Comparative Evaluation of Protective Effects of Single and Combined Administration of Silymarin, *Morus alba* L and *Olea europaea* L Leaves Extracts on CCl₄-Induced Hepatotoxicity: Preliminary Study

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Received: 10 Sept. 2018 / Accepted: 30 Oct. 2018 / Publication date: 10 Nov. 2018

**ABSTRACT**

The individual hepatoprotective effects of herbs such as silymarin (Sily), *Morus alba* L leaves (MLE) and *Olea europaea* L leaves (OLE) extracts have been investigated in hepatotoxic damage rats. However, few studies on the hepatoprotective role of these herbs when administered in combination against liver damage were conducted. The present investigation examined the capacity of MLE and OLE either alone or in combination with silymarin (Sily), as reference drug, to protect rats hepatocytes against toxicity induced by CCl₄. Adult male Sprague Dawely rats were randomly divided into seven groups of ten rats each, including normal control and carbon tetrachloride (CCl₄) groups. The remaining five groups were divided according to the received treatment into: Sily (100 mg/kg, P.O), MLE (600 mg/kg, P.O), OLE (200 mg/kg, P.O), Sily + MLE and Sily + OLE treated groups. The oral administration of different extracts was started two weeks prior CCl₄ administration and continued till the end of the experiment (10 weeks). For induction of liver injury, 2.0 ml/kg of 20% CCl₄ (in olive oil) was administered orally twice a week for 8 weeks. The serum levels of alanine aminotransferase activity (ALT), alkaline phosphatase activity, total cholesterol and triglycerides levels as well as hepatic glutathione (GSH), malondialdehyde (MDA), and nitrite/nitrate concentrations in rats' livers were measured. Biochemical observations were also supplemented with histopathological examination of haematoxylin and eosin staining liver sections.

While single Sily treatment for 10 weeks could restore serum ALT activity to the basal value, single MLE and OLE as well as combined treatments accentuated CCl₄–induced elevation of serum ALT activity. Single treatments for 10 weeks could attenuate CCl₄–induced elevation in serum ALP activity to reach nearly the basal value. However combined treatments failed to induce any alteration on CCl₄–induced elevation in serum ALP activity.

Administration of single and combined OLE treatments for 10 weeks potentiated CCl₄–induced increase in serum triglycerides level. Both Single and combined treatments could restore serum cholesterol level to the basal value at 6 and 10 weeks. Moreover, combined OLE treatment for 10 weeks could reduce serum cholesterol than basal value. While all treatments regimens had no influence on CCl₄–induced increase in hepatic MDA and NO metabolites contents, only single MLE treatment could restore hepatic MDA content to the normal value and reduce hepatic NO metabolites than basal value. Single OLE treatment could restore hepatic NO metabolites to the basal value as well. Single MLE and combined treatments increased in the hepatic GSH content. Histopathological examinations of CCl₄ group revealed severe liver damage and those of single MLE and OLE groups showed significant regression in the hepatic damage and decreased punctate necrosis as thin strands of fibroblasts proliferated around the hepatocytes were detected. Combined treatments showed moderate fibrosis. The data indicated that single MLE and OLE ameliorate CCl₄–induced liver injury compared to combined treatments.

**Keywords:** silymarin - *Morus alba*-leaves extract- Olive leaves extract - CCl₄

**Introduction**

The liver is one of the most important organs in the body, performing a fundamental role in the regulation of diverse processes, among which the metabolism, secretion, storage, and detoxification of endogenous and exogenous substances. Despite enormous advances in modern medicine, there are no
completely effective drugs that stimulate hepatic function, that offer complete protection of the organ. Thus, it is necessary to identify pharmaceutical alternatives liver diseases, with the aim of these alternatives being more effective and less toxic. Historically, plant-derived extracts have been used in traditional medicine for maintaining health, enhancing overall immune status, as well as prevention and treatment of different diseases (Spencer and Jacobs, 1999).

Silymarin (Sily) is the extract of Silybum marianum, or milk thistle, and consists of seven flavonoglycans and a flavonoid (Kim et al., 2003). Silybum marianum Linn. (Family; Asteraceae) has been used to treat liver diseases since 16th century. It is well established that Sily has been utilized medicinally to cure liver diseases including viral hepatitis, cirrhosis and alcoholic liver disorders (Saller et al., 2007, Federico et al., 2017). Experimental studies demonstrated that Sily has antioxidant, free radical scavenging properties and improve the antioxidative defense by preventing glutathione (GSH) depletion and antifibrotic activity (Trappoliere et al., 2009, Karimi et al., 2011). Besides the antioxidant effect, Sily indicates effective anti-inflammatory (Nazemian, 2010), antineoplastic (Ramakrishnan et al., 2009), immunomodulating (Taghiabadi et al., 2012) and membrane stabilizing properties in different animal and human studies (Muthumani and Milton, 2014).

Morus alba L. (Mulberry) leaf belongs to the Moraceae family, distributed mainly in the temperate and subtropical regions in the northern hemisphere. It has been traditionally used in Asian countries as herbal tea as well as herbal medicine. According to the oldest Korean medical book (Dong-ui-bo-gam), it is called “Sang-Yeop” and introduced to be beneficial in alleviating beriberi, edema and pains (Hu, 2003). Moreover, the aqueous extract of mulberry leaves, rich in flavonoids, acts as the scavenger of blood lipid radicals and potent antioxidant (Li et al., 2005; Butt et al., 2008). Recent studies have reported that it shows antiatherosclerosis, antihypertension (Naowaboot et al., 2009; Yang et al., 2012), antiobesity (Oh et al., 2009), antidiabetic (Katsube et al., 2010) and liver protection (Hsu et al., 2012).

Olive tree (Olea europaea L, Oleaceae) is a longevous plant, anciently known in the Mediterranean basin (Melillo, 1994) commonly known as olive, with noticeable pharmacological activities (Chebbi Mahjoub et al., 2011). The therapeutic use of the olive plant has even been mentioned in Holy books. Olive leaf extract (OLE) could be used as potentially safe natural additives for cosmetics, functional food and medicine. Studies had found more antioxidants in olive leaves than in olive oil (Yuan et al, 2015). Olive leaves extract exerts antioxidant (Visioli and Galli, 2002), anti-inflammatory (De la Puerta et al., 2000) and anticancer effects (Abaza et al., 2007). The olive leaf is believed to have antihypertensive, hypocholesterolemic and hypoglycaemic effects (El and Karakaya, 2009; Wainstein et al., 2012).

The individual hepatoprotective effects of herbs such as Sily, MLE and OLE have been investigated in hepatotoxic damage; however, few studies on the hepatoprotective role of these plants extracts when administered in combination were conducted. The aim of the present study was to find out the eventual protective effect of each MLE and OLE either alone or in combined treatment with the reference drug (Sily) against CCl4-induced hepatotoxicity in rats.

Materials and Methods

Plant Materials:

I) One kilogram of Morus alba L. leaves was collected from the farm of Applied Research Center of Medicinal Plants (ARCMP), Kafer El Gabl (Abo-Elhol) region at National Organization for Drug Control and Research (NODCR), Egypt.

II) One Kilogram of Olea europaea L. leaves was collected from El-Saharanwi road, Egypt.

The plant material (leaves) from each species were authenticated, based on its microscopic and macroscopic characteristics, and scientifically defined by Pharmacognosy Department, National Organization for Drug Control and Research (NODCAR). The leaves identifications were according to T Ack Holom (1974) and Boulos (1999). The plant material (leaves) from each species were cleaned and dried in air, ground to a fine powder and kept in tightly closed amber colored glass containers protected from light at low temperature.
Preparation of the Polyphenolic *Morus alba* L. Leaves Extract.

Air-dried (powder) leaves of *Morus alba* (1kg) were extracted twice with 70% (v/v) aqueous ethanol and kept at 4 ºC for 24 h. The obtained extract was concentrated under reduced pressure (at a bath temperature of 60 ºC) and dried in a vacuum evaporator. The residue was dissolved in distilled water and stored in a refrigerator for experimentation.

Preparation of the Polyphenolic *Olea europaea* L (Olive leaves) Extract

The methods of Al-Attar and Abu Zeid (2013) were used for the preparation of olive leaves extracts. The air-dried olive leaves (750 grams) were ground into fine powder. The powder was extracted with 70% aqueous ethanol several times till exhaustion. The collective ethanol extract was filtered, and the filtrate was concentrated to dryness under reduced pressure (at temperature 60 ºC). The extract was prepared every 2 weeks and stored in a refrigerator for experimentation.

Measurement of Extract Total Phenolic Content

Total phenolic content of the extract was assessed by the Folin-Ciocalteu method. Briefly, 20 μl extract 85% plus 1.58 ml deionized water and 100 μl Folin- Ciocalteu reagents were mixed. After 30 s, 30 μl Na2CO3 was added to the mixture. Then, the mixture was incubated at 20 ºC for 2 h. Finally, the absorbance was read in 765 nm.

Experimental Animals

Male Sprague Dawley rats (300~350 g) were obtained from the animal house of NODCAR. The animals were acclimated for 1 week prior to the experiments, and housed in an air-conditioned animal room with a 12/12h light/dark cycle at a temperature of 22 ± 1 ºC. The animals were provided with a laboratory diet and water *ad libitum*. All experimental protocols involving the use of animals were conducted in accordance with National Institutes of Health (NIH) guidelines and approved by the Committee in NODCAR. Assurance of considering the guidelines is the job of the Scientific Board of the General Division of Pharmacology.

Experimental Design:

Adult male rats of Sprague-Dawley strain were randomly divided into seven groups with ten animals in each. The first group was served as control group received only vehicle; olive oil (0.5 ml/kg bw) and fed with a normal diet. The second group, the toxicant (CCl4) group received oral administration of 2 ml CCl4/kg bw (20% CCl4/olive oil) twice a week for 8 weeks. The remaining five groups were divided according to the received treatment into single treatment groups and combined groups. The single groups were, Silygroup (100 mg/kg bw), MLE group (600 mg/kg bw) and OLE group (200 mg/kg). For combined treatments, the groups were Sily + MLE group and Sily + OLE. All treatments were given orally through a feeding tube daily for 10 weeks. These single and combined treated groups were also received 2 ml CCl4/kg bw (20% CCl4/olive oil) orally twice a week for 8 weeks. All animals were anesthetized, 24 h of the last treatment, blood was collected by retro-orbital venous plexus puncture procedure and serum was obtained by blood centrifugation at 1500 × g for 10 min, at 4 ºC. Rats were then sacrificed humanely twenty-four hours (10 weeks post-treatment). The liver was dissected out and used for biochemical estimation and histopathological examination. The serum and liver samples were stored at - 80 ºC.

The biochemical assays on the rat's blood were conducted at 6 and 10 weeks to confirm the hepatoprotective effects of Sily, MLE and OLE singly or in combined treatments.

Collection of Livers for Hepatic Tissue Oxidative Stress Markers

After blood collection, the livers were identified and carefully dissected out en bloc from each rat. The right lobe of the liver was rinsed in ice cold 1.15% KCl solution in order to preserve the oxidative enzyme activities of the liver and to prevent the breakdown of the hepatic antioxidant biomarkers before being stored.
Determination of Serum Hepatic Function Parameters

Serum ALT was determined colorimetrically according to Reitman and Frankel (1957) using spectrophotometer (Heñios TM-α, thermo scientific, North Carolina, USA). Alkaline phosphatase activity was determined according to the colorimetric method of Babson et al., (1966) using commercial kit (Quimica Clinica Aplicada S. A., Spain). The principle is based on that serum alkaline phosphatase hydrolyzes a colorless substrate of phenolphthalein monophosphate giving rise to phosphoric acid and phenolphthalein which, at alkaline pH values, turns into a pink color that can be photometrically determined at 550 nm. According to the manufacturer, triglyceride level was measured by a triglyceride assay kit (Bio-diagnostic, Egypt) based on Fossati and Prencipe (1982) method. Serum cholesterol was determined by quantitative-enzymatic-colorimetric determination of total cholesterol in serum using commercial kit (Stanbio Laboratory, USA). The method is based on the Allain et al., (1974). The produced aquinoneimine chromogen, with the absorption maxima at 500 nm was determine using spectrophotometer (Heñios TM-α, thermo scientific, North Carolina, USA). The intensity of the final color is proportional to total cholesterol concentration.

Determination of Liver Tissue Reduced Glutathione Level (GSH)

The reduced glutathione (GSH) content in the liver tissue was estimated according to the method described by Ellman (1959) with some modification as described by (Nurrochmad et al., 2010). To the homogenate 10% TCA was added and centrifuged. One milliliter of the supernatant was treated with 0.5 ml of Ellman’s reagent (19.8 mg of 5, 5-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium citrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance of the developed color was read immediately at 412 nm on spectrophotometer (Heñios TM-α, thermo scientific, North Carolina, USA). It was expressed as μmol GSH/g tissue.

Estimation of Lipid Peroxidation Assay (TBARS)

Malondialdehyde in liver homogenate was determined by reaction with thiobarbituric acid (TBA). Briefly, 1.0 ml reaction assay was consisted of 0.58 ml phosphate buffer (0.1 M; pH 7.4), 0.2 ml liver supernatant, 0.2 ml ascorbic acid (100 mM), and 0.02 ml ferric chloride (100 mM). After incubation for 1hr at 37°C, the reaction was stopped by addition of 1.0 ml of trichloroacetic acid (10%). Following addition of 1.0 ml 0.67% thiobarbituric acid, all the tubes were placed in boiling water bath for 20 min and then shifted to crushed ice-bath. After cooling, the samples were extracted with n-butanol and centrifuged. The optical density of the butanol layer was determined at 535 and 520 nm using spectrophotometer (Heñios TM-α, thermo scientific, North Carolina, USA). The difference between the two determinations was calculated as TBA value. The concentrations were expressed as nmol TBARS/g wet tissue (Mihara and Uchiyama, 1978).

Determination of Liver Nitrate/Nitrite Content

Total nitrate/nitrite metabolite in liver was performed according to Miranda et al., (2001) as an indication of nitric oxide (NO) production. Before NO estimation, liver homogenate were de-proteinized by adding absolute ethanol in double volume of the sample. Experiment was performed by adding equal volumes of sample, saturated solution of VCl3 (200 mg VCl3 (Sigma-Aldrich) in 25 ml of 1 M HCl), Griess reagents (1:1 mixture of 0.1% N-(1-naphthyl) ethylene diamine in de-ionized H2O and 2% sulfanilamide (Sigma-Aldrich) in 5% HCl and premixed immediately prior application). The absorbance at 540 was measured using a UV-Vis spectrophotometer (Heñios TM-α, thermo scientific, North Carolina, USA). The results were expressed as µmol/gm tissue.

Histopathological Examination

For qualitative analysis of liver histology, the tissue samples were fixed for 48 h in 10% formalin-saline and dehydrated successfully in different mixtures of ethyl alcohol-water, cleaned in xylene, and embedded in paraffin. Paraffin liver sections of 4-5μm thickness were hematoxylin-eosin
stained. These stained sections were examined under a photomicroscope by a single observer in a blinded-manner for histopathological examination.

Statistical Analysis.

Data were analyzed with one-way analysis of variance followed by a post hoc least significant difference (LSD) test in order to measure statistical significance of the differences observed (SPSS). All data are presented as the mean ± standard error of the mean (SEM) and P values of 0.05 or less were considered to be statistically significant.

Results

Table 1: The Preliminary Phytochemical Screening of Morus Alba and Olea europaea Extract:

<table>
<thead>
<tr>
<th></th>
<th>Carbohydrates and/or glycosides</th>
<th>Flavonoids</th>
<th>Polyphenols and/or tannins</th>
<th>Sterols and/or triterpenes</th>
<th>Resins</th>
<th>Alkaloids</th>
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<tr>
<td>Morus Alba</td>
<td>++</td>
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<td>-</td>
<td>traces</td>
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<tr>
<td>Olea europaea</td>
<td>++</td>
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The Preliminary Phytochemical Screening of Morus alba L. Leaves Extract, showed the presence of flavonoids (++), polyphenols (+), sterols and/or triterpenes (++), carbohydrates and/or glycosides (+++) (Table 1).

The Preliminary Phytochemical Screening of Olea europaea Leaves Extract showed the presence of flavonoids (+), sterols and/or triterpenes (+), carbohydrates and/or glycosides (++), phenolic acid and tannins (+++) (Table 1).

Both MLE and OLE extracts were free from volatile oils and anthraquinones. OLE contains resins and MLE contains alkaloids (Harborne, 1998).

Effect of Extracts on Liver Function:

The obtained results revealed that serum ALT activity of CCl₄ group was significantly elevated by 41% (P<0.05) as compared to control group. After 6 weeks of treatments serum ALT activities of Sily and MLE single treated groups showed insignificant alteration while single OLE, MLE and OLE combined treated groups showed pronounced increase in their serum ALT activities by 39%, 60% and 41% (all P<0.001), respectively as compared to CCl₄ group (Fig. 1).

After 10 weeks of treatment, although single Sily treated group showed normal level of serum ALT activity, MLE and OLE either in single or combined treated groups still exhibited significant increase in their serum ALT activities (Fig. 2).

At 6 weeks, CCl₄ had no influence on ALP activity. Meanwhile both Sily and OLE single treatments induced significant decrease in ALP activity by 59 (P<0.01) and 62% (P<0.05) respectively, single MLE and both combined treatments induced significant increment in serum ALP activity by 42.5% (P<0.01), 83% (P<0.001) and 44% (P<0.001), respectively compared to CCl₄ group (Fig. 3).

After 8 weeks of CCl₄ administration, serum ALP activity of CCl₄ group exhibited pronounced increase by 70% (P<0.001) as compared to control group. While combined treatment failed to induce any alteration on CCl₄-induced increase in ALP activity, single treatments could attenuate such increase by 38% (P<0.001), 18% (P<0.01) and 20.5% (P<0.01) for Sily, MLE and OLE, respectively as compared to CCl₄ group (Fig. 4).

Serum lipid concentrations were determined to evaluate the effects of the treatments on lipid profiles. After the induction of liver injury, CCl₄ had no influence on serum triglycerides level. After 6 weeks of treatments, only single MLE and OLE combined treatments induced increments in serum triglycerides levels by 47% (P<0.05) and 145% (P<0.001), respectively as compared to CCl₄ group (Fig 5).

Although CCl₄ induced insignificant increase in triglycerides level, single and combined OLE treatment induced pronounced increase in serum triglycerides level by 115% and 130% (both P<0.001), respectively as compared to CCl₄ group (Fig 6). On the other hand, combined MLE for 10 weeks induced significant increase in serum triglycerides level by 77% (P<0.001) as compared to single Sily group (Fig. 6).
Although CCl₄ and all the utilized extracts regimen induced insignificant alteration in serum cholesterol level, only single MLE treatment for 6 weeks and OLE combined treatment for 10 weeks could induce significant decrease in serum cholesterol level by 26 and 27% (both, P<0.05), respectively as compared to control group (Figs 7&8). In addition, single Sily and combined MLE treatments induced significant decrement in serum cholesterol level by 28% and 32% (both P<0.05) as compared to CCl₄ (Fig.8).

**Fig. 1**: Effect of single and combined silymarin, morous and olive leaves extracts for 6 weeks on ALT activity.

**Fig. 2**: Effect of single and combined silymarin, morous and olive leaves extracts for 10 weeks on ALT activity.

**Fig. 3**: Effect of single and combined silymarin, morous and olive leaves extracts for 6 weeks on serum alkaline phosphate activity.
**Fig. 4:** Effect of single and combined silymarin, morous and olive leaves extracts for 10 weeks on serum alkaline phosphate activity.

**Fig 5:** Effect of single and combined silymarin, morous and olive leaves extracts for 6 weeks on serum triglycerids level.

**Fig. 6:** Effect of single and combined silymarin, morous and olive leaves extracts for 10 weeks on serum triglycerids level.
Fig. 7: Effect of single and combined silymarin, morous and olive leaves extracts for 6 weeks on serum cholesterol level.

Fig. 8: Effect of single and combined silymarin, morous and olive leaves extracts for 10 weeks on serum cholesterol level.

Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD) test
(a) Significantly different from control group at P<0.05.
(b) Significantly different from CCl4 group at P<0.05.
© Significantly different from Sily+CCl4 group at P<0.05.
(d) Significantly different from MLE+CCl4 group at P<0.05.
(e) Significantly different from OLE+CCl4 group at P<0.05.
(F) Significantly different from Sily+MLE+CCl4 group at P<0.05.

Effects of Extracts on Hepatic MDA, GSH and Nitrate/Nitrite Contents

The end product of lipid peroxidation, MDA levels, was significantly higher by 148% (P<0.001) in the CCl4 group than control group (Fig.9). While single MLE pretreatment for 10 weeks could restore the hepatic MDA content to the basal value, each of single OLE and MLE combined treatment induced accentuation of CCl4-induce increase in the hepatic MDA content by 25.5% and 26% (P<0.05) as compared to CCl4 group (Fig.9). Surprisingly single Sily treatment had no influence on CCl4-induced elevation in the hepatic MDA content (Fig.9).

The data represented in figure (10) revealed that CCl4 decreased the hepatic GSH content however, this decrement was statistically insignificant Hepatic GSH content of single Sily and MLE treated rats exhibited pronounced increase by 244% and 192%, respectively (both P<0.001) as well as MLE and OLE combined treatments efficiently increased hepatic GSH content by 217% and 210% (both P<0.001, Fig. 10), respectively as compared to CCl4 group.

Hepatic NO metabolites level of CCl4 group exhibited significant increase by 42%(P<0.05) as compared to control group. Single Sily and both combined treatments failed to induce any alteration on CCl4-induced increase in the hepatic NO metabolite content. Nevertheless, single OLE treatment could restore hepatic NO metabolite content to the basal value (Fig.11). Interestingly, single MLE pretreatment could decrease the hepatic NO metabolites content less than normal value by 37% (P<0.001) as compared to control group (Fig.11).
Fig. 9: Effect of single and combined silymarin, morous and olive leaves extracts for 10 weeks on hepatic MDA content.

Fig. 10: Effect of single and combined silymarin, morous and olive leaves extracts for 10 weeks on hepatic GSH content.

Fig. 11: Effect of single and combined silymarin, morous and olive leaves extracts for 10 weeks on hepatic nitrate/nitrite content.

Data are represented as Mean±SEM (n=10)
Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD) test
(a) Significantly different from control group at P<0.05.
(b) Significantly different from CCl4 group at P<0.05.
© Significantly different from Sily+CCl4 group at P<0.05.
(d) Significantly different from MLE+CCl4 group at P<0.05.
(e) Significantly different from OLE+CCl4 group at P<0.05.
(F) Significantly different from Sily+MLE+CCl4 group at P<0.05.
Liver Pathology

The results of histopathological examination provided further endorsement for subchronic CCl₄ induced hepatotoxicity. As shown in Figure (12), typical photomicrographs of the control group depicted normal histoarchitecture pattern of hepatic lobules consists of centrilobular (central) vein and radiating array of regular hepatocytes of well-preserved cytoplasmic and nuclear appearance (Fig.12). In comparison, CCl₄ toxicity for 8 weeks involved remarkable distortions of liver architecture which included portal fibrosis as represented by presence of multiple fibrotic nodules and extensive fibrosis predominantly in the periportal areas (Fig.13). In addition, considerable degree of necrosis and apoptosis diffused throughout hepatic parenchyma with prominent periportal inflammatory and kupffer cells infiltration, congestion of blood vessels and fatty globules accumulation. Hepatocytes in the vicinity of hepatic lesions, particularly those situated immediately alongside the lesion border, showed features of pronounced steatosis (fig 13).

Liver section of rats treated with Sily, MLE or OLE alone prior CCl₄ exposure showed regression of the hepatic injury (Figs 14&15). However, thin strands of fibroblasts around lobules of hepatocytes and collagen deposits were present but they were reduced in extent compared to CCl₄. Regarding MLE and OLE combined treatment, microscopic examination of these rat’s livers showed moderate fibrosis and steatosis and apoptosis of hepatocytes were still present (Figs 16&17).

Fig. 12: Liver of rat from control negative group showing the normal histological structure of hepatic lobule (H & E X 400).

Fig. 13: Liver section of rat treated with CCl₄ showing portal fibrosis with briding of fibroblasts around lobules of hepatocytes. Notice apoptosis of hepatocytes (H & E X 100).

Fig. 14: Liver of rat treated with MLE+CCl₄ showing portal fibrosis with briding of fibroblasts around lobules of hepatocytes. (H & E X 100).

Fig. 15: Liver section of rat treated with OLE+CCl₄ showing strands of fibroblasts around lobules of hepatocytes. Notice fatty change of hepatocytes (H & E X 100).

Fig. 16: Liver of rat treated with Sily+MLE+CCl₄ showing collagen fibers deposition around the hepatic lobules. Notice fatty change of hepatocytes (H & E X 100).

Fig. 17: Liver section of rat treated with Sily+OLE+CCl₄ showing thin strands of fibroblasts and steatosis of hepatocytes (H & E X 100).
Discussion

Carbon tetrachloride (CCl₄) is a model for studying free radical-induced liver injury and screening hepatoprotective drugs. Numerous studies have reported the involvement of oxidative stress in CCl₄ induced liver damage and the hepatoprotective effects mediated by different antioxidant agents. The mechanism of CCl₄-induced hepatic injury involves the biotransformation of CCl₄ by cytochrome P450 to trichloromethyl radicals (CCl₃:3) which generates highly reactive free radicals such as trichloromethylperoxy (CCl₃OO·). These free radicals react readily with polyunsaturated fatty acids leading to free radical chain reaction as well as membrane lipid peroxidation that disrupts the membrane integrity (Manibusan et al., 2007). Disruption of cell membrane integrity leads to cell injury, cellular necrosis and chronic liver injury with the result of liver fibrosis (Wasser and Tan, 1999).

The increase serum aminotransferases level is known to reflect the severity of liver injury (Lin et al., 1996). Pervious results revealed that during 2 weeks of CCl₄ intoxication, serum aminotransferases exhibited pronounced increases reaching more than 100 folds (Hou et al., 2014). Nevertheless, these increases decline to reach 3 times during two or three months (Muriel et al., 2005; Hou et al., 2014) and continued to be similar or lower to those of the control after 4 months (Muriel et al., 2005).

In the present study, disruption of the hepatic structural integrity by subchronic CCl₄ administration for 8 weeks is reflected by slight increases in the serum activities of ALT and ALP. The results of histopathological examination provided further endorsement for the subchronic CCl₄ intoxication involved remarkable distortions of liver architecture. Livers of CCl₄ animals showed the presence of multiple fibrotic nodules and extensive fibrosis predominantly in the periportal areas, prominent periportal inflammatory and kupffer cells infiltration, congestion of blood vessels and considerable degree of apoptosis diffused throughout hepatic parenchyma. It is well established that continuous exposure to CCl₄ leads to the development of severe hepatic fibrosis (Shyu et al., 2008).

In the present study, CCl₄ induced increase in both MDA and NO metabolite contents. It is well known that, increased NO production attributes to NF-κB-induced iNOS expression following CCl₄ challenge (Chamulitrat et al., 1995). The produced NO reacts with superoxide anion O₂⁻ to produce peroxynitrite (ONOO⁻) (Hon et al., 2002). Peroxynitrite increases lipid peroxidation leading to oxidative stress, hampering mitochondria and release of Cytochrome C from the mitochondria and consequently apoptosis (Crompton, 2000). Elevation in the level of end products of lipid peroxidation (MDA) in livers of rats treated with CCl₄, an observation similar to earlier reports, is attributed to enhanced lipid peroxidation (Drewa et al., 2002), leading to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals (Wang et al., 2004). Disrupted lipid metabolism following CCl₄ administration was also confirmed in the current study by the observed increase in serum triglycerides level (Sturgill and Lambert, 1997) and further supported by histopathological liver examination revealing presence of steatosis. It is well known that steatosis results from the abnormal accumulation of triglycerides within the hepatocyte (Fromenty and Pessayre, 1995).

In order to ascertain whether MLE and OLE either in a single or in combined treatment would reduce and ameliorate CCl₄ induced liver damage, rats were pre-treated with 100 mg/kg Sily (standard reference drug), 600 mg /kg b. wt. MLE and 200 mg/kg OLE for 2 weeks before challenging them with CCl₄ treatment (2ml/kg b. wt. /twice a week/ 8 weeks) and continued for another 10 weeks.

Previous studies showed that Sily has been to exert (1) antioxidant activity Sily is an ROS scavenger and also reduces the loss of endogenous antioxidant enzymes such as glutathione reductase and peroxidase, catalase and superoxide dismutase (Kwon et al., 2013). Silymarin also affects intracellular glutathione, which prevents lipoperoxidation of membranes; (2) anti-inflammatory activity [Sily interferes with the NF-kB-induced inflammatory cascade (Dehmlow et al., 1996)]; and (3) antifibrotic activity (Sily has been shown to reduce liver collagen deposition in CCl₄ induced liver fibrosis (Li et al., 2012), chronic alcoholic liver damage and NASH (Kim et al., 2012). In general, Sily has antioxidant, anti-fibrotic, anti-proliferative, immunomodulatory, and antiviral properties (Vargas-Mendoza et al., 2014). Silymarin protection can be attributed its antioxidant and membrane-stabilizing actions (Mouereille et al., 1989). The effect of Sily to prevent CCl₄-induced lipid peroxidation and hepatotoxicity (Lettéron et al., 1990; Muriel and Mourelle, 1990) is attributed to its ability to normalize the levels of the transaminases that are elevated in hepatotoxicity (Sharma et al.,
peroxidases 1 and 4 (Gpx1 and Gpx4). These gene expression changes suggest that mulberry upregulates the expression of genes could be related combined MLE treated rats may explain their partial protective effects in alleviating liver fibrosis and significantly in the hepatic NO production, single MLE and OLE treatments could restore it to the basal value. While Sily and both combined treatments failed to inactivation of antioxidant enzymes as well as generation of the highly toxic derivative, peroxynitrite, involved in pathogenesis of acute hepatotoxicity mainly through depletion of hepatic GSH and/or triterpenes (++).

This study demonstrated that Sily treatment for 10 weeks could restore serum ALT, ALP, triglycerides and cholesterol to their basal values and increased hepatic GSH, however it failed to induce any alteration on CCl4-induced increase in the hepatic MDA and total NO metabolite contents. In addition, histopathological examination of Sily rats' livers showed thin strands of fibroblasts around lobules of hepatocytes and steatosis of hepatocytes. Inhibition of hepatotoxin binding to receptor sites on the hepatocyte membrane; increase in the level of GSH in the liver, stimulatory effect on ribosomal RNA polymerase and finally protein synthesis leading to increased hepatocyte regeneration, are considered as Sily hepatoprotective mechanisms of action (Dixit et al., 2007).

As previous results revealed that during 2 weeks of CCl4 intoxication, serum ALT and AST exhibited pronounced increases reaching more than100 folds (Hou et al., 2014). Nevertheless, these increases decline to reach 3 times during two or three months (Muriel et al., 2005; Hou et al., 2014) and continued to be similar or lower to those of the control after 4 months (Muriel et al., 2005). Surprisingly, sera of rats pretreated with MLE and OLE either as single or combined treatments exhibited elevation in ALT activity. One can reasonably infer from these results that these extracts pretreatment regimens delayed the onset of hepatic fibrosis and the development of CCl4 hepatotoxicity. It worth noting that deferoxamine delays the development of the hepatotoxicity of acetaminophen (Schnellmann et al., 1999), and using mice expressing Bcl-2 in their hepatocytes for protection against hepatocyte mitochondrial dysfunction delays early stages of fibrogenesis (Mitchell et al., 2009). However, these delay of fibrinogenis onset and transit protection is eventually overwhelmed when ROS are further produced by extramitochondrial compartments and nonhepatocytic cells.

This study demonstrated that single MLE and OLE could restore ALP and cholesterol to the normal values after 10 weeks. Kojima et al. (2010) tested the hypolipidemic effects of MLE in healthy non-diabetic human subjects and found no significant differences in serum total cholesterol, HDL- and LDL-cholesterols and triglyceride levels after 6 and 12 weeks (Kojima et al., 2010), however the hyperlipidemic effects of mulberry leaf tea may not be similar between normal and diabetic conditions (Wilson and Islam, 2015). Regarding OLE, the present study revealed that single and combined OLE treatment increased serum triglycerides level. Consistently, Eidi et al., (2009) observed insignificant increase in serum triglycerides level and decrease in serum total cholesterol level in rats treated with 100 and 250 mg OLE for only 14 days (Eidi et al., 2009).

While Sily (the reference drug), single OLE and combined treatments had no influence on CCl4-induced elevation in hepatic MDA content, single MLE effectively minimized MDA levels, to almost basal levels (normal value) indicating the ability of MLE to alleviate oxidative stress. The antioxidant and free radical scavenging activity of MLE could be due to their flavonoids and phenolic compounds constituents. It has been suggested that the protective effect of plant extracts against CCl4-induced liver damage may be attributed to the presence of constituents including flavonoids and triterpenoids (Tran et al., 2001; Gupta et al., 2004). Flavonoids are known to be antioxidants, free radical scavengers and anti-lipoperoxidants which cause hepatoprotection (Al-Qarawi, et al., 2004; Mankani et al., 2005). The present phytochemical screening showed MLE contain flavonoids (++ and) andsterols and/or triterpenes (++).

Hepatic injury has been shown to be associated with increased level of nitric oxide (NO). NO is involved in pathogenesis of acute hepatotoxicity mainly through depletion of hepatic GSH and inactivation of antioxidant enzymes as well as generation of the highly toxic derivative, peroxynitrite, through reaction with superoxide (Clancy and Abramson, 1995). The current study demonstrated that while Sily and both combined treatments failed to induce any alteration on CCl4-induced increase in the hepatic NO production, single MLE and OLE treatments could restore it to the basal value.

Each of single Sily and MLE treatments as well as the combined treatments increased significantly in the hepatic GSH content. The elevation of hepatic GSH content among single and combined MLE treated rats may explain their partial protective effects in alleviating liver fibrosis and could be related to the flavonoids and polyphenolic contents of MLE. Mulberry administration upregulates the expression of genes involved in the response to oxidative stress like glutathione peroxidases 1 and 4 (Gpx1 and Gpx4). These gene expression changes suggest that mulberry
administration reduces oxidative stress (Kobayashi et al., 2010). Thus, the observed increase in hepatic GSH level in MLE administered rats could be attributed to MLE administration up regulated the expression of genes involved in the response to oxidative stress (Kobayashi et al., 2010).

Histopathological examination of single MLE and OLE treated rat's livers showed regression of hepatic lesion, alleviated and improved signs of hepatocellular injuries compared to CCl₄ group. Reductions in liver tissue damage were obvious by decreased punctate and focal necrosis as well as thin strands of fibroblasts proliferated around the hepatocytes were detected in both MLE and OLE groups. However, we did not observe protective role of combined treatments against CCl₄ induced toxicity in the current animal model. Moderate fibrosis, steatosis and apoptosis of hepatocytes were still present in the combined treated groups.

The present study showed that both MLE and OLE had protective effects against CCl₄ toxicity in hepatic tissues. Moreover, MLE was found to be more effective than OLE in the CCl₄ -induced changes in GSH level and MDA content. Because synergism was not observed in the overall spectrum of biochemical and histological changes in the liver, we thought that Sily and each of MLE and OLE may not have synergistic effects against CCl₄ induced oxidative damage in hepatic tissue. Combined treatments did not have stronger effects than their separate effect against CCl₄ induced liver damage. Livers of these combined pretreated animals showed moderate fibrosis, steatosis and apoptosis of hepatocytes. It seems that it was related to the antioxidant dose, because high doses of some antioxidants do not have a protective effect, and can exacerbate tissue damage (Azarkish et al., 2013).

Acknowledgments

The authors acknowledge Ass. Prof. Gouda Talat Mohamed and Dr. Marwa El-Said Hassan, Applied Research center of Medicinal Plants (ARCMO), at National Organization for Drug Control and Research, for their co-operation and kind help for the identification and extraction of the plants, and prof. Dr. Sahar Darwish Histopathology Department. The assistance of the technical staff in the Department of Developmental Pharmacology is also acknowledged.

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