. Delimited results showed that the CCl4-treated group feeding on the basal diet alone had a significant (P<0.05) increment in activities of liver enzymes (ALT, AST and ALP), compared to a normal control group. However, combining a supplemented diet with the three different colors mulberry fruits (white, black, and red) and their combination with the by CCl_4 injection, results in a significant (p<0.05) lowering in the activity of liver enzymes. The best improvement results were reported in rats treated with the combination of the three different colors mulberry fruits.

Effect of Supplemented Diet with White, Black, and Red Mulberry Fruits on the Serum Concentrations of TP, Alb, TBL, DBL and IDBL in Rats with Hepatotoxicity:

The obtained results in Table (6), exhibit the effect of supplemented diet with white, black, and red mulberry fruits and their mixture on the serum levels of TP, Alb, TBL, DBL and IDBL in Rats with Hepatotoxicity. The results showed that the CCL4-treated group feeding on the basal diet alone had a significant (P<0.05) decrease in the serum levels of TP and Alb, and increase in the levels of TBL, DBL and IDBL, compared to a normal control group. However, combining a supplemented diet with the three different colors mulberry fruits (white, black, and red) and their mixture with the injection of CCl4, results in a significant (p<0.05) ameliorates serum levels of TP, Alb, TBL, DBL and IDBL, compared with the positive control group. In addition, the results showed that the supplemented diet with red mulberry increases the improvement rate of serum concentration of the above parameters.

Table (5): Effect of supplemented diet with White, Black, and Red Mulberry Fruits on the activity of ALT, AST and ALP enzymes in rats with hepatotoxicity.

Groups / Parameters	− ve	+ ve	Tread group with			
			White	Black	Red	Combination
ALT (u/ml)	32.47 ± 0.4^{4d}	101.50 ± 2.58^{a}	73.14 ± 0.74^{b}	72.06 ± 1.36^{b}	70.04 ± 1.12^{b}	55.840 ± 1.538^{c}
AST(u/ml)	44.07 ± 0.5^{2f}	119.94 ± 0.8^{3a}	87.76 ± 1.6^{4b}	83.57 ± 0.91^{c}	78.23 ± 1.8^{3d}	61.810 ± 1.046^{e}
ALP (ng/ml)	1.97 ± 0.06^{d}	9.23 ± 0.14^{a}	6.79 ± 0.2^{7b}	6.38 ± 0.06^{b}	6.41 ± 0.11^{b}	4.031 ± 0.107^{c}

Data are expressed as the mean ± SE; Mean values with different superscript letters at the same row are significantly different at P < 0.05

Table (6): Effect of Supplemented Diet with White, Black, and Red Mulberry Fruits on the Serum Concentrations of TP, Alb, TBL, DBL and IDBL in Rats with Hepatotoxicity

Groups / Parameters	− ve	+ ve	Tread group with			
			White	Black	Red	Combination
TP (gm/dl)	11.59 ± 0.20^{a}	8.40 ± 0.19^{d}	8.50 ± 0.26^{d}	9.55 ± 0.25^{c}	10.61 ± 0.23^{b}	10.62 ± 0.23^{b}
Alb (gm/dl)	5.65 ± 0.27^{a}	4.71 ± 0.14^{d}	5.06 ± 0.40^{c}	5.40 ± 0.05^{b}	5.52 ± 0.11^{ab}	5.39 ± 0.05^{b}
TBL (gm/dl)	0.75 ± 0.12^{d}	0.95 ± 0.13^{a}	0.81 ± 0.11^{b}	0.67 ± 0.21^{c}	0.59 ± 0.11^{d}	0.61 ± 0.12^{d}
DBL (gm/dl)	0.20 ± 0.11^{c}	0.40 ± 0.16^{a}	0.39 ± 0.12^{a}	0.29 ± 0.11^{b}	0.22 ± 0.11^{c}	0.22 ± 0.13^{c}
IDBL (gm/dl)	0.39 ± 0.12^{c}	0.53 ± 0.16^{a}	0.45 ± 0.11^{b}	0.38 ± 0.12^{c}	0.39 ± 0.12^{c}	0.40 ± 0.13^{c}

Data are expressed as the mean ± SE; Mean values with different superscript letters at the same row are significantly different at P < 0.05

Effect of Supplemented Diet with White, Black, and Red Mulberry Fruits on the Serum levels of TL, TC, TG, HDL-c, LDL-c and VLDL-c in Rats with Hepatotoxicity:

Results in Table (**7**) demonstrate the effect of the CCL4-treated rats feeding on the basal diet alone and/or feeding on a supplemented diet with white, black, and red mulberry fruits and their mixture at ratio of 1:1:1 on the serum levels of TL, TC, TG, HDL-c, LDL-c and VLDL-c. In comparison to the negative control group, IP injection of CCl4 induced a significant (P<0.05) increase in serum concentrations of TL, TC, TG, LDL-c and VLDL-c and decrease in the level of HDL-c. However, in comparison to the positive control group, feeding on the supplemented diet with white, black, and red mulberry fruits and their mixture resulted in significantly lower in serum levels of TL, TC, TG, LDL-c and VLDL-c and increase in the level of HDL-c. A better improvement in serum levels of TL, TC, TG, HDL-c, LDL-c and VLDL-c, was discovered in treated hepatotoxicity rats with the mixture of the three different colors mulberry fruits.

Table (7): Effect of Supplemented Diet with White, Black, and Red Mulberry Fruits on the Serum Concentrations of TL, TC, TG, HDL-c, LDL-c and VLDL-c in Rats with Hepatotoxicity

Groups / Parameters	− ve	+ ve	Tread group with			
			White	Black	Red	Combination
TL (mg/dl)	403.71±4.82^{e}	643.29±6.62^{a}	575.0±9.37^{b}	508.00±1.8^{c}	440.71±3.9^{d}	439.70±3.97^{d}
TC (mg/dl)	64.06±0.68^{e}	118.38±0.84^{a}	91.80±1.14^{b}	89.71±1.24^{b}	81.17±0.43^{c}	71.46±0.32^{d}
TG (mg/dl)	82.00±0.34^{f}	130.90±1.10^{a}	107.02±1.41^{b}	101.34±1.07^{c}	95.73±1.18^{d}	88.95±0.9^{1e}
HDL-c (mg/dl)	32.14±0.44^{a}	11.64±0.5$^{1\,d}$	19.71±0.38^{c}	20.83±0.29^{c}	20.51±0.69^{c}	26.31±0.63^{b}
LDL-c (mg/dl)	15.50±0.40^{f}	54.22±0.61^{a}	35.39±1.15^{b}	32.58±0.83^{c}	30.16±0.22^{d}	20.78±0.4^{8e}
VLDL-c (mg/dl)	16.40±1.02^{d}	26.18±1.33^{a}	21.40±1.25^{b}	20.27±1.07^{b}	19.15±0.77^{c}	17.79±0.49^{d}

Data are expressed as the mean ± SE; Mean values with different superscript letters at the same row are significantly different at P < 0.05

Effect of Supplemented Diet with White, Black, and Red Mulberry Fruits on the Serum Concentrations of MDA and the Activity of CAT, SOD, GSH and GPx Enzymes in Rats with Hepatotoxicity:

Table (8) represents the effect of three different colors mulberry fruits and their mixture on the protection of rats from an oxidative imbalance resulting from toxicity caused by CCl4. Serum levels of MDA and the activities of antioxidant enzymes (CAT, SOD, GSH and GPx) were used as indicators of this effect. Our results revealed that IP injection of CCl4 induced a significant ($p<0.05$) rise in serum MDA level, and lower activity of AT, SOD, GSH and GPx enzymes, in comparison to the negative control group. Combining the three different colors mulberry fruits and their mixture in the diet with the IP administration of CCl4, significantly reduced serum levels of MDA and increased the activity of the mentioned antioxidant enzymes, compared to positive control rats fed on the basal diet alone. The superior result in serum concentration of MDA and activity of antioxidant enzymes was discovered in treated hepatotoxicity rats with the mixture of the three different colors mulberry fruits.

Table (8): Effect of Supplemented Diet with White, Black, and Red Mulberry Fruits on the Serum Concentrations of MDA and the activity of CAT, SOD, GSH and GPx Enzymes in rats with Hepatotoxicity.

Groups / Parameters	− ve	+ ve	Tread group with			
			White	Black	Red	Combination
MDA (nmol/L)	0.82±0.0^{1e}	4.69±0.06^{a}	2.65±0.16^{b}	2.75±0.09^{b}	2.36±0.0^{9c}	1.59±0.10^{d}
CAT (mmol/dl)	4.58±0.03^{a}	1.01±0.0^{2e}	2.27±0.07^{d}	2.60±0.09^{c}	2.73±0.22^{c}	3.69±0.02^{b}
SOD (mmol/L)	3.83±0.01^{a}	0.95±0.01^{e}	2.39±0.17^{cd}	2.28±0.15^{d}	2.61±0.0^{7c}	3.16±0.09^{b}
GSH (mmol/dl)	5.58±0.0^{3a}	1.08±0.03^{f}	2.95±0.08^{e}	3.35±0.10^{d}	3.78±0.0^{8c}	4.70±0.04^{b}
GPX (mmol/dl)	7.21±0.1^{1a}	2.06±0.14^{e}	3.86±0.10^{d}	4.13±0.05^{c}	4.20±0.11^{c}	5.92±0.03^{b}

Data are expressed as the mean ± SE; Mean values with different superscript letters at the same row are significantly different at P < 0.05

Histopathological Examination

Histopathologically, the liver of rats from negative control group demonstrated the normal histological structure of lobules as exhibit in **Photo 1**. Microscopical examination of liver sections from positive control rats discovered marked histopathological changes as pseudo-lobulation of hepatic parenchyma due to proliferation of fibrous tissue septa that connect the portal areas (**Photo 2**). In addition, there were intense mononuclear inflammatory cells infiltration especially at the portal areas and ballooning with existence of numerous intracytoplasmic vacuoles as showed in **Photo 3**. Concerning the liver of treated rats with CCl_4 + 5% of dried white Mulberries exhibited marked improvement as well as, the hepatocytes were apparently normal or exhibiting slight swelling and congested central veins and hepatic sinusoids (**Photo 4**). Some of the portal areas showed mild inflammatory edema and mild

fibroplasia in some instances (**Photo 5**). Meanwhile, the liver of treated rats with CCl_4 + 5% of dried black Mulberries were apparently normal in some sections, while other sections exhibited minor congestion in both hepatic vessels and sinusoids as shown in **Photo 6**. Liver sections from treated rats with CCl_4 + 5% of red Mulberries showed mild hepatocellular degeneration and mild portal edema (**Photo 7**). However, liver sections from treated rats with CCl_4 + 5% of white, black and red Mulberries mixture revealed moderate diffuse hepatocellular vacuolation (**Photo 8**) and other sections showed mild portal edema and mild hepatocellular swelling (**Photo 9**).

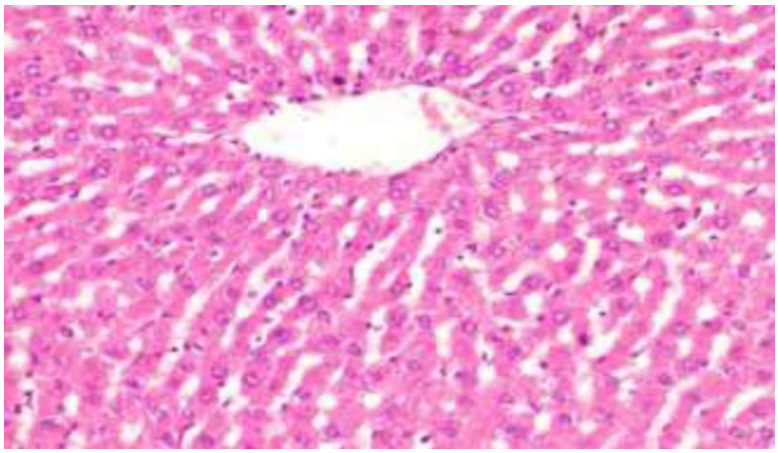

Photo 1: Photomicrograph of liver sections from rats of negative control group showing the normal histological architecture of liver (H & E X 400).

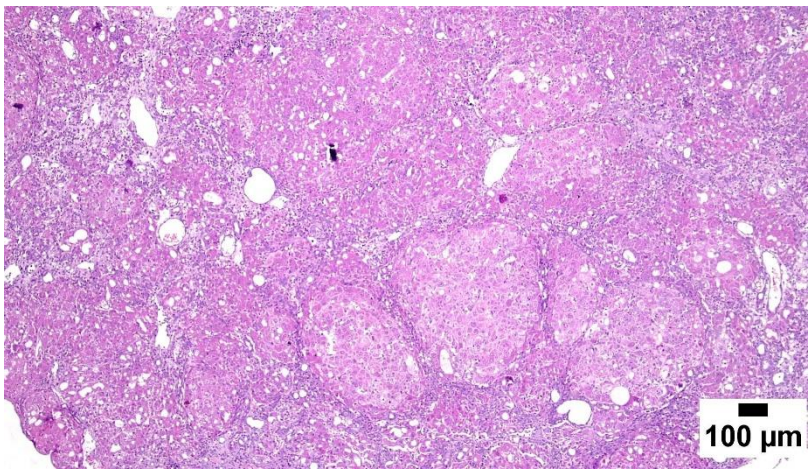

Photo 2: Photomicrograph of livers from rats of positive control group showing pseudo-lobulation of hepatic parenchyma with mononuclear inflammatory cells infiltration (H&E).

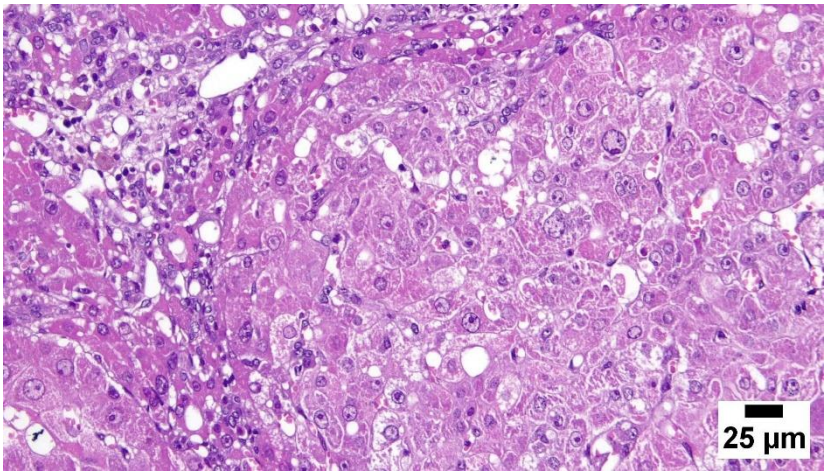

Photo 3: Photomicrograph of livers from rats of positive control group showing marked hepatocellular ballooning, proliferation of fibrous septa and mononuclear inflammatory cells infiltration (arrow) (H&E).

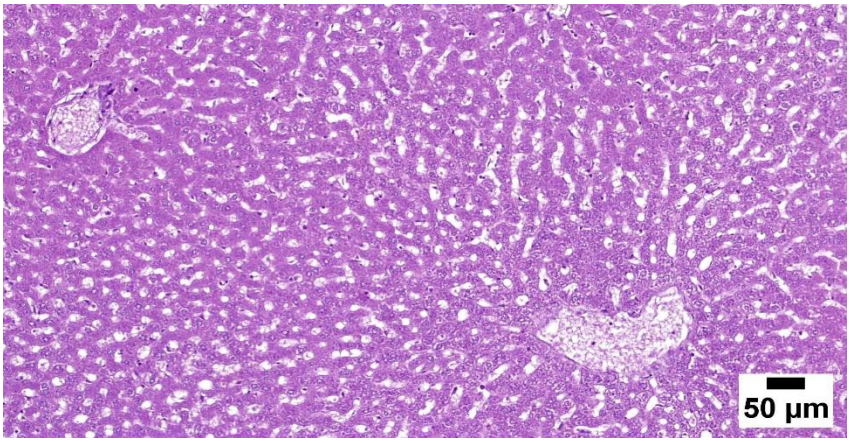

Photo 4: Photomicrograph liver of rats from treated rats with CCl_4 + 5% of dried white Mulberries showing congested central veins and hepatic sinusoids with apparently normal hepatocytes (H&E).

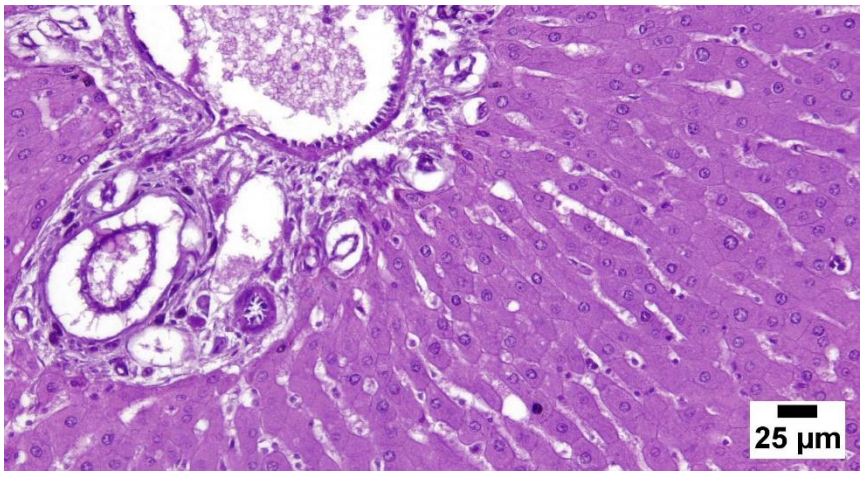

Photo 5: Photomicrograph liver of rats from treated rats with CCl_4 + 5% of dried white Mulberries showing magnification portal edema (arrow) with mild mononuclear inflammatory cells infiltration (H&E).

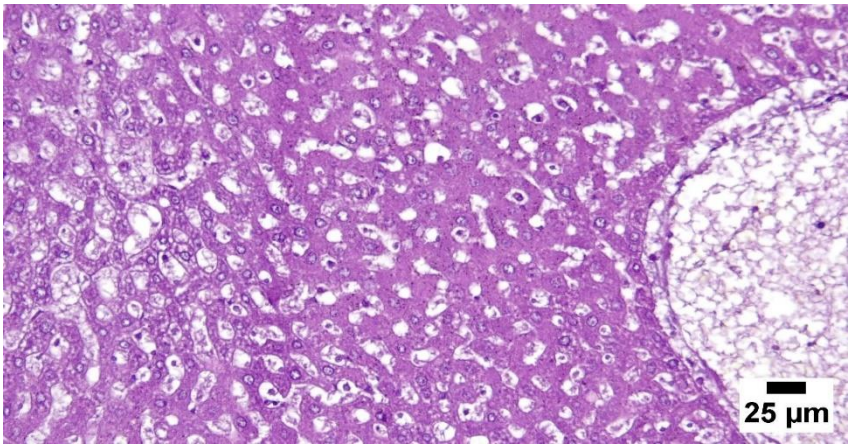

Photo 6: Photomicrograph treated rats with CCl_4 + 5% of dried black Mulberries showing minor congestion in hepatic vessels and sinusoids. (H&E).

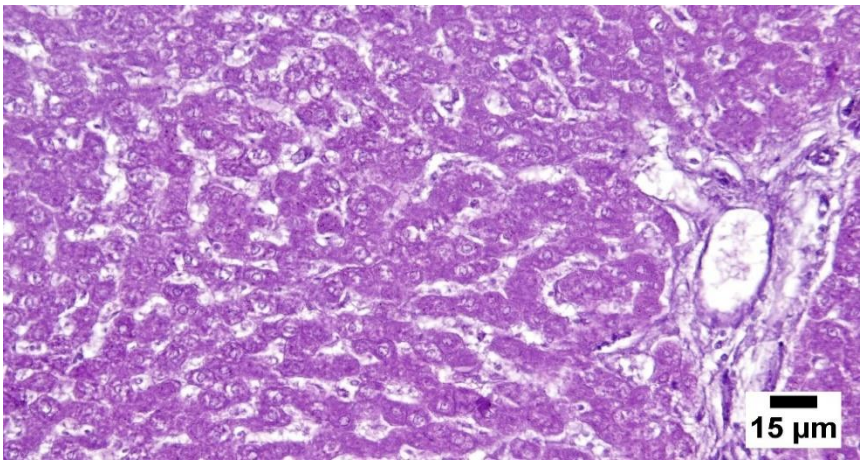

Photo 7: Photomicrograph liver of rats from treated rats with CCl_4 + 5% of dried red showing mild hepatocellular degeneration and portal edema. (H&E).

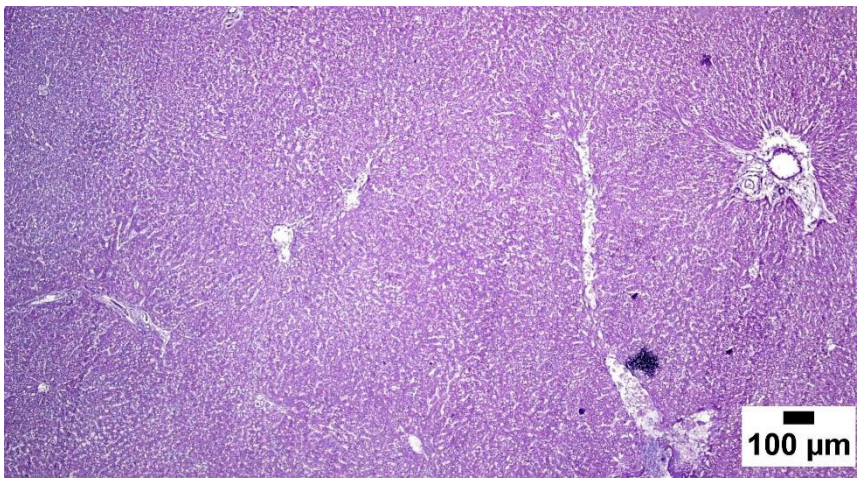

Photo 8: Photomicrograph liver of treated rats with CCl_4 + 5% of white, black and red Mulberries mixture showing mild diffuse hepatocellular vacuolation (H&E).

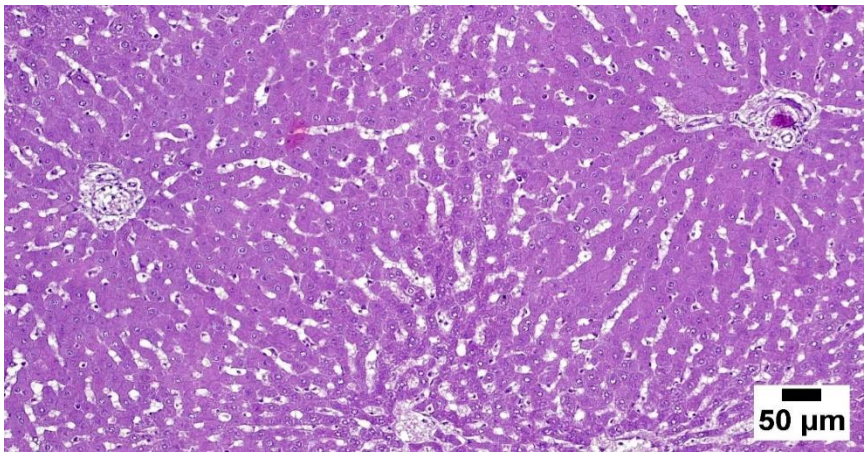

Photo 9: Photomicrograph liver of treated rats with CCl_4 + 5% of white, black and red Mulberries mixture showing mild portal edema and mild hepatocellular swelling (H&E).

Discussion

Our results showed that the protein, fat, carbohydrate and fiber contents of White Mulberry fruits were 14.40%, 0.39%, 23.80%, and 14.20%, and they were 16.23%, 0.35%, 24.31%, and 13.90% in Black Mulberry fruits, while they were 15.16%, 0.41%, 23.11%, and 12.95%, respectively, for Red Mulberry fruits. The results of White Mulberry are almost in line with **Munir *et al.*, (2018)**, who discovered that the protein and fiber were 13–15%, 12–14%, **Ercisli and Orhan (2007)**, found higher fat than our results it was 1.10%, and **Ghanem *et al.*, (2022)**, showed lower carbohydrate than our results it was 15.46%. The outcomes of Black Mulberry concur with **Khattak and Rahman (2015)**, who recorded that the fiber was between 11.6 to 14.45%, while the content of protein was lower than our results it was between 8.6 to 13.15%, the content of fat was higher than our results it was between 2.7 to 4.7%, and **Ghanem *et al.*, (2022)**, showed lower carbohydrate than our results it was 14.31%. The results of Red Mulberry are quite similar to **Abbasi *et al.*, (2015)**, in the content of protein, while was low in the content of carbohydrate, who found that the protein and carbohydrate were 13.1% and 70.7%, and were higher in the content of fiber, and lower in the content of fat than **Khalil and Embaby (2017)**, who showed that the fiber, and fat were 2.92%, and 1.35%, respectively. Our results showed that the PH value of White, Black and Red Mulberry fruits was 5.88, 6.13 and 5.98, respectively. These outcomes consistent with those mentioned by **Aljane and Sdiri (2016)**, They showed that the PH value of White, Black Mulberry was 5.98, 5.56, respectively, while **Khalil and Embaby (2017)**, found that the PH value of Red Mulberry was 6. Our results showed that the total phenolics (mg/100g), flavonoids, anthocyanin, carotenoids, and antioxidant capacity contents of White Mulberry fruits were 1352 mg, 188.99 mg, 25.21 mg, 0.57 mg, and 125.53 mg, and they were 1375 mg, 265 mg, 28.10 mg, 0.85 mg, and 175 mg in Black Mulberry fruits, while they were 1035 mg, 241 mg, 31.28 mg, 1.28 mg, and 133 mg, respectively, for Red Mulberry fruits. The results of White Mulberry are in conformity with **Raman *et al.*, (2016)**, in the content of flavonoids, and **Kim and Lee (2020)**, in the content of anthocyanin who showed that the content of flavonoids and anthocyanin were 187.23 mg and 0.95–28.61 mg, respectively. **Lin and Tang (2007)**, showed that the content of phenolics were higher than our results, and also **Owon *et al.*, (2016)**, in the content of carotenoids, which were 1515.9 mg and 13.7 mg, respectively. The results of Black Mulberry are in agreement with **Tomas *et al.*, (2015)**, in the content of phenolics, who showed it was 1375 mg, while our results are almost in line with **Khattak and Rahman (2015)**, who showed that the flavonoids were ranged from 63.7to 244.0 mg, and also with **Wang *et al.*, (2022)**, in the contents of anthocyanin and antioxidant capacity, who showed they were 21.17–239.46 mg and 28.48–100.11 mg, respectively. The results of Red Mulberry are higher than **Khalil and Embaby (2017)**, in the contents of phenolics and anthocyanin, who showed they were 162.84 mg and 26.10 mg, respectively, and higher than **Rania *et al.*, (2022)**, in the content of flavonoids who showed they were 34.16 mg. The differences between Mulberry fruits depends on many factors, such as degree of maturity at harvest, genetic differences, environmental conditions **(Rodrigues *et al.*, 2019)**, climate, topography, soil conditions (**Huang *et al.*, 2013**), plant nutrition, time of harvest, and growing location **(Skrovankova *et al.*, 2015)** during fruit development.

The hepatoprotective effect of White, Red and Black Mulberry fruits against carbon tetrachloride

(CCl_4) - induced hepatotoxicity in rats was examined. That method has been verified by exploring its effect on some biological parameters such as changes in body weight, liver functions, lipid profile, lipid peroxidation levels and the activities of antioxidant enzymes. In addition to histopathological examination of liver tissues. The existing results documented that intraperitoneal injection (IP) with CCl_4 gives rise to a significant decrease in food consumed, body weight and HDL-c levels and activity of antioxidant enzymes tested (CAT, SOD, GSH and GPx), decrease in serum concentrations of total proteins (TP), albumin (Alb) .In addition, there is a significant increase in serum activities of liver enzymes (AST, ALT and ALP), serum concentrations of total, direct and indirect bilirubin, TL, TC, TG, LDL-c and VLDL-c levels and MDA. The histopathology examination pronounced changes as pseudo-lobulation of hepatic parenchyma due to proliferation of fibrous tissue septa that connect the portal areas, intense mononuclear inflammatory cells infiltration especially at the portal areas and ballooning with existence of numerous intracytoplasmic vacuoles.

The results were in conformity with **Mnaa *et al.*, (2015)**, who said that toxic CCl_4 showed fibrosis, inflammatory cells infiltration and ballooning degeneration in the hepatocytes. **Vanitha *et al.*, (2007)**, mentioned that CCl_4 causes hepatotoxic effects by producing centrilobular necrosis and steatosis. This consequence of strength is due to the free radicals, which are composed from trichloromethyl (CCL_3) and proxy trichloromethyl ($OOCCL_3$) radicals, which causes liver damage according to **Al Amin and Menezes, (2022)**. **Shahidi and Zhong (2010)**, who stated that CCl_4 causes hepatic injury and fibrosis which increased lipid peroxidation, generation of free radicals, and the depletion of antioxidant status. In addition, **Taj *et al.*, (2014)**, exhibit that CCl_4 -treated rats showed marked elevation of serum level of hepatic enzymes (ALT, AST, ALP) and other biochemical parameters as (total, direct and indirect Bilirubin) thus indicating liver injury. Further, **Negm *et al.*, (2020)**, mentioned that CCl_4 caused decrease in the body weight of rats, increase in serum activity of TC, TG, LDL-c, VLDL-c, and decrease in serum activity of the levels of HDL-c. **Beyaz *et al.*, (2022)** and **Boro and Das (2019)**, proved that the CCl_4 - treated rats have a significant increase in serum levels of MDA and depletion in the antioxidant enzymes activities of SOD, CAT, GSH and GPx enzymes, and decrease in serum activity of the levels of TP and Alb.

With regard to the accomplishment of Mulberry fruits on hepatotoxicity rats, the present findings state that feeding hepatotoxicity rats on a diet contains different types of Mulberry fruits (White, Black, Red, and the combination of the three Mulberry fruits) results in a significant increase in FI, FBW, BWG and RBWG %, comparable to the positive control group fed on the basal diet alone. In addition, adding the Mulberry fruits (White, Black, Red, and the combination of the three Mulberry fruits) to the diet successfully recovers liver functions and preserves their tissues from deterioration, as well as recovers the activities of the antioxidant enzymes and decreases the levels of lipid peroxidation. Also, Mulberry fruits ameliorates serum levels of TP, Alb, TBL, DBL, and IDBL, and serum levels of TL, TC, TG, HDL-c, LDL-c and VLDL-c compared with that treated by CCl_4 and fed on the basal diet alone. The highest results were established in treated hepatotoxicity rats with the combination of the three Mulberry fruits group follow it, respectively the Red, Black, and White Mulberry was the lowest. The results of the histological examination exhibited marked improvement in the hepatocytes with Mulberry fruits.

The current study was in agreement with the results of **Mnaa *et al.*, (2015)**, who said that body weight gain and feed efficiency ratio were improved in the White and Black Mulberry feeding rats, Liver enzymes ALT and AST showed significant amelioration for mixture mulberries, black and white berries, respectively, increased serum levels of HDL-c, and decreased significantly serum levels of TC, TG and LDL-c, and the antioxidant activity significantly for black and white Mulberry decreasing serum levels of MDA compared to CCl_4 group. As well, **Deniz *et al.*, (2018)**, illustrated that Black Mulberry extract reductions in the serum activities of ALT, AST and TBL concentration, and significantly increased the activities of GPx and SOD compared to the CCl_4 group. **Rehab and Hany (2015)**, concur with our results who showed that red mulberry have a significant increase in body weight gain, feed intake and FER, decrease serum activities of AST, ALT, ALP, and serum levels of TB, while increase serum levels of TP, and HDL-c, and decrease the serum levels of TC, TG, LDL, V-LDL and increase the serum activities of SOD. These results suggested that red mulberry prevents liver injury by decreased oxidative stress.

Our results were in conformity with **Elmasry and Moawad (2021)**, who reported that, CCl_4 caused decrease in the body weight of rats. Treated group with CCl_4 elevated levels of plasma ALT, AST, and ALP, MDA, and decline the hepatic GSH level and GPx enzyme activity, while the administration of black mulberry extracts along with CCl_4 resulted in significantly protect the liver from injury by reducing the biomarkers of liver (ALT, AST, ALP), and MDA, and increase in hepatic GSH and GPx. **Hanaa *et al.*, (2020)**, consistent with our results who found that the

administration of black mulberry, white mulberry alleviated the serum levels of AST, ALT, ALP and total and direct bilirubin, and reduce the serum TC, TG, LDL-c and the activity of MDA, and increase the levels of albumin, HDL-c, the activity of CAT.

Previous studies have demonstrated that mulberry extract contains large amounts of favonoids, phenolic compounds, and anthocyanins **(Bao *et al.*, 2016 and Munagala *et al.*, 2017).** The results presented by **Chang *et al.*, (2011)** illustrate that ethanolic extract of mulberry exhibited radical scavenging and reducing activity due to its high content of phenolic compounds such as maclurin, isoquercitrin, and resveratrol. Flavonoids can protect the liver from toxins through their anti-inflammatory, antioxidant, anti-cancer, and antifibrogenic pharmacological activities (**Sahu *et al.*, 2023**). Eleven flavonoid compounds (luteoin, naringin, rutin, hespirdin, rosmarinic, quercetrin, quercetin, hespirtin, kaempferol, apegenin and 7-hydroxy flavone) were identified and quantified by **Hanaa *et al.*, (2020).** Anthocyanins have the ability to neutralize free radicals, prevent the process of lipid peroxidation, and regulate the release of pro-inflammatory mediators (**Szymanowska and Baraniak, 2019**). Resveratrol may alleviate liver injury through reduce the oxidative stress (**Meng *et al.*, 2019**). Naringenin reduce the hepatic toxicity by inhibit the oxidative stress (**Das *et al.*, 2016). Osama *et al.*, (2022)** concluded that rutin and quercetin either alone or in combination may have potential preventive effects against doxorubicin (DXR)-induced hepatotoxicity through inhibiting oxidative stress, inflammation, and apoptosis as well as modulating the Nrf2 expression. Carotenoids such as lycopene can improved the hepatotoxicity by acting as an antioxidant (**Bandeira *et al.*, 2017**). Anthocyanins from the extract of bilberry in reducing the hepatotoxic and pro-inflammatory effects of drugs, as well as preventing the development or alleviation of the progression of various chronic liver diseases (e.g., ALD, NASH, and NAFLD) (**Popović *et al.*, 2019**). **Yang and Jo (2018),** mentioned that Mulberry extract (MB) has the potential to ameliorate NAFLD in HFD-fed rats that suppressed the hepatic injury markers including serum ALT and AST, as well as hepatic TG level as a hepatic lipid parameter, regulating dyslipidemia and liver steatosis by inhibits oxidative stress and ROS. **Park *et al.*, (2020)**, reported that, Mulberry water extracts and silk amino acids improved the cell survival in hepatocellular carcinoma HepG2 cells treated with D-galactosamine and attenuated acute liver damage by reducing oxidative stress, reduced liver damage indexes such as serum AST and ALT, TG levels, MDA, and inflammation, and increased the antioxidant activity of SOD and GPx. **Kim *et al.*, (2021)**, revealed that Mulberry anthocyanin decreased the activity of the liver enzyme ALT and AST, as an indicator of liver damage, suggesting that it has hepatoprotective effects. The anti-hepatosteatosis effects of mulberry were attributed to its ability to inhibit fatty acid and triglyceride synthesis and promote fatty acid oxidation **(Song *et al.*, 2016)**. **Noh and Yoon (2022)**, showed that Mulberry ethanol extract (MBEE) improved the hepatic steatosis and lowered the ALT and AST activities, hepatic TG, cholesterol and LDL-C levels.

Conclusion: The present study demonstrated the ameliorative effects of white, red, and black mulberry and their mixture against CCl_4-induced-hepatotoxicity as showed that mulberry fruits enhanced liver functions, lipid profile, and the activities of antioxidant enzymes, these conclusions need to be strengthened in future studies investigating the health benefits of mulberry fruits.

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