

Hepatoprotective effect of the unsaponifiable matter from olive, linseed and sesame oils against carbon tetrachloride-induced liver injury in rats

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SUMMARY: In the present study, the hepatoprotective activity of the unsaponifiable matter (UNSAP) of olive oil, linseed, and sesame oils against CCl₄-induced liver toxicity in rats was investigated. In a preliminary antioxidant study, UNSAP showed pronounced DPPH radical scavenging activity (IC₅₀ 6.2-10.8 mg/mL). The constituents of UNSAP were determined by GC-MS. The subcutaneous administration of CCl₄ caused liver injury. The hepatoprotective effect of UNSAP was comparable to that of α -tocopherol, a standard antioxidant agent. The co-administration of the investigated UNSAP normalized the activities of serum marker enzymes, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Furthermore, the serum alkaline phosphatase (ALP) activity and hepatic malondialdehyde (MDA) level were found to be alleviated by pre-treatment with the UNSAP. A histopathological evaluation showed marked improvement in the liver of UNSAP- and α -Tocopherol-treated animals. The hepatoprotective effect could be attributed to the antioxidant characteristics of UNSAP.

KEYWORDS: CCl₄; Hepatotoxicity; Histopathology; Rat; Unsaponifiable matter

RESUMEN: Efecto hepatoprotector del insaponificable de los aceites de oliva, linaza y sésamo contra la lesión hepática inducida por tetracloruro de carbono en ratas. En el presente estudio, se investigó la actividad hepatoprotectora de la materia insaponificable (UNSAP) de los aceites de oliva, linaza y sésamo contra la toxicidad hepática inducida por CCl₄ en ratas. En un estudio preliminar de antioxidantes, UNSAP mostró una pronunciada actividad de captación de radicales DPPH (IC₅₀ 6.2-10.8 mg / mL). Los constituyentes del UNSAP fueron determinados por GC-MS. La administración de CCl₄, por vía subcutánea, causó lesión hepática. El efecto hepatoprotector del UNSAP fue comparable al del α -Tocoferol, un agente antioxidante estándar. La administración conjunta del UNSAP normalizó las actividades de las enzimas marcadoras séricas, alanina aminotransferasa (ALT) y aspartato aminotransferasa (AST). Además, se encontró que la actividad de la fosfatasa alcalina en suero (ALP) y el nivel de malondialdehído hepático (MDA) se mitigan con el tratamiento previo con UNSAP. La evaluación histopatológica mostró una mejoría notable en el hígado de los animales tratados con UNSAP y α -Tocopherol. El efecto hepatoprotector podría atribuirse a las características antioxidantes de UNSAP.

PALABRAS CLAVE: CCl₄; Hepatotoxicidad; Histopatología; Materia insaponificable; Rata

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1. INTRODUCTION

The liver has a wide range of vital functions for the human body. It detoxifies toxic substances that cause oxidative damages. Liver injury induced by carbon tetrachloride (CCl₄) is a commonly used model for screening the hepatoprotective activity of natural compounds. The toxicity of CCl₄ is a result of its biotransformation to the highly reactive tri-chloro-methyl, and tri-chloro-methyl peroxy free radicals. Both radicals cause the oxidative degradation of lipids, loss in integrity of cell membranes, and damage to hepatic tissue (Zhou *et al.*, 2010). Free radicals in living cells caused structural damage, cytotoxic effects, and health problems, but they are eliminated by antioxidant systems. Plant bioactive compounds include hydrophilic antioxidants (such as polyphenols and vitamin C) and lipophilic antioxidants (such as carotenoids and tocopherols) that are used to overcome those harmful effects (Nicco and Batteux, 2018).

Crude linseed oil, extra virgin olive oil, and crude sesame oil are traditionally edible oils. Feeding piglets a 5% flaxseed oil diet for 3 weeks alleviated liver damage induced by lipopolysaccharide and reduced serum ALT, AST and ALP activities (Wang *et al.*, 2018). These effects were ascribed to the high level of α -linolenic acid in this oil (Hendawi *et al.*, 2016). Treating male rats with olive oil at 1 mL/kg body weight (b.w) orally for 4 weeks diminished the toxic effects of CCl₄ (Al-Seeni *et al.*, 2016). The beneficial effect of extra virgin olive oil was attributed to squalene. It quenches free radicals and prevents lipid peroxidation (Reddy and Couvreur, 2009). Olive oil can be separated into a larger fraction (saponifiable) and a minor fraction that is composed of two types of components, the UNSAP fraction and the soluble part (phenolic compounds). Hydroxytyrosol is one of the major phenolic compounds of olive oil that exhibits a radical scavenging activity and protective effect against liver damage caused by a high fat diet (Echeverría *et al.*, 2017).

Sesame oil unsaponifiables (sesamin, sesamol, sesamol, and γ -tocopherol) have antioxidant activity (Fukuda *et al.*, 1986). The supplementation of sesame oil at 0.5 mL/kg b.w/day, p.o. for 14 days attenuated hepatic injury in mice induced by the long-term treatment of Ketoconazole (Periasamy *et al.*, 2016).

The UNSAP fraction represents a “fingerprint” of each oil. It is present in considerable amounts in crude oils. It contains hydrocarbons, sterols, triterpene alcohols, and tocopherols (Fontanel, 2013). These nonglyceride components have antioxidant activity and play an important role in protecting against many diseases (Narasinga, 2001). Feeding male rats (100±5 g) one g of UNSAP from ghee, orally, for 8 days caused a marked decrease in the proliferation of lymphocytes (derived from spleen or blood), and did not affect the percent aggregation or rate of aggregation of platelets (Niranjan *et al.*, 1999). The No Observed Adverse Effect

Level (NOAEL) of α -tocopherol in a 13-week oral (gavage) toxicity study in rats was 125 mg/kg b.w/day (European Food Safety Authority, 2015). A pretreatment of human hepatoma (HepG2) cells with UNSAP from rice bran oil prevented the oxidative damage of tert-butyl hydroperoxide, as determined by cell cytotoxicity (Ham *et al.*, 2016).

There are limited data in the literature on the hepatoprotective effect of UNSAP. The current study was designed to evaluate the antioxidant activity and hepatoprotective effect of UNSAP from linseed oil, extra virgin olive oil, and crude sesame seed oil in CCl₄ intoxicated rats.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Crude oils, kits and chemicals

Linseed oil, extra virgin olive oil, and sesame oil were obtained from Tanta Oil and Soap Company (Egypt), Agriculture Research Center, Giza, Egypt, and private Mill, Cairo, Egypt, respectively.

Kits used for the determination of ALT, AST, and ALP were obtained from the Spinreact Company (Girona, Spain). Kits used for the determination of superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), and MDA were obtained from the Biodiagnostic Company for Diagnostic and Research Reagents (Cairo, Egypt). α -tocopherol was purchased from Hoffman La Roche (Basel, Switzerland). All other chemicals and reagents were of analytical grade.

2.1.2. Experimental animals

Forty-two Sprague-Dawley male rats weighing 170±10 g were obtained from The Holding Company for Biological Products and Vaccines, Egypt. The study was carried out in accordance with ethical procedures approved by the Institutional Animal Care and Use Committee of Cairo University, Giza, Egypt (Approval number CU II F 31 17). All institutional and national guidelines for the care and use of laboratory animals were followed.

2.2. Methods

2.2.1. Quality characteristics of oils

Acid value, peroxide value, and UNSAP of the investigated crude oils were determined according to AOCS (2009).

2.2.2. Extraction of UNSAP

Extraction of UNSAP from the oils was carried out according to AOCS (2009) under conditions which prevent oxidation during the saponification

process by using pyrogallol as antioxidant as follows: The oil sample (100 g) was saponified with pyrogallol ethanolic potassium hydroxide solution (1 N) by heating under reflux for 2 h. After cooling, distilled water was added and mixed well. The unsaponifiable matter was extracted twice with diethyl ether using a separating funnel. The upper layers were collected, combined and washed with distilled water until they became alkali free before drying over sodium sulphate, anhydrous. The solution was evaporated under vacuum using a rotary evaporator at 40 °C. The extraction of UNSAP from each oil was repeated to prepare the required quantity of UNSAP for analysis and the biological experiment.

2.2.3. Gas chromatographic analysis of the UNSAP

The UNSAP were analyzed using the gas chromatograph Thermo Scientific, Trace GC ultra/ISQ, USA, equipped with single quadrupole MS, and TG-5MS-fused silica capillary column (30 m, 0.25 mm, 0.1 mm film thickness). The column temperature was programmed from 260 to 300 °C while the injection temperature was set at 280 °C. The helium flow rate was set at 1 mL/min. The identification of peaks was based on the retention time of standard substances and MS spectra of the NIST Mass Spectral Database. The calculation of percent composition of the identified components was based on peak area.

2.2.4. DPPH radical scavenging activity

The DPPH radical scavenging activity of the investigated UNSAP was determined according to Ramadan *et al.*, (2008). Two hundred microliters of butanol solution of the UNSAP or the standard antioxidant (α -tocopherol) at different concentrations were added to 0.8 mL of a DPPH methanolic solution. The mixture was shaken and left at room temperature for 30 min in the dark. The absorbance of the resulting solution was then measured at 517 nm. The antiradical activity was expressed as IC₅₀ (mg/mL), the dose required to inhibit 50% of the radicals. A lower IC₅₀ value corresponds to a higher antioxidant activity. The ability to inhibit the DPPH radicals was calculated using the following equation:

$$\text{Inhibition \%} = (A_0 - A_s) / (A_0) \times 100 \quad (1)$$

where A₀ is the absorbance of the blank, and A_s is the absorbance of the sample. All samples were analyzed in three replicates.

2.2.5. Experimental design

The rats were housed in individual cages in the animal house of the National Nutrition Institute, Ministry of Health and Population, Egypt. A basal diet was prepared according to AOAC (2005) and water was given

ad-libitum. The animals were kept under normal laboratory conditions for 1 week for adaptation. The rats were randomly divided into seven equal groups, each of six rats. The experimental design of Jain *et al.*, (2008) was followed with some modifications as follows:

G1: Negative control group received olive oil (1 mL/kg b.w, subcutaneous (s.c.)) on days 2, 3, 7, 8.

G2: Positive CCl₄ control group received a 1:1 mixture of CCl₄, and olive oil (2 mL/kg b.w, s.c.) on days 2, 3, 7, and 8.

G3 and G4: α -tocopherol groups were treated with the standard α -tocopherol, (10 and 50 mg/kg b.w, respectively, oral administration (p.o.)) daily for 10 days and also received the same CCl₄-olive oil mixture (1:1, 2 mL/kg b.w, s.c.) on days 2, 3, 7 and 8, after 30 min of administration of α -tocopherol.

The remaining groups (G5, G6 and G7) were administered the investigated UNSAP orally, every day for 10 days at a dose equivalent to the antioxidant activity (DPPH radical scavenging activity, IC₅₀) of 10 mg α -tocopherol.

G5: received 500 mg UNSAP from linseed oil/ Kg b.w

G6: received 600 mg UNSAP from olive oil/ kg b.w

G7 received 900 mg UNSAP from sesame oil/ kg b.w

The rats in these groups (G5, G6 and G7) received the same CCl₄-olive oil mixture (1:1, 2 mL/kg b.w, s.c.) on days 2, 3, 7, and 8, after 30 min of administration of the UNSAP.

On day 11, the rats were weighed, blood samples were collected, and diethyl ether anaesthetized animals were scarified by cervical dislocation.

2.2.6. Blood samples

Animals were fasted for 8 h before blood sampling. Blood was collected from the portal hepatic vein in serum and heparin glass tubes. The serum samples were obtained after centrifugation of the coagulated blood at 950 x g, for 15 min at 4 °C. Serum samples were then stored at -20 °C, until analysis. The heparinized whole blood was centrifuged at 950 x g for 15 min at 4 °C; the plasma layer was collected and stored at -20 °C until analysis. The erythrocyte pellets were washed three times with cold physiological saline (0.9% w/v), and stored at -80 °C until analysis.

2.2.7. Preparation of tissue supernatant

Immediately after scarifying the rats, the liver organ of each rat was dissected out, washed with cold physiological saline (0.9% w/v), blotted dry, and weighed. Liver tissue samples were homogenized with a cold buffer (50 mM potassium phosphate, pH 7.5), and centrifuged at 950 x g for 15 min. The supernatant was collected, and stored at -80 °C for biochemical analysis.

2.2.8. Measurement of serum ALT, AST and ALP

The determination of blood serum AST, ALT, and ALP activities were carried out using commercial kits according to the manufacturers' protocols.

2.2.9. Measurement of antioxidant enzyme activities and MDA

Whole blood was used for the determination of GSH. Blood plasma was used for the determination of MDA level and CAT activity while blood erythrocyte lysate was used for the determination of SOD activity. The clear supernatant of liver homogenate was used for the measurement of GSH, SOD, CAT activities, and MDA level. Biochemical determinations were carried out using kits according to the manufacturers' protocols.

2.2.10. Histopathological evaluation of liver tissue

The liver specimens were taken from the rats and fixed with 10% neutral formalin for 72 h before proceeding with the preparation of 5 μ m-thick paraffin sections. These sections were sequentially stained with Hematoxylin and Eosin (H & E) for histopathological examination using light microscope according to Bancroft *et al.*, (1996).

2.2.11. Statistical analyses

Results were reported as mean \pm S.D. The data were analyzed by one-way ANOVA and Tukey's test. A bivariate analysis of the data was carried out by Pearson's correlation test. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed with Minitab Statistics software version 17 (Minitab Inc., USA).

3. RESULTS AND DISCUSSION

3.1. Quality characteristics of oils and chemical composition of the extracted UNSAP

The freshness of the investigated oils was confirmed and their acid and peroxide values were compatible with the Codex Alimentarius (2015). Linseed oil had the lowest content of UNSAP fraction (0.8%). Olive oil and sesame seed oils were characterized by a higher content of UNSAP fraction, which amounted to 1.7 and 2.5%, respectively (Table 1). These results are in agreement with those reported by Fontanel (2013).

Gas chromatographic analysis data of the extracted UNSAP are shown in Table 1. GC-MS data indicated that UNSAP from linseed oil contained γ -tocopherol (5.43%), sterols (13.91%), and hydrocarbons. α -tocopherol was not detected in the UNSAP of linseed oil as reported by

Gliszczynska-Swiglo *et al.*, (2007). Squalene represented the main component (96.14%) of olive oil UNSAP. Negligible levels (0.8%) of sterols, and α -tocopherol were found in this UNSAP. Olive oil represents the highest source of squalene, compared to other oils (Reddy and Couvreur, 2009). The UNSAP from sesame oil contained sterols (43.86%), sesamin (28.95%), and γ -tocopherol (18.44%). Gamma-tocopherol is the major tocopherol in sesame oil (Codex Alimentarius, 2015), while sesamin is considered the predominant constituent of lignans in crude sesame oil (Moazzami *et al.*, 2007). In addition, phytol is present in considerable quantities in UNSAP from linseed oil and sesame oil as reported by Krauß and Vetter (2018).

3.2. In vitro radical scavenging activity by DPPH assay

The scavenging activity of the investigated UNSAP against DPPH radicals is shown in Table 2. α -tocopherol was used as a positive control.

In the present study, the antioxidant activity of the UNSAP from linseed oil ($IC_{50} = 6.2$ mg/mL) was close to that of the UNSAP from olive oil (7.3 mg/mL). The radical scavenging activity of the UNSAP from sesame oil ($IC_{50} = 10.8$ mg/mL) represented less than 50% of the activity of the other investigated UNSAP. Farhoosh *et al.*, (2013) reported that the UNSAP fraction of the sesame oil had no scavenging activity against the DPPH free radicals at the concentrations studied. The IC_{50} value of α -tocopherol was found to be 121.32 μ g/mL, which is in agreement with that reported by Yildiztekin *et al.*, (2016). This means that the antioxidant activity of α -tocopherol was 50, 60, and 90 times that of the UNSAP of linseed oil, olive oil and sesame seed oil, respectively. This ratio helps in selecting the hepatoprotective dose levels of the investigated UNSAP. The hepatoprotective activity of a dose of 10 mg α -tocopherol/Kg b.w/day was evaluated in comparison with the doses of 500 mg UNSAP of linseed oil/Kg b.w/day, 600 mg UNSAP of olive oil/Kg b.w/day, and 900 mg UNSAP of sesame oil/Kg b.w/day. One should keep in mind that each investigated UNSAP is a mixture of several compounds and hence may affect, in part, its efficiency as antioxidant compared to pure α -tocopherol.

3.3. Effect of α -tocopherol and UNSAP on liver functions of CCl₄-intoxicated rats

The activities of the AST, ALT, and ALP enzymes in the serum of the rats are presented in Table 3.

The administration of CCl₄ induced hepatic injury as evidenced by a significant ($p < 0.05$) elevation of serum AST, ALT, and ALP activities (~ three-fold) compared to the normal control group is shown in Table 3. The pre-treatment of CCl₄ intoxicated rats

TABLE 1. Quality characteristics of oils and GC-MS identified components of unsaponifiable matter

Parameter	Linseed oil	Olive oil	Sesame oil
Acid value (mg KOH/g Oil)	0.26±0.01	0.57±0.01	0.90±0.02 ^a
Peroxide value (mEq O ₂ /kg oil)	3.25±0.04	6.50±0.05	2.80±0.15
Unsaponifiable matter %	0.80±0.03	1.70±0.05	2.50±0.01
Unsaponifiable components (Relative Area %)			
RT ^b	Name	MW ^c	
30.90	Phytol	296	2.66 ND ^d 4.02
41.67	Tricosane	324	ND ND 0.66
43.73	Tetracosane	338	ND ND 0.77
50.12	Tetratriacontane	478	36.63 ND ND
51.38	Squalene	410	ND 96.14 0.88
51.86	Cycloeucaleanol	426	ND 0.58 ND
52.12	Pentatriacontane	492	1.43 ND ND
53.10	Triacotane	422	2.10 ND ND
54.61	Tritriacontane	464	24.92 ND ND
55.56	γ-Tocopherol	416	ND 5.43 18.44
56.80	α-Tocopherol	430	ND 0.80 ND
57.56	Rutin	610	ND ND 1.05
57.63	Tetracontane	562	10.30 ND ND
57.76	Sesamin	354	ND ND 28.95
58.46	Campesterol	400	5.84 ND 12.99
58.83	Stigmasterol	412	1.87 ND 3.85
59.82	β-Sitosterol	414	6.20 0.79 27.02
60.00	Cycloartenol	426	ND ND 1.38
60.03	Dotriacontane	450	2.62 ND ND
62.04	24-methylenecycloartanol	440	ND 0.77 ND
63.36	6-[3-[3-[3-(5,11 dioxoindeno [1,2-c]isoquinolin-6-yl) propylamino] propyl] indeno [1,2-c] isoquinoline-5,11-dione	648	ND 0.91 ND

^aMean ± Standard Deviation (n=3).^bRT: Retention time in minutes.^cMW: Molecular weight.^dND: Not detected.

with the investigated UNSAP kept their AST and ALT activities not significantly ($p < 0.05$) different from those of the control group. The pre-treatment with the UNSAP from linseed oil and olive oil significantly ($p < 0.05$) reduced the activity of ALP compared with the CCl₄ group. Likewise, the UNSAP from sesame seed oil suppressed significantly ($p < 0.05$) the ALP activity of CCl₄ intoxicated rats to the normal level. These results indicate that the investigated UNSAP have noticeable antioxidant efficacy against CCl₄ radicals.

The UNSAP doses were sufficient to overcome the destructive effect of CCl₄ on liver enzymes. The antioxidant activity of each UNSAP could be attributed to the synergistic effect of its constituents.

The administration of α-tocopherol (a positive control) at both investigated doses in CCl₄-treated rats significantly ($p < 0.05$) lowered the activity of AST compared with the CCl₄-treated group and

TABLE 2. DPPH radical scavenging activity of the investigated unsaponifiable matter

Unsaponifiable matter	IC ₅₀ ^a (mg/mL)
Linseed oil	6.20±0.08 ^b
Olive oil	7.30± 0.16
Sesame oil	10.80± 0.35

^aIC₅₀ value denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals. IC₅₀ value of α-tocopherol was found to be 121.32 ± 0.48 (μg/mL).

^bMean ± Standard Deviation (n=3).

reduced ALT activity to the normal level. However, the rats pre-treated with α-tocopherol at 50 mg/Kg b.w significantly decreased the activity of ALP, compared to the CCl₄ group. Although the nutritional value of α-tocopherol is higher than the other forms of tocopherols, γ-tocopherol is biologically active

TABLE 3. Liver function and oxidative stress biomarkers in the blood of CCl₄ - intoxicated rats treated with unsaponifiable matter and α -tocopherol

Parameter Group	AST (U/L)	ALT (U/L)	ALP (U/L)	GSH (mg/dL)	SOD (U/mL)	CAT (U/L)	MDA (nmol/mL)
Control	82.33 ± 3.06 ^c	65.00 ± 4.36 ^b	119.00 ± 11.79 ^d	26.85 ± 0.98 ^a	365.38 ± 4.81 ^a	435.30 ± 29.50 ^b	9.23 ± 1.44 ^{bc}
CCl ₄	239.30 ± 35.20 ^a	160.67 ± 17.01 ^a	283.67 ± 3.21 ^a	17.52 ± 1.70 ^c	328.80 ± 27.10 ^b	286.30 ± 19.50 ^c	19.56 ± 0.75 ^a
α -Tocopherol (10 mg/Kg b.w) + CCl ₄	157.67 ± 15.28 ^b	69.30 ± 18.60 ^b	237.33 ± 25.80 ^{ab}	19.04 ± 0.86 ^{bc}	355.30 ± 4.24 ^{ab}	275.00 ± 47.30 ^c	7.77 ± 2.74 ^c
α -Tocopherol (50 mg/Kg b.w) + CCl ₄	158.33 ± 2.52 ^b	55.67 ± 10.97 ^b	230.70 ± 31.90 ^{bc}	22.88 ± 0.67 ^{ab}	358.11 ± 4.77 ^{ab}	434.70 ± 34.20 ^b	7.93 ± 1.42 ^c
Linseed oil UNSAP (500 mg/Kg b.w) + CCl ₄	125.67 ± 15.53 ^{bc}	52.33 ± 14.98 ^b	215.67 ± 4.93 ^{bc}	19.38 ± 1.97 ^{bc}	328.13 ± 5.39 ^b	513.00 ± 39.10 ^a	13.42 ± 0.94 ^b
Olive oil UNSAP (600 mg/Kg b.w) + CCl ₄	121.00 ± 4.36 ^{bc}	61.00 ± 7.21 ^b	187.67 ± 10.02 ^c	24.27 ± 2.17 ^{ab}	350.33 ± 2.47 ^{ab}	308.36 ± 15.57 ^c	13.90 ± 3.25 ^b
Sesame oil UNSAP (900 mg/Kg b.w) + CCl ₄	127.3 ± 19.30 ^{bc}	69.67 ± 6.66 ^b	123.00 ± 9.64 ^d	23.46 ± 3.38 ^{ab}	364.31 ± 7.54 ^a	320.90 ± 23.40 ^b	13.08 ± 1.62 ^{bc}

Data are expressed as Mean \pm Standard Deviation (n=6). Significant differences among the means were determined by analysis of variance and Tukey's test. Means with the same letter in the same column are not significantly different at 0.05 level of significance.

and protects against chronic diseases (Gliszczynska-Swiglo *et al.*, 2007).

3.4. Effect of α -tocopherol and UNSAP on the oxidative status of the blood of CCl₄ intoxicated rats

The antioxidant effects of α -tocopherol and UNSAP on the CCl₄-intoxicated rats are shown in Table 3. Treating rats with CCl₄ caused significant ($p < 0.05$) decreases in the GSH levels, SOD and CAT activities, and significant ($p < 0.05$) increase in serum MDA compared to those of the normal control group (Table 3). This indicates the impairment of the anti-oxidative system due to the CCl₄ treatment. GSH, SOD and CAT represent the defense system against oxidative stress (Beji *et al.*, 2018). Pre-treatment with α -tocopherol at a low dose (10 mg/Kg b.w) as well as the UNSAP of linseed oil did not prevent the GSH depletion induced by CCl₄. However, pre-treatment with α -tocopherol at a high dose or the UNSAP of olive oil or the UNSAP of sesame oil significantly ($p < 0.05$) normalized GSH levels, indicating a curative effect. Blood GSH levels fluctuated during different stages of liver injury since this level of GSH is a balance between secretion from the liver and elimination through the kidneys (Lai *et al.*, 2018). Pre-treatment with sesame oil UNSAP prevented CCl₄ toxicity and increased SOD activity to the normal value. Moreover, the efficiency of the investigated UNSAP on GSH level and SOD activity was comparable to that of the reference α -tocopherol at a high dose. Pre-treatment with α -tocopherol at a low dose or UNSAP of olive oil did not significantly ($p < 0.05$) restore CAT activity to the normal value. The administration of α -tocopherol at 50 mg/Kg b.w or UNSAP of linseed

oil or UNSAP of sesame oil significantly ($p < 0.05$) increased CAT activity to the normal level.

MDA is one of the main lipid peroxidation products. Pre-treatments of CCl₄ intoxicated rats with the investigated UNSAP or α -tocopherol at both investigated doses significantly reduced ($p < 0.05$) MDA to the normal level.

3.5. Effect of UNSAP on oxidative status of liver tissue of CCl₄-intoxicated rats

The effects of the investigated treatments on the activities of SOD and CAT in the liver and the levels of GSH and MDA are shown in Table 4.

The activities of liver SOD, and CAT in the CCl₄-treated group were significantly ($p < 0.05$) decreased compared to the normal group. The results revealed a significant ($p < 0.05$) increase in the MDA level (five times) in the CCl₄-treated group compared to the control. The free radical of trichloro-methyl increases the MDA level that damages the antioxidant defense of tissues (Johnston and Kroening, 1998). GSH is essential for keeping the reduced status of tissues. Tissue injury causes severe depletion in its levels. In this study, a significant decrease in the GSH level in the liver tissue by approximately 40% was recorded after the administration of CCl₄ in comparison with the rats of the control group (Table 4).

The pre-treatment with α -tocopherol at 50 mg/Kg b.w significantly increased ($p < 0.05$) the GSH level compared to that of the CCl₄-treated group. At the same time, treating CCl₄-intoxicated rats with the UNSAP of linseed oil, or the UNSAP of sesame oil significantly ($p < 0.05$) increased the GSH level of liver homogenate to the normal level.

On the other hand, the administration of α -tocopherol at 50 mg/Kg b.w or UNSAP of olive

TABLE 4. Oxidative stress biomarkers in the liver tissue of CCl₄ intoxicated rats treated with unsaponifiable matter and α -tocopherol

Group	GSH (mg/g tissue)	SOD (U/mg tissue)	CAT (U/g tissue)	MDA (nmol/g tissue)
Control	115.54 ±2.28 ^a	24.42 ±0.90 ^a	155.55 ±6.73 ^{ab}	23.82 ±0.85 ^d
CCl ₄	71.97 ±7.83 ^c	8.32 ±1.01 ^d	129.37 ±2.45 ^c	115.21 ±10.56 ^a
α -Tocopherol (10 mg/Kg b.w) + CCl ₄	80.30 ±1.23 ^{bc}	19.14 ± 3.12 ^{bc}	143.66 ±11.49 ^{bc}	44.18 ±9.17 ^{bc}
α -Tocopherol (50 mg/Kg b.w) + CCl ₄	93.13 ±5.12 ^b	22.33 ± 1.20 ^{ab}	171.44 ±5.10 ^a	27.53 ±0.92 ^{cd}
Linseed oil UNSAP (500 mg/Kg b.w) + CCl ₄	118.60 ±9.08 ^a	18.78 ±0.59 ^{bc}	132.13 ±4.56 ^c	28.35 ±3.43 ^{cd}
Olive oil UNSAP (600 mg/Kg b.w) + CCl ₄	76.21 ±10.26 ^{bc}	20.10 ±2.78 ^{abc}	156.85 ±3.43 ^{ab}	47.09 ±3.95 ^b
Sesame oil UNSAP (900 mg/Kg b.w) + CCl ₄	115.17 ±8.94 ^a	15.58 ±0.78 ^c	144.20 ±9.32 ^{bc}	51.96 ±7.62 ^b

Data are expressed as Mean \pm Standard Deviation (n=6). Significant differences among the means were determined by analysis of variance and Tukey's test. Means with the same letter in the same column are not significantly different at 0.05 level of significance.

TABLE 5. Pearson's correlation coefficient (r) between liver function enzymes and the oxidative stress biomarkers in the different groups studied

Parameter	AST	ALT	ALP	Blood MDA	Liver MDA	Blood GSH	Liver GSH	Blood SOD	Liver SOD	Blood CAT
ALT	0.746**									
ALP	0.794**	0.529*								
Blood MDA	0.499*	0.629**	0.241							
Liver MDA	0.779**	0.933**	0.530*	0.721**						
Blood GSH	-0.705**	-0.515*	-0.748**	-0.302	-0.538*					
Liver GSH	-0.662**	-0.491*	-0.633**	-0.287	-0.556**	0.386				
Blood SOD	-0.579**	-0.381	-0.602**	-0.529*	-0.382	0.672**	0.279			
Liver SOD	-0.774**	-0.811**	-0.493*	-0.709**	-0.888**	0.602**	0.355	0.499*		
Blood CAT	-0.433*	-0.469*	-0.207	-0.205	-0.615**	0.215	0.643**	-0.042	0.498*	
Liver CAT	-0.412	-0.529*	-0.313	-0.585**	-0.549**	0.619**	0.042	0.605**	0.706**	0.158

*Significant at $p < 0.05$.

**Significant at $p < 0.01$.

oil in CCl₄-treated rats significantly ($p < 0.05$) increased the activities of SOD and CAT in the liver tissue to the normal value. Hydroxytyrosol, the major phenolic compound in extra virgin olive oil, has the ability to activate GSH biosynthesis and attenuate the increase of TBARs (Illesca *et al.*, 2019). Treatments with α -tocopherol showed a dose-dependent increase in the GSH levels and activities of SOD and CAT compared to the control group.

In the present study, the MDA level of liver tissue was significantly reduced after the administration of α -tocopherol or the investigated UNSAP. The administration of α -tocopherol at a high dose as well as the UNSAP of linseed oil in CCl₄-treated rats significantly ($p < 0.05$) decreased the MDA level of liver tissue to the normal value.

The data showed that all UNSAP studied contained several compounds at varied concentrations. The antioxidant effect of these UNSAP is, actually, the accumulative effect of its constituents and could not be directly attributed to one single compound in the mixture.

The correlation between the investigated parameters using Pearson's Correlation is shown in Table 5.

The results revealed significant ($p < 0.01$) positive correlations among the activities of liver function enzymes. The results indicated an inverse ($p < 0.01$) correlation between the activities of liver function enzymes and SOD and GSH levels. In addition, the CAT activity in the liver had a significant ($p < 0.01$) positive correlation with the activity of SOD in the liver and blood. A significant ($p < 0.01$) positive correlation between the MDA levels in the liver

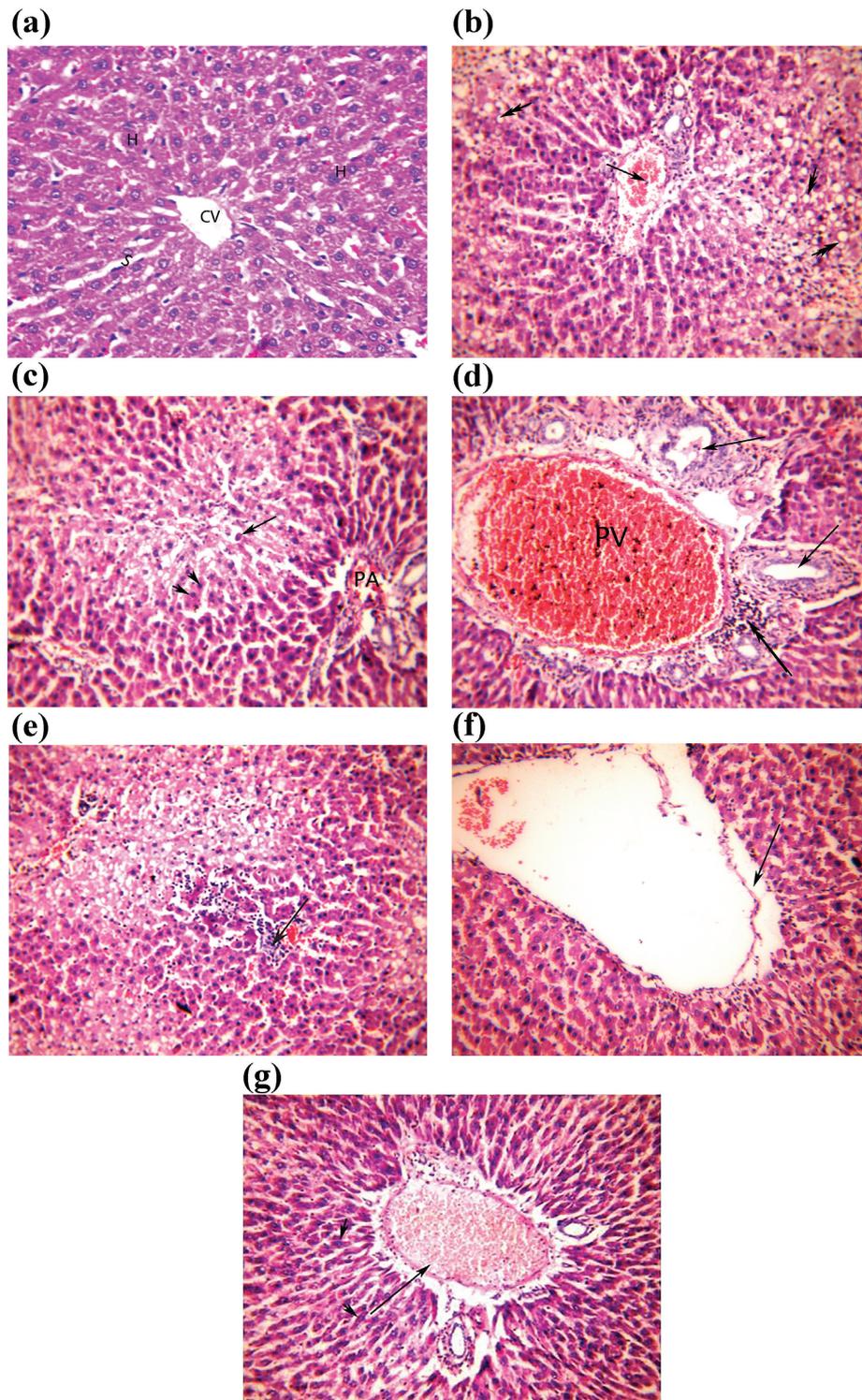


FIGURE 1. Photomicrographs of liver sections of CCl₄ treated rats administered with Unsaponifiable matter and α-tocopherol (a-g). (a) Control group showing normal hepatocytes (H), normal sized sinusoids (S) and central vein (CV). (b) CCl₄ intoxicated rats, showing steatotic hepatocytes (arrowhead), and congested portal vein (arrow). (c) CCl₄ intoxicated rats treated with α-tocopherol (10 mg/kg b.w) showing hyaline body (arrow), apoptotic hepatocytes (arrowhead) and restricted portal area (PA). (d) CCl₄ intoxicated rats treated with α-tocopherol (50 mg/kg b.w) showing proliferated bile duct (arrow), periportal inflammation (double arrow) and dilated congested portal vein (PV). (e) CCl₄ intoxicated rats treated with the unsaponifiable matter of linseed oil, showing inflammatory cell aggregates (arrow). (f) CCl₄ intoxicated rats treated with the unsaponifiable matter of olive oil, showing improvement of most hepatocytes, and lacerated wall of central vein (arrow). (g) CCl₄ intoxicated rats treated with the unsaponifiable matter of sesame oil, showing dilated, congested blood vessel (arrow), where most hepatocytes display normal (arrowhead). (Haematoxylin & Eosin×200)

and blood was observed. On the other hand, the activity of hepatic SOD significantly ($p < 0.01$) and negatively correlated with the MDA levels in both blood and liver. However, no statistical significant correlation was found between the GSH levels in the liver and blood of the investigated rats. The same trend was observed in the CAT levels in the liver and blood.

3.6. Histopathological examination

A microscopical examination of the hepatic tissues of the control group revealed normal liver structure with a normal lobular pattern. The hepatocytes showed well-defined central nuclei and abundant cytoplasm. Magnification at 200x enabled the observation of the normality of the central vein and portal venous channels with normal-sized sinusoids. Bile ducts were lined with normal cuboidal epithelium. (Figure 1a).

A histopathological examination of the hepatic tissues of the CCl₄ group showed a weaker architecture, with edema. Most of the hepatic vasculature revealed congestion, dilatation, and inflammation. Hepatocytes showed steatosis and severe cytoplasmic vacuolation (Figure 1b). These results are in accordance with those obtained by Beji *et al.*, (2018).

The treatment of intoxicated rats with α -tocopherol at 10 mg/Kg b.w revealed a mild degree of improvement, where disturbed architecture with expanded portal areas was observed. A hyaline body with a fragmented nucleus and apoptotic hepatocytes were also seen (Figure 1c). Increasing the dose level of α -tocopherol to 50 mg/Kg b.w caused a moderate degree of improvement, where wide areas of hepatocytes displayed normal features. However, necrotic areas with hyaline body were observed (Figure 1-d). This finding was consistent with that found by Liu *et al.*, (2011).

A histological picture of the hepatocytes of UNSAP of the linseed oil group indicated mild to moderate curative effects. The examination revealed areas of intact hepatocytes alternated with areas of hepatocytes with vacuolated cytoplasm or fatty degeneration (Figure 1e). Insignificant pathological changes were detected in animals treated with the UNSAP of olive oil, where most of the hepatocytes displayed normal histological patterns, indicating a marked improvement. However, 30% of the animals displayed congested central veins with lacerated walls together with mild inflammation (Figure 1f). A pre-treatment with the UNSAP of sesame oil ameliorated the CCl₄-induced liver injury, and showed a mild to pronounced degree of improvement. The liver sections of 50% of animals within this group showed a dilated congested blood vessel accompanied by an inflammatory cell infiltrate (Figure 1-g). The protective effect of UNSAP against CCl₄-induced liver injury was superior compared to that

of α -tocopherol. The histopathological examination of the liver of CCl₄-intoxicated rats treated with UNSAP attenuated hepatocytes degeneration and restored its normal functions. The mechanism of hepatoprotection by UNSAP is due to its antioxidant activity.

4. CONCLUSIONS

The investigated UNSAP were found to be effectively hepatoprotective, as evidenced by biochemical, and histopathological studies. The efficiency of those UNSAP could be due to an interaction between its components which far exceeds its antioxidant activity.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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