Antioxidant and hepatoprotective potentials of olive (Olea europaea L. var. Sigoise) leaves against carbon tetrachloride-induced hepatic damage in rats, and investigation of its constituents by high-performance liquid chromatography-mass spectrometry

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Abstract
The present work explored the preventive potential of ethanolic extract of Olea europaea L. (EEOE) leaves against CCl4-induced liver injury in rats. The fingerprint chromatogram of EEOE was determined by HPLC-MS analysis. The antioxidative potential of EEOE was determined by adopting three approved in vitro methods. The EEOE was orally given at a dose of 400 mg/kg, once a day, for 15 days continuously, succeeded by intraperitoneal (i.p.) injection of CCl4 (0.2%). The hepatoprotective potential was evaluated by estimating biochemical parameters including alanine aminotransferase, aspartate aminotransferase, alkaline phospha
tase, total bilirubin, total cholesterol, and triglycerides in the bloodstream. In vivo, the antioxidant ability against CCl4-induced liver injury in rats was assessed by estimating the levels of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), total reduced glutathione, and lipid peroxidation in the liver tissues. Further, histological analyses were performed to evaluate the degree of hepatic damage. Nine compounds were identified in the EEOE, principally oleuropein, luteolin-7-O-glucoside, and apigenin. The EEOE exhibited strong radical scavenging activity in DPPH assay. The EEOE significantly decreased the augmentation of serum cholesterol, TG, ALT, AST, ALP, and total bilirubin contents. It also restored hepatic SOD, CAT, GSH-Px activities, and glutathione (GSH) amount, and lowered lipid peroxidation amount comparable to the standard silymarin. The biochemical data were complemented with histological conclusions. These findings are indicative of the protective potential of the EEOE against CCl4-induced hepatotoxicity, which is possibly related to the potent antioxidative capacity of its phenolic compounds.

Introduction

The liver is consistently the main destination for the assimilation of medicines and toxic compounds (Ben Hsouna et al., 2018). One of the most determinedly used systems for xenobiotic-induced oxidative hepatotoxicity is CCl4-induced liver injury. Cytochrome P450-dependent monooxygenases metabolise CCl4 into cytotoxic ‘CCl3 and ‘OOCCl3 in hepatic parenchyma cells (Lu et al., 2016). Involving unpaired electrons, these radicals react with protein thiols, and provoke peroxidation of membrane lipids, leading to the impairment of mitochondria and nuclei, thus contributing to defective hepatocytes activities, and finally, to cell necrosis (Ferreira et al., 2007). Although there is immense progress in modern medicines for the treatment of hepatic disorders, they have, sometimes, undesirable effects, particularly in long-term administration. Therefore, establishing new and functional foods has essential implications to treat liver illness (Lu et al., 2016).

Olive (Olea europaea L.) leaves are rich in phenolic compounds, especially in tyrosol, hydroxytyrosol, vanillin, rutin, apigenin glucoside, luteolin glucoside, apigenin rutinoside, verbascoside,
caffeic acid, luteolin, diosmetin, and oleuropein (Meirinhos et al., 2005; Silva et al., 2006; Talhaoui et al., 2014). Various other works have also indicated that olive leaves have noticeable pharmacological actions. Single or repeated administration of olive leaves ethanolic extract to rats did not provoke mortality or any manifestations of toxicity. Biochemical and histopathological evaluations also did not display any abnormalities, thus implying that olive leaves ethanolic extract does not induce toxicity (Ustuner et al., 2018). Ustuner et al. (2018) also revealed that olive leaf extract decreased hepatotoxicity in rats. Likewise, Mahmoudi et al. (2018) demonstrated that olive leaf extracts displayed hepatoprotective potential against bisphenol A-induced hepatic injury. It has previously been observed that pre-treatment with olive fruit pulp extract ameliorated CCl4-induced alterations in hepatocyte morphology, and decreased the lipid amounts in CCl4-intoxicated animals. Corroborating its hepatoprotective effect, the extract exhibited significant \textit{in vitro} antioxidant potentials (Kang and Koppula, 2014).

Since the capacity of ethanolic extract of olive leaves var. Sigoise from Algeria to relieve CCl4-induced oxidative impairments has not been studied so far, the present work examined its preventing power against oxidative stress-induced hepatic injury in CCl4-intoxicated rats by investigating some biochemical and physiological markers, and exploring the histological changes. Further, the phenolic profile of olive leaves ethanol extract was also analysed by HPLC-PDA-ESI-MS.

**Materials and methods**

**Extraction**

Olive leaves var. Sigoise were harvested in November 2018 from Chlef (36° 10’ 0.001” N, 1° 19’ 59.999” E), and taxonomically authenticated (voucher no. FSNVDB 18.197) by Mr. O. Naji, a botanist at Hassiba Ben Bouali University of Chlef, Algeria. Olive leaves' ethanolic extract was prepared as previously reported by Deng et al. (2012) with slight modifications. The dehydrated milled leaves were macerated in ethanol for 24 h at a 1:3 sample-to-solvent ratio. The extract was defatted with hexane, filtered using Whatman filter paper (No. 1), then evaporated under vacuum at 45°C in a rotary evaporator. The resulting solution was lyophilised before the EEOE (ethanolic extract of \textit{O. europaea}) was obtained. The yield of dry extract was 13.64% (w/w dry leaves).

**Phytochemical investigations**

**Total phenolic content**

The total phenolic content (TPC) was determined using the colorimetric Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 100 μL solution of 1 mg/mL extract in ethanol was mixed with 750 μL of Folin-Ciocalteu reagent (10%). The mixture was incubated for 5 min. Next, 750 μL of 7.5% Na2CO3 was added. After 30 min, the absorbance was read at 725 nm. Gallic acid served as standard to construct the calibration curve. The TPC was expressed as micrograms of gallic acid equivalents per milligrams of extract (μg GAE/mg extract).

**Total flavonoids content**

The total flavonoids content was determined as previously described (Zhishen et al., 1999). Quercetin served as standard to construct the calibration curve. To 100 μL of extract (1 mg/mL), 100 μL of 2% AlCl3 was added. The mixture was incubated at 25°C for 30 min. The absorbance was measured at 510 nm. The TFC was expressed as micrograms of quercetin equivalents per milligrams of extract (μg QE/mg extract).

**Tannin content**

A solution of 1 mg/mL extract was assayed for tannin content as previously described (Julkunen-Tiitto, 1985). Briefly, 50 μL of the extract solution was added with 1.5 mL of 4% vanillin and 750 μL of HCl. After incubation for 20 min, the absorbance was read at 500 nm. Catechin served as standard to construct the calibration curve. The tannin content was expressed as micrograms of catechin equivalents per milligrams of extract (μg CE/mg extract).

**HPLC-PDA-ESI-MS analysis of the EEOE**

An extract solution of 2 mg/mL was passed through a 0.45 μm nylon filter. HPLC investigations were achieved on a Thermo Finnigan Surveyor Plus HPLC device supplied with an autosampler and a photodiode array detector using a Gemini C18 110 Å (150 × 2 mm, 5 μm) column. The system was connected to an LCQ Advantage max ion trap mass spectrometer with an electrospray ionisation source. HPLC settings were as follows (Silva et al., 2006): solvent A, water: phosphoric acid (99.9: 0.1);
solvent B, water: acetonitrile: phosphoric acid (59.9:40:0.1); gradient, from 0% B to 20% after 15 min, 70% after 70 min, and 100% after 85 min; injection volume, 5 µL; flow rate, 0.7 mL/min; column temperature, 40°C. The MS settings were: ionisation mode, negative; capillary temperature, 350°C; drying gas, nitrogen; capillary voltage, 4500 V; fragmentor voltage, 135 V; nebuliser pressure, 35 Psi; full scan acquisition, from 100 to 1600 m/z; flow rate, 10 L/min.

In vitro antioxidative action

β-carotene bleaching assay (BCB)

The EEOE effect on the oxidation of β-carotene/linoleate was analysed as previously reported (Chaouche et al., 2014). The outcomes were given as IC\textsubscript{50} values (µg/mL). Butylated hydroxytoluene served as standard.

Ferric reducing antioxidant power (FRAP)

The EEOE was assayed for reducing power as previously reported (Moein et al., 2008). The effective concentration, EC\textsubscript{50} (µg/mL), is the amount of EEOE giving an absorbance of 0.5. Ascorbic acid served as standard.

Free radical scavenging ability (DPPH assay)

The procedure of Blois (1958) was used to evaluate the free radical scavenging action of the EEOE as IC\textsubscript{50} (µg/mL), which is the quantity of sample needed to diminish the absorbance of DPPH by 50%. Ascorbic acid served as standard.

Biological investigations

Animals

The in vivo assessments were based on the Institutional Animal Care Committee of the National Administration of Algerian Higher Education and Scientific Research (approval no: 98-11 law of August 22, 1998). Wistar albino rats (200 ± 20 g) were used. The rats were caged under regular temperature (25 ± 2°C) and light-dark cycles (12/12 h). Food and water were given ad libitum.

Acute toxicity study

A single increasing dose of the EEOE, ranging from 10 to 2000 g/kg b.w. was given per os to six groups of rats (ten animals each) to evaluate acute toxicity. During the first hour, we reported the signs of toxicity. The rats were observed for 14 days (Lorke, 1983). The present work demonstrated the safety of the extract up to 2000 mg/kg.

In vivo procedure

Animals were partitioned into four groups of six rats each, and managed for 15 days following the procedure of Raj and Gothandam (2014). Rats in group I (control) received normal saline (25 mL/kg, p.o.) for 15 successive days; group II (CCl\textsubscript{4}) received normal saline (25 mL/kg, p.o.) for 15 successive days; group III (positive control) received reference drug silymarin (flavonolignans) (Sigma Aldrich Chemicals, Co.) (100 mg/kg, p.o.) for 15 successive days; and group IV (test group) received EEOE (400 mg/kg) for 15 successive days. One hour after saline administration, silymarin, and EEOE, rats of groups II, III, and IV received CCl\textsubscript{4} (0.2% in olive oil) at a dosage of 10 mL/kg, i.p., and rats of group I received olive oil (10 mL/kg, i.p.).

Serum sample preparation

Blood samples were collected by retro-orbital puncture 24 h after CCl\textsubscript{4} delivery. Blood samples were centrifuged at 2500 rpm. Serum was stored at -80°C until further investigations.

Serum biomarkers

Serum amounts of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, total cholesterol, and triglycerides were evaluated using commercial diagnostic kits (Spinreact, Spain) following the manufacturer's instructions.

Liver sample preparation

After washing with 0.9% NaCl, liver specimens (1 g) were blended with 10 mL of phosphate buffer (100 mM, pH 7). Hepatic homogenates were centrifuged at 4000 rpm and 4°C for 15 min.

Total protein content

Tissue homogenates were assayed for protein content as previously reported (Lowry et al., 1951). Bovine serum albumin served as standard.

Lipid peroxidation determination

The supernatants of hepatic homogenates were assessed for lipid peroxidation using the thiobarbituric acid reactive substances (TBARS) assay which determines the malondialdehyde
Liver homogenate (1 mL) was reacted with thiobarbituric acid-trichloroacetic acid (2 mL) (15% TCA in 0.25 N HCl and 0.375% TBA). The mixture was boiled at 100°C for 15 min, and centrifuged (1000 rpm, 15 min). The absorbance of hepatic MDA formed was evaluated at 530 nm, and expressed as nmol/mg protein.

Reduced glutathione determination
Liver specimens were assessed for reduced glutathione (GSH) as previously reported (Ellman, 1959). The approach is based on the generation of a yellow chromophore deriving from the reduction of DTNB \(5,5'-\text{dithiobis}(2\text{-nitrobenzoate})\) in the presence of sulfhydryl groups. Reduced glutathione amounts were expressed as µg GSH/mg protein.

Antioxidant enzymes assays
Catalase activity determination
Liver homogenates were analysed for catalase activity (CAT) as previously reported (Johansson and Häkan Borg, 1988). Specimens were incubated with methanol and \(\text{H}_2\text{O}_2\) in a buffered solution (250 mM, pH 7.0). Buffer was used as blank. After incubation for 20 min, the reaction was terminated by the addition of KOH (7.8 M). The \(\text{H}_2\text{O}_2\) reduction rate was measured spectrophotometrically at 240 nm. One unit of CAT was determined as µmol of \(\text{H}_2\text{O}_2\) disintegrated/min/mg protein.

Superoxide dismutase activity determination
The SOD activity was determined as reported by Kakkar et al. (1972). Liver homogenates were mixed with 0.052 M sodium pyrophosphate buffer (pH 8.3), 186 µM phenazine methosulphate, 300 µM nitro blue tetrazolium, and 750 µM NADH. The mixture was incubated at 30°C for 90 s. The reaction was terminated by the addition of glacial acetic acid, and then, the mixture was agitated with \(n\)-butanol. The absorbance of the chromogen developed was measured at 560 nm against \(n\)-butanol. One unit of SOD activity is the enzyme quantity needed to stop chromogen development by 50%.

Glutathione peroxidase activity determination
Samples were analysed for GSH-Px activity as previously reported (Flohé and Gunzler, 1984). Liver homogenates were reacted with 0.1 M phosphate buffer and 4 mM GSH. After incubation for 10 min at 37°C, \(\text{H}_2\text{O}_2\) (5 mM) was added to the mixture. The reaction was terminated by adding perchloric acid. The absorbance was measured at 412 nm. The GSH-Px activity was expressed as µg of glutathione oxidized/minute/mg protein.

Histopathological examinations
Liver specimens were fixed in formalin (10%), and ingrained in paraffin. Next, 5 µm thick portions were obtained, dyed with Hematoxylin-Eosin (H&E) stain, and examined for histological modifications in the hepatic tissues using a microscope (Olympus CH20, Japan).

Statistical analysis
Data were reported as mean ± S.D. The variations between groups were detected by one-way analysis of variance (ANOVA), followed by Tukey’s post-hoc test using IBM SPSS Statistics 21.0 software. Significance levels were set at the 5% level.

Results
Total phenolic, flavonoid, and tannin contents
Phytochemical screening of the EEOE highlighted its total phenolic (541.40 ± 7.97 µg GAE/mg extract), flavonoid (249.33 ± 1.43 µg QE/mg extract), and tannin (47.99 ± 4.09 µg of CE/mg extract) contents.

Phenolic profiling of EEOE
Chromatographic investigation using HPLC-PDA-ESI-MS demonstrated the presence of different phenolic compounds. The major peaks were investigated under negative ESI-MS mode. Nine compounds (Figure 1) were found: hydroxytyrosol, tyrosol, vanillin, verbascoside, oleuropein, apigenin-7-O-rutinoside, luteolin-7-O-glucoside, luteolin, and apigenin by correlating their retention times, UV-Vis absorption data, and MS fragmentation patterns with those available in the literature. Table 1 provides data regarding the description of the peaks, including retention times, UV-Vis absorption spectra, and ESI/MS data.

In vitro antioxidant activity
To evaluate the capacity of the EEOE to scavenge 50% of free radicals, DPPH and BCB assays were carried out. The FRAP method is
Figure 1. HPLC-PDA chromatogram of ethanolic extract of *O. europaea* (EEOE) leaves.

Table 1. HPLC-PDA and ESI-MS data of the detected compounds in ethanolic extract of *O. europaea* (EEOE) leaves.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Molecular formula</th>
<th>R&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>UV-Vis (λ&lt;sub&gt;max&lt;/sub&gt;)</th>
<th>Area (%)</th>
<th>[M−H]&lt;sup&gt;−&lt;/sup&gt; (m/z)</th>
<th>Tentative identification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C&lt;sub&gt;8&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>14.059</td>
<td>279</td>
<td>2.194</td>
<td>153</td>
<td>Hydroxytyrosol</td>
<td>D’Antuono et al. (2016)</td>
</tr>
<tr>
<td>2</td>
<td>C&lt;sub&gt;8&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>14.763</td>
<td>277</td>
<td>1.236</td>
<td>137</td>
<td>Tyrosol</td>
<td>Tasioula-Margari and Tsabolatidou (2015)</td>
</tr>
<tr>
<td>3</td>
<td>C&lt;sub&gt;4&lt;/sub&gt;H&lt;sub&gt;9&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>26.745</td>
<td>281, 331</td>
<td>2.807</td>
<td>151</td>
<td>Vanillin</td>
<td>Sanz et al. (2012)</td>
</tr>
<tr>
<td>4</td>
<td>C&lt;sub&gt;29&lt;/sub&gt;H&lt;sub&gt;36&lt;/sub&gt;O&lt;sub&gt;15&lt;/sub&gt;</td>
<td>27.807</td>
<td>267, 273, 331</td>
<td>2.205</td>
<td>623</td>
<td>Verbascoside</td>
<td>Cardinali et al. (2012)</td>
</tr>
<tr>
<td>5</td>
<td>C&lt;sub&gt;25&lt;/sub&gt;H&lt;sub&gt;32&lt;/sub&gt;O&lt;sub&gt;13&lt;/sub&gt;</td>
<td>29.393</td>
<td>226, 280</td>
<td>27.344</td>
<td>539</td>
<td>Oleuropein</td>
<td>Sanz et al. (2012)</td>
</tr>
<tr>
<td>6</td>
<td>C&lt;sub&gt;27&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;O&lt;sub&gt;14&lt;/sub&gt;</td>
<td>32.411</td>
<td>277, 331</td>
<td>1.944</td>
<td>577</td>
<td>Apigenin-7-O-rutinoside</td>
<td>Brito et al. (2014)</td>
</tr>
<tr>
<td>7</td>
<td>C&lt;sub&gt;21&lt;/sub&gt;H&lt;sub&gt;20&lt;/sub&gt;O&lt;sub&gt;11&lt;/sub&gt;</td>
<td>33.288</td>
<td>245, 251, 348</td>
<td>13.750</td>
<td>447</td>
<td>Luteolin-7-O-glucoside</td>
<td>Mylonaki et al. (2008)</td>
</tr>
<tr>
<td>8</td>
<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>35.668</td>
<td>257, 266, 338</td>
<td>4.547</td>
<td>285</td>
<td>Luteolin</td>
<td>Mitreski et al. (2014)</td>
</tr>
<tr>
<td>9</td>
<td>C&lt;sub&gt;13&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
<td>38.142</td>
<td>268, 337</td>
<td>11.481</td>
<td>269</td>
<td>Apigenin</td>
<td>Mitreski et al. (2014)</td>
</tr>
</tbody>
</table>

Antioxidative activity of the EEOE correlated with ascorbic acid and BHT which served as standards.

The DPPH is a stable free radical with maximum absorption at 517 nm that can fix an electron provided from an antioxidant, and thus, it is convenient to evaluate the free radical scavenging capacity of compounds. The data presented in Table 2 revealed that the EEOE efficiently scavenged DPPH free radicals with an IC<sub>50</sub> of 147.802 ± 3.11 µg/mL. This value was inferior to that of ascorbic acid (100.893 ± 3.73 µg/mL) (*p* < 0.01).

Table 2. Antioxidative capacity of ethanolic extract of *O. europaea* (EEOE) leaves.

<table>
<thead>
<tr>
<th>Antioxidant activity</th>
<th>DPPH IC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
<th>BCB IC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
<th>FRAP EC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEOE</td>
<td>147.802 ± 3.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>146.946 ± 5.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.761 ± 1.58&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>100.893 ± 3.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>36.441 ± 2.50&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BHT</td>
<td>ND</td>
<td>129.641 ± 2.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are mean ± SD of triplicate measurements (*n* = 3). ND: not detected. Means followed by different superscripts within the column are significantly different (*p* < 0.01).

The lipid radical scavenging ability was examined using the β-carotene/linoleate technique. In this method, the oxidation of linoleate generates lipid radicals thus resulting in β-carotene discoloration;
this causes a reduction in absorbance at 470 nm. In the present work, the EEOE demonstrated an enhanced inhibition potential of β-carotene oxidation with an IC₅₀ value of 146.946 ± 5.23 µg/mL. This value was inferior to that of BHT (129.641 ± 2.43 µg/mL).

An important mechanism of antioxidant capacity is chelating capacity. Therefore, it is beneficial to determine the capacity of the sample to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). From the iron-chelating results, it was evident that the EEOE had an improved reducing power, and able to efficaciously chelate Fe (III) ions, with EC₅₀ value of 36.441 ± 2.50 µg/mL. The EC₅₀ of ascorbic acid was 16.761 ± 1.58 µg/mL.

Blood biochemical markers

The findings of serum biochemical measurements are given in Table 3. The hepatic damage caused by CCl₄ was exhibited by the significant (p < 0.001) augmentation in total cholesterol (1.03 ± 0.03 mmol/L), triglycerides (0.98 ± 0.07 mmol/L), ALT (299 ± 38.66 IU/L), AST (395.66 ± 9.70 IU/L), ALP (253.5 ± 6.18 IU/L), and total bilirubin levels (7.52 ± 1.34 mg/dL) in CCl₄-treated animals in comparison with the control animals. Interestingly, EEOE treatment induced a significant (p < 0.001) decrease in the amounts of ALT (114 ± 4.51 IU/L), AST (187.33 ± 5.16 IU/L), ALP (208.83 ± 2.31 IU/L), and total bilirubin (1.61 ± 0.27 mg/dL) in correlation with CCl₄-treated animals. The EEOE at 400 mg/kg exhibited significant inhibition of liver damage akin to the reference drug.

Table 3. Impact of ethanolic extract of O. europaea (EEOE) leaves on serum biochemical parameters, and antioxidative enzymes activities of control and treated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>CCl₄</th>
<th>Silymarin (100 mg/kg, p.o.) + CCl₄</th>
<th>EEOE (400 mg/kg, p.o.) + CCl₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>42.16 ± 1.09</td>
<td>117.33 ± 3.07***</td>
<td>60.16 ± 6.58***</td>
<td>89.33 ± 6.34****</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>58.66 ± 5.53</td>
<td>90.83 ± 8.48***</td>
<td>64 ± 2.68***</td>
<td>74.58 ± 4.58****</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>114.5 ± 7.94</td>
<td>395.66 ± 9.70***</td>
<td>126.5 ± 5.20****</td>
<td>187.33 ± 5.16****</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>39.66 ± 6.47</td>
<td>114 ± 4.51***</td>
<td>68.66 ± 7.68***</td>
<td>83 ± 3.16***</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>188.33 ± 3.66</td>
<td>253.5 ± 6.18***</td>
<td>199.16 ± 3.81****</td>
<td>208.83 ± 2.31****</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.29 ± 0.03</td>
<td>7.52 ± 1.34***</td>
<td>0.75 ± 0.11***</td>
<td>1.61 ± 0.27***</td>
</tr>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>1.85 ± 0.11</td>
<td>3.95 ± 0.07***</td>
<td>2.26 ± 0.09****</td>
<td>2.79 ± 0.07****</td>
</tr>
<tr>
<td>GSH (µg/mg protein)</td>
<td>75.32 ± 2.86</td>
<td>42.93 ± 2.07***</td>
<td>64.77 ± 0.99*****</td>
<td>56.67 ± 2.10****</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>150.30 ± 3.19</td>
<td>72.67 ± 3.46***</td>
<td>123.93 ± 2.92****</td>
<td>93.17 ± 4.27****</td>
</tr>
<tr>
<td>CAT (U/mg protein)</td>
<td>31.57 ± 0.67</td>
<td>14.10 ± 0.67***</td>
<td>23.92 ± 0.51****</td>
<td>18.71 ± 1.13****</td>
</tr>
<tr>
<td>GSH-Px (U/mg protein)</td>
<td>17.31 ± 0.37</td>
<td>5.28 ± 0.25***</td>
<td>13.91 ± 0.33****</td>
<td>9.93 ± 0.37****</td>
</tr>
</tbody>
</table>

Data are mean ± S.D. of six animals (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001 values as compared to control group, #p < 0.05, ##p < 0.01, ###p < 0.001 values as compared to CCl₄ toxic group.

Lipid peroxidation and reduced glutathione levels

Malondialdehyde (MDA) is broadly considered an indicator for lipid peroxidation caused by oxidative stress. It is apparent from Table 3 that a single application of CCl₄ resulted in a substantial augmentation in hepatic MDA levels, with the value of 3.95 ± 0.07 nmol/mg protein as compared to the control group (1.85 ± 0.11 nmol/mg protein). EEOE pre-administration markedly attenuated the CCl₄-induced increase of MDA levels, and the corresponding value was 2.79 ± 0.07 nmol/mg protein (p < 0.001). The standard silymarin treated group displayed comparable data (2.26 ± 0.09 nmol/mg protein). CCl₄ significantly (p < 0.001) reduced the level of hepatic reduced glutathione (GSH) (42.93 ± 2.07 µg/mg protein) which was restored by EEOE treatment (56.67 ± 2.10 µg/mg protein) (Table 3).

Antioxidant enzymes activities

To estimate the protective capacity of EEOE on the CCl₄-induced hepatic oxidative impairment in animals, we checked the hepatic antioxidative enzymes activities. In contrast to the normal control rats, CCl₄ significantly (p < 0.001) lessened hepatic...
SOD, CAT, and GSH-Px amounts in the CCl₄-treated animals (Table 3). Nevertheless, the use of EEOE significantly (p < 0.01) re-established the amounts of the tissue antioxidants nearly normal in comparison to CCl₄-treated rats. Furthermore, the EEOE was observed to possess antioxidative potential in the hepatic homogenate akin to the standard drug-treated group.

**Histopathological analyses**

Figure 2 shows the findings of the histological investigations of liver specimens stained with H&E in control, CCl₄, silymarin + CCl₄, and EEOE + CCl₄ treatments. The liver sections in the control group revealed the existence of hepatocytes with distinguished cytoplasm, pronounced nucleus, and prominent central vein (Figure 2A). Conversely, the CCl₄-treated group displayed hepatic sections with remarkable structural damages as evidenced by extensive necrosis of hepatocytes in the centrilobular area, destruction of cellular borders, congestion in the central vein, damaged lobular structure, and inflammatory cells influx (Figure 2B). Microscopic observation of liver tissues showed that pre-treatment with EEOE protected hepatic cells from CCl₄-induced toxicity. Improvement in liver morphology with the preservation of the parenchymal structure, and protection from hepatic cells deterioration and centrilobular necrosis was also observed (Figure 2C). These animals showed normal hepatocytes similar to the normal animals and the silymarin-treated group (Figure 2D).

**Figure 2.** Photomicrographs of H&E stained sections of liver from (A) normal control group, (B) CCl₄ group, (C) silymarin (100 mg/kg) + CCl₄ group, (D) EEOE (400 mg/kg) + CCl₄ group (40×). H: hepatocytes, MI: mononuclear infiltration, CV: central vein, CN: central necrosis, S: sinusoids. Scale bar: 50 µm.

**Discussion**

Hepatotoxic agents induce damage to the liver, and cause disorders in the metabolism. CCl₄ has been broadly used as experimental chemical agent to induced hepatotoxicity. The NADPH-Cyt p450 system metabolises CCl₄ to trichloromethyl radical (Jasemine et al., 2007). This free radical adheres to essential biological molecules like polysaccharides, proteins, lipids, and nucleic acids, thus leading to DNA disintegration and hepatocellular membrane damage via the lipid peroxidation process. The generated cellular defect is manifested by modifications in haematological and biochemical markers (Ben Hsouna et al., 2018).

Hepatocytes have high amounts of AST, ALT, and ALP which are measured in the estimation of hepatic damage. Free radicals-induced hepatocyte membrane deterioration contributes to hepatic enzyme efflux into the extracellular space. Thus, the
estimation of blood amounts of AST, ALT, and ALP could reveal the hepatic status (Deng et al., 2012). The present work evaluated the hepatoprotective activity of EEOE by investigating its preventive and antioxidant potentials against CCl₄-induced oxidative liver damage in rats. Therefore, increased amounts of biochemical markers like ALT, AST, ALP, and bilirubin, as well as the significant increment in total cholesterol and triglycerides could indicate cellular membrane impairment in the CCl₄-treated rats. Our results demonstrated that polyphenol-rich EEOE (400 mg/kg) improved the liver markers quantity close to normal, thus indicating a promising hepatoprotective capacity of EEOE. The increase in ALP activity and total bilirubin level was depleted by EEOE treatment. These data are likely to be related to the capacity of EEOE to control biliary impairment in rats with CCl₄-induced liver damage.

Many authors have related the preventive capacity of plant extracts against hepatic injury to their phytoconstituent-associated antioxidant potential. ROS-induced oxidative stress has a pivotal role in hepatic injury. It could provoke liver damage by triggering lipid peroxidation, disturbing biomembrane architecture, and affecting the enzyme function (Ben Hsouna et al., 2018). To overcome the damaging actions of free radicals, organisms developed enzymatic and non-enzymatic antioxidative systems. Enzymatic complexes involve SOD, CAT, and GSH-Px that preserve tissues from oxidative injury through a radical scavenging system. Thus, these antioxidant enzymes play a vital role in hepatic detoxification (Huo et al., 2011). Superoxide dismutase (SOD) converts superoxide radical (O₂⁻) into hydrogen peroxide (H₂O₂) and oxygen. Catalase (CAT) decomposes H₂O₂ to generate water and oxygen. Glutathione peroxidase (GSH-Px) catalyses the reduction of H₂O₂ by GSH (Raj and Gothandam, 2014).

In the present work, CCl₄ treatment led to a significant reduction in the amounts of antioxidant enzymes (SOD, CAT, and GSH-Px), thus demonstrating severe hepatic oxidative stress status (Huo et al., 2011). The use of EEOE (400 mg/kg) resulted in the restoration of the actions of SOD, CAT, and GSH-Px comparable to silymarin. These results agree with those of Ustuner et al. (2018) who also found that olive leaves regulated the antioxidant enzymes activities of CCl₄-treated rats, thus affirming its antioxidant potential.

A non-enzymatic system, liver GSH, is involved in the detoxification of CCl₄. Also, it conserves cells from oxidative stress-induced deterioration. Reduced glutathione (GSH) provides an electron to ROS, thus transforming them into non-toxic compounds (Raj and Gothandam, 2014). The findings of the present work suggested that CCl₄ significantly diminished the GSH level. Nevertheless, the use of EEOE at 400 mg/kg has re-established the GSH to near-normal levels.

Carbon tetrachloride intoxication-induced reactive species accelerate the lipid peroxidation process by removing a hydrogen atom from the unsaturated fatty acids. Malondialdehyde is the end product of this mechanism, and the increase of MDA in liver tissue is an indicator of hepatic damage (Ben Hsouna et al., 2018).

A fundamental antioxidative mechanism to stop the lipid peroxidation chain reaction is free radical scavenging. The present work displayed a significant increment in the MDA amount in response to CCl₄ treatment. The EEOE (400 mg/kg) decreased the MDA to near-normal levels. In agreement with these data, we could infer that the hepatoprotective capacity of EEOE could be associated with their antioxidative and free radical inhibiting ability by prohibiting the linking of free radicals to hepatocyte membrane, thus avoiding lipid peroxidation. Several investigations have reported that the hepatoprotective activity could be relevant to the antioxidative process responsible for the ROS scavenging and the blockage of free radicals such as hydroxyl, alkyl, and lipid peroxides (Hsu et al., 2009). In the present work, EEOE appeared to exhibit marked radical-scavenging and metal-chelating capacity, thus, it could reverse pathological impairment created by the CCl₄-generated free radicals.

Earlier reports displayed that olive leaves possess potent antioxidant power (Ferreira et al., 2007). It seems potential that the preventive ability of EEOE is related to its phytochemical compounds which were identified by HPLC-MS and previously described in olive leaves (Meirinhos et al., 2005). Many phytochemical constituents, including polyphenols, have antioxidant potential, which is associated with the protection of the biological system from damaging oxidation reactions. The phytochemical screening of olive leaves extract showed that it has high contents of total phenolics, flavonoids, and tannins.
It was reported that phenolic constituents possess antioxidant characteristics since they have hydroxyl groups with mobile hydrogen. These components prohibit the dissociation of hydroperoxides into free radicals which improve the antioxidant capacity of plants’ extracts (Ben Hsouna et al., 2018). Results of the present work demonstrated the hepatoprotective and antioxidant potential of polyphenol-rich EEOE against CCl₄-induced hepatic damage in rats. Raj and Gothandam (2014) have also noted that the protective activity of polyphenols from Amorphophallus commutatus var. wayanadensis against CCl₄-induced liver toxicity is related to their antioxidant activity. In compliance with the present results, previous studies demonstrated that Syzygium jambos extract lowered the expression of heat shock protein HSP-16.2 gene besides its scavenging reactive species. S. jambos extract-treated group significantly decreased hepatic markers amounts in comparison with the CCl₄-intoxicated animals (Sobeh et al., 2018a). These results are in agreement with Sobeh et al. (2018b) who showed that the antioxidant and hepatoprotective activities of S. samarangense were likely related to the abundance of flavonoids in the extract. They also found that these antioxidants displayed noticeable scavenging potential of free radicals, inhibiting superoxide anion-producing enzymes, and suppressing the enzymes that are responsible for ROS production. Furthermore, these molecules are chelators of transition metals. The results of the present work agree with Sobeh et al. (2018c) who suggested that the antioxidant, hepatoprotective, anti-inflammatory, and pain-killing abilities of S. aqueum leaf extract were likely to be related to the formation of various hydrogen and ionic bonds with proteins. As a result, polyphenol-bounded proteins modified their 3D structures, thus changing their biological activities.

Phenolic profiling of EEOE revealed the presence of hydroxytyrosol, tyrosol, vanillin, verbascoside, oleuropein, apigenin-7-O-rutinoside, luteolin-7-O-glucoside, luteolin, and apigenin. The significant hepatoprotective and antioxidant capability of EEOE could be attributed to its polyphenolic composition. This agrees with another study that was performed to explore the therapeutic action of olive leaves extract containing 20% oleuropein on CCl₄-induced hepatic injury (Ustuner et al., 2018). They suggested that olive leaf extract reduced hepatic toxicity by lowering the MDA levels, regulating antioxidant enzymes activity, and decreasing DNA impairment. Also, olive leaf extract decreased ALP, AST, and ALT levels, and raised SOD and CAT activities of blood samples.

In agreement with the present results, previous studies have also demonstrated that oleuropein and hydroxytyrosol restored the increased amounts of TG and hepatic enzymes by improving SOD and CAT activities. Besides, they reported that the olive leaves extracts diminished the expression of NF-κB and TNF-α (Mahmoudi et al., 2018). A previous study had noted that hydroxytyrosol and tyrosol reduced TCDD-induced liver injury by suppressing CYP1A1 expression, and boosting the liver antioxidant enzymes. These compounds suppressed apoptosis through the inhibition of Bax expression, and the induction of Bcl-2 expression (Kalaiselvan et al., 2015).

Makni et al. (2011) revealed in their study that vanillin inhibited the reduction of protein synthesis and the augmentation in serum ALT and AST amounts, and attenuated TNF-α, IL-1β, and IL-6 expression amounts. They also revealed in their study that this compound prevented liver lipid peroxidation, formation of protein carbonyl, and preserved the antioxidative systems. Another significant finding was that oleuropein inhibited the stimulation of hepatic stellate cells caused by the TNF-β1, besides the activation of caspase-3. These results were likely to be related to the induction of heme oxygenase-1 by NF-E2-related factor 2 (Domitrović et al., 2012). Further reports demonstrated that luteolin inhibited the evolution of hepatic fibrosis by suppressing fibrosis-related genes in HSC, and inhibiting TGF-β and PDGF signalling pathways (Li et al., 2014). Finally, apigenin has been described for its potent antioxidant effect and hepatoprotective potential. As mentioned by Rašković et al. (2017), apigenin-treated animals exhibited reduced ALT and ALP activities. Paracetamol-induced histopathological alterations were also reduced by apigenin. Apigenin also reversed the elevation in MDA level. In addition, apigenin also enhanced the enzyme antioxidant defence systems.

Conclusion

The present work demonstrated that the phenolic-rich ethanol extract of olive leaves decreased CCl₄-induced liver damage by recovering antioxidant enzymes activities, lowering lipid.
peroxidation, and restoring the levels of liver markers. These data implied that the hepatoprotective capacity of the extract was conceivably related to its antioxidative action. However, additional investigations are imperative to ascertain the molecular mechanisms of action of olive leaves’ active constituents.

References


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