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Research article

Phytochemical profiling, antioxidant potential and protective effect of leaves extract of tunisian *Vitis vinifera* autochthonous accessions against acute CCl_4-injured hepatotoxicity in mice

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ABSTRACT

Vitis vinifera leaves (VVL) are agro-industrial waste. In the current study, the phytochemical profile of *V. vinifera* leaves extracts (VVLE) of two Tunisian autochthonous accessions was determined via LC-UV-ESI/MS, and their antioxidant and hepatoprotective properties were also assessed. Mice were pretreated orally with VVLE (7.5, 15 and 30 mg/kg) for 7 days, and then received acutely and by *i.p.* a solution CCl_4 at 12% in sunflower oil (v/v). Serum levels of hepatic markers, oxidative stress indicators in liver tissue and histological changes were assessed. LC-UV-ESI/MS analysis revealed four phenolic compounds identified in both extracts with quercetin-3-*O*-glucuronide being the dominant constituent (23.32 ± 1.06 *vs.* 10.24 ± 0.12 mg/g DM, $p < 0.05$ for wild and cultivated accessions, respectively). The Antioxidant activity revealed a significant difference between the genotypes. Moreover, the VVLE of the wild "Nefza-I" ecotype was the most active based on antioxidant assays. Furthermore, the results showed that pre-treatment, especially with VVLE, of the wild ecotype "Nefza-I", attenuated CCl_4-induced acute liver injury in a dose-dependent manner, as demonstrated by the decrease in the activities of hepatic serum function markers. This was also evidenced by a decrease in the levels of lipoperoxidation and histological damage in the liver, as well as a restoration of antioxidant enzyme activities (SOD and catalase) and an increase in the hepatic glutathione content. Our results demonstrate that VVLE possesses protective effects on CCl_4-induced liver injury. Overall, the wild ecotype "Nefza-I" extract could serve as an effective protector against CCl_4-induced hepatocellular oxidative stress.

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1. Introduction

The liver, a metabolic hub of the human body, responsible for regulating several critical physiological processes such as the detoxification of biologically harmful substances, digestion, storage, excretion, secretion and metabolism [1]. The liver could be a subject of exposure to various exogenous substances such as drugs, alcohol, heavy metals, pesticides, viruses, bacteria, parasites and environmental toxicants, which generally may trigger hepatic dysfunction and deleterious health damages that could lead to death [2, 3].

In many cases, pathological changes in the liver were seen following the excessive exposure to CCl_4 leading to hepatic fibrosis which subsequently progress to cirrhosis, autoimmune hepatitis and lastly to hepatocellular carcinoma [4]. Liver diseases are among the most challenging diseases to design potential therapies, for and a major public health issue worldwide (i.e., ranked fifth as a major common cause of death) [5]. Previous research revealed CCl_4 as a noxious hepatotoxic chemical causing the induction of acute hepatic damage in different types of animal models. CCl_4 direct or indirect stimulation of hepatocytes, is very similar compared to hepatic injuries in humans [6]. It is well accepted that CCl_4, a potent hepatotoxic chemical, is reduced by hepatic cellular cytochrome P450 2E1 (CYP2E1) in endoplasmic reticulum to produce the highly reactive free radicals including trichloromethyl ($\bullet CCl_3$), trichloromethyl peroxy radical ($\bullet OOCCl_3$) and reactive oxygen species (ROS). However, excess production of these reactive free radicals within the tissues ultimately leads to oxidative stress, which plays a crucial role in the pathogenesis of liver injury [7].

ROS's capacity to interact with different types of biomolecules in the cell [10] (e.g., proteins, lipids, nucleic acids), results in the destruction of cell structure (i.e., membranes), contributing physiologic dysfunction, lipid peroxidation, and ultimately leading to hepatic damage. Advanced effects of CCl_4 cause hepatocellular necrosis and inflammation, mitochondrial dysfunction, release of liver enzymes and hepatocyte apoptosis [8,9]. Conventional therapies for liver diseases using synthetic medicines like tiopronin, glutathione, and biphenyl dimethyl-ester are common, costly ineffective, and at the same time are of major side effects. For instance, long-term use of tiopronin revealed as a cause of rare complications such as proteinuria and potential risk of kidney injury [10]. From this perspective, a safer and more efficient alternative is timely needed. Recently, a trending toward natural product substitutes has opened room for researches aiming to present natural sources of antioxidants as validated suitable treatments for hepatic diseases. Thus, researchers paid particular interest to evaluate the hepatoprotective effect of natural bioactive compounds from different medicinal plants. Particularly, grapevine (*Vitis vinifera* L.) is a plant originating from the Mediterranean area, is an economically, medicinally and nutritionally important fruit species cultivated across the globe, particularly in North Africa, and more precisely in Tunisia, and this since the Carthage Phoenician period. This plant has become, for a long time, an important and inseparable element of the Western societies' mode. Indeed, the different underused bioresources generated by winemaking industry and cultivation of grapevines itself are represented by pomace, berries, pulp, seeds, stems, skins and leaves. Grapevine leaves are considered a waste product of agricultural production and winemaking and should be valorized normally in the framework of a circular economy and sustainable development. However, grapevine leaves have received wide attention from the scientific community based on its substantial nutritional value, as a promising and inexpensive source of secondary metabolites with great promoting potential benefits to human health [11]. Previous phytochemical investigations have shown that grapevine leaves are known to be rich and varied matrix of secondary bioactive metabolites including phenolic acids (both hydroxybenzoic and hydroxycinnamic), flavonoids such as flavanones, flavonols, flavones, flavan-3-ols, proanthocyanidins, anthocyanins, resveratrol and others stilbenes derivates [12–14]. Owing to the traditional applications, this winery by-product is considered as a potential added-value product, that could be further developed into a useful treatment/preventive for multiple disorders, including bleeding, hemorrhage, stomachaches, diarrhea, pain, gout, inflammation-related disorders, hepatitis, diabetes, chronic bronchitis and some circulatory diseases [12,15]. Similarly, evidence suggests that grapevine leaves possess a plethora of beneficial properties such as antioxidative, antimicrobial, anti-inflammatory, neuroprotective, hepatoprotective, cytotoxic, gastroprotective, anti-obesity and antiviral against coronavirus 2 (SARS-CoV-2) and Herpes simplex virus type 1 (HSV-1) [12,16–19]. To the best of our knowledge, Tunisian autochthonous accessions have previously been genetically characterized, and several studies have been done at the molecular level [20,21]. However, no thorough phytochemical and hepatoprotective data about these local genotypes, in particular on CCl_4-induced hepatotoxicity is available so far. In the current study, we aim to characterize the phytochemical profile of VVLE produced by simple maceration and obtained from two native accessions grown in Tunisia, then evaluate, *in vitro*, their antioxidant properties. Furthermore, assessing *in vivo*, the protective capability of VVL against CCl_4 induced-acute-liver toxicity in Swiss-albino male mice, through the determination of biochemical indicators, histopathology, and behavioral observations. This study underlines a broader goal of searching for novel natural ingredients with potential use as food supplies, and/or new openings for innovative therapy and biomedical applications.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade methanol, water, acetonitrile, formic acid, hydrochloric acid (HCl), Sulfuric acid (H_2SO_4), hydrogen peroxide (H_2O_2), carbon tetrachloride (CCl_4), gallic acid, catechin, ascorbic acid, DPPH (2, 2-diphenyl-1-picrylhydrazyl), BHT (2,6-ditert-butyl-4-hydroxy-boxylic acid), (±)-6-Hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid (Trolox), bovine catalase, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), ethylenediamine tetraacetic acid (EDTA), ammonium molybdate, ferrous chloride ($FeCl_2$) and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), as well as phenolic standards, contain quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside, quercetin-7-*O*-glucuronide and rutin were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Serum ALT, AST, ALP, LDH and GGT contents were determined using commercially available test kits (Biomaghreb Ariana Tunis, Tunisia).

2.2. Plant material

In August 2019, two Tunisian autochthonous *Vitis vinifera* accessions, one wild grapevine ecotype ("Nefza-I") and one grapevine from cultivar ("Marsaoui"), growing under Mediterranean conditions in the north of Tunisia (Latitude 36° 51′ 15″ N; Longitude 10° 10′ 26″ E; altitude: 28 m). They were grown in the same experimental vineyard within the same cultural conditions and agronomic practices, without irrigation and soil maintenance. These grapevine genotypes are cultivated in the national germplasm collection of the Horticulture Laboratory, located at the National Institute of Agricultural Research (INRAT, Tunisia).

For each accession, leaves were collected during the grapevine harvest period. The collected biomass was cleaned under cold distilled water, air-dried at room temperature under shady conditions, freeze-dried and then ground to a fine powder using an electric blender, and the lyophilized powder was encapsulated under vacuum and stored in polyethylene plastic bags at room temperature in dry place priori to use. The plants were taxonomically identified by Pr Mounira HARBI at the INRAT. A voucher specimen of leaves of each accession was deposited at the herbarium of the laboratory (LBE, Bizerta) under following numbers: VvwN-I/LBE 20-001 and VvcM/LBE 20-002.

2.3. Preparation of extract

The dried powdered materials of VVL (2 g) were extracted by maceration using 20 ml of a mixture of methanol and water (70–30%, v/v) and stirred for 24 h at room temperature (25 ± 1 °C). After centrifugation at 3460×*g* for 20 min the supernatants were collected and dried using a rotary evaporator and freeze-dried in a lyophilizer to obtain powders, which were stored in glass vials in a refrigerator at 4 °C until analysis. Finally, the extracted powder was dissolved in physiological saline (NaCl 0.9%) to prepare the VVLE for the biological test.

2.4. Phytochemical analysis of VVLE by LC-PDA-ESI/MS

The chemical composition present in the hydromethanolic extracts of VVL was identified using LC-PDA-ESI/MS (LC–MS 2020 system, Shimadzu, Tokyo, Japan). For polyphenols separation was performed on 20 μL sample injections on to Gemini 3 μm C18 130 Å reversed phase column (Phenomenex, Torrance, CA, USA) of 150 × 4.6 mm i.d. Mobile phase elution was made with a flow rate of 600 μL/min using a gradient mobile phase; mixture of (A) 0.5% formic acid in aqueous solution and (B) acetonitrile and the oven temperature of the column was fixed at 30 °C. The following elution gradient was used: 0–15.5 min: linear gradient from 90:10 (A:B) to 50:50; 15.5–16 min: linear gradient from 50:50 to 10:90; 16–20.5 min: isocratic elution at 10:90; 20.5–21 min: linear gradient from 10:90 to 90:10; 21–25 min: isocratic elution at 90:10 (equilibration step). In addition, double detection was ensured by a photodiode array detector (PDA) in the 200–400 nm wavelength range and mass spectrometry (MS). MS settings were as follows: negative mode electrospray source (ESI) with 1.5 L/min nebulization gas flow, 12 L/min drying gas flow, 300 °C heat block temperature, 250 °C desolvation line temperature, −4 kV probe voltage. To avoid contamination of the electrospray source, *Vitis vinifera* leaves extracts were filtered, then diluted twenty fold. MS data acquisition was performed in the selected ion monitoring (SIM). The peaks were identified according to their retention time (t_R), *m/z* ratio, mass spectrum, and comparisons with standards. For quantitative analysis, a 5-level linear calibration curve was obtained by injection of known concentrations (from 10^{-4} to 10^{-2} mg/mL) of standards. Method sensitivity was assessed by determining the limits of detection (LOD) and quantification (LOQ), defined as the concentrations leading to signal-to-noise (S/N) values of 3 and 10, respectively. The determination of LOD and LOQ values was achieved with standards in the following concentration ranges: rutin ($R^2 = 0.99$; LOD = 0.3 ppm; LOQ = 0.99 ppm); quercetin-3-*O*-glucuronide ($R^2 = 0.99$; LOD = 3.8 ppm; LOQ = 13 ppm); quercetin-7-*O*-glucuronide ($R^2 = 0.99$; LOD = 1.8 ppm; LOQ = 6.3 ppm); quercetin-3-*O*-glucoside ($R^2 = 0.99$; LOD = 1.3 ppm; LOQ = 4.2 ppm). Results were expressed in milligrams per gram of dry matter (DM).

2.5. In vitro antioxidant evaluation

2.5.1. Determination of total antioxidant capacity (TAC)

The total antioxidant capacity (TAC) of plant extracts was estimated by phosphomolybdenum reducing method described by Prieto et al. [22]. Briefly, an aliquot of 0.1 mL sample solution was combined with 1 mL reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in water bath at 95 °C for 90 min. Followed by incubation, the samples were left to stand and cooled for 6 min at room temperature. Thus, the absorbance was measured by a BioRad SmartSpec ™ 3000 Scan UV–visible spectrophotometer at 695 nm against a blank (1 ml of reagent solution and 0.1 ml of extraction buffer). A standard curve was plotted using standard solution of ascorbic acid (0–400 μg/ml) and the results were carried out in triplicates were expressed as mg equivalent ascorbic acid (standard) per gram of dry weight (DM) (AAE/g of DM).

2.5.2. DPPH (1, 1-diphenyl-2-picryl-hydrazyl) radical scavenging activity

The radical scavenging ability of VVLE was assessed *in vitro* by the standardized method based on the scavenging potential of the stable DPPH free radical [23]. About 40 μL of different concentrations (0.001–20 mg/mL) of the extracts were added to 1 mL of DPPH radical was prepared freshly in methanol (final concentration of DPPH was 100 μM). Reaction mixtures were shaken vigorously and incubated for 30 min in absence of light at 37 ± 2 °C. Finally, the UV–visible spectrophotometer BioRad SmartSpec ™ 3000 was used for the measurement of the decrease in absorbance of the solution at 517 nm. The synthetic antioxidant Trolox (a water-soluble analog of vitamin E) was used as a reference.

The half-maximal inhibitory concentration (IC_{50}) in mg/kg, which refers to the smallest concentration of an antioxidant agent (among others our extracts) required for providing 50% inhibition of the DPPH radical was determined and the assay was performed in triplicates and the percentage scavenging activity was calculated using the following equation:

% DPPH Scavenging activity = $[(A_0 - A_1)/A_0]*100$, where, A_0 is the absorbance recorded at 517 nm of the reagent of the control (blank, without extract) and A_1 is the absorbance of the test sample.

2.5.3. Ferric ion reducing antioxidant power (FRAP) assay

The total reducing power of VVLE was determined by using the protocol devised by Berker et al. [24]. A volume of 1 mL of extract at different concentrations (10–250 μg/mL) was mixed with an equal volume of phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%, m/v). The mixture obtained is vortexed and incubated at 50 °C for 20 min followed by the addition of 2.5 ml of trichloroacetic acid (10%, m/v) and centrifuged at 650 g for 10 min. Then, 2.5 ml of the upper layer of solution was mixed with 2.5 ml of distilled water and 0.5 mL of 0.1% $FeCl_3$ (m/v) then allowed to stand for 10 min at room temperature. The extraction buffer was used as a blank and ascorbic acid in concentrations (10–150 μg/mL) was used as a standard in this assay. An increase in the absorbance of the reaction mixture indicates an increase in the reducing power of both the extracts and ascorbic acid. The IC_{50} value (μg/mL) providing 0.5 of absorbance was calculated from the graph of absorbance at 700 nm (BioRad SmartSpec™ 3000 Scan UV–visible spectrophotometer) against extract concentration in the solution. The experiments were all repeated three times.

2.5.4. Ferrous ion chelating activity assay

The chelating capacity of VVLE was determined by measuring the formation of the Fe^{2+}-ferrozine complex as described by Le et al. [25] and compared with that of the reference chelator agent EDTA. 500 μL of extracts or standard chelator (EDTA) at different concentrations were added to 100 μL of ferrous chloride ($FeCl_2$) (0.6 mM) and 900 μL of methanol. After 5 min, 100 μL of ferrozine (5 mM in methanol) was added to the reaction medium to initiate the reaction. After this part of the protocol, the obtained samples were incubated for 10 min at room temperature after vigorous stirring to allow the complexation of the residual iron. The absorbance of the Fe^{2+}-ferrozine complex was read at 562 nm using a UV–visible spectrophotometer BioRad SmartSpec ™ 3000. The chelating ability was calculated following the equation:

Chelating activity (%) = 100 x (A0 – A1)/A0), where A_0 is the control absorbance and A_1 is the absorbance in the presence of extracts. All the experiments were run three times.

2.6. In vivo studies

2.6.1. Experimental design

Healthy adult male Swiss albino mice (*Mus musculus*) approximately 6 weeks old (weighing 25–30 g; housed 10 per cage), with no sign of diseases or injury were used throughout this investigation. All animals were procured from Tunis Pasteur Institute (Tunisia) and were handled in accordance with both the ATSAL's (Tunisian Society for the Care and Use of Laboratory Animals) recommendations and the European Convention (2010) for the protection and use of vertebrate animals. All procedures involving animals have been approved by the regional bioethics review committee (Ethical approval No. 0121/2022 ATSAL), whose recommendations are mainly aimed at reducing both the number of animals used and their suffering during handling. All mice were reared under basal standard rations and permitted water and standard rodent food (pellet, Badr, 7060 Utique, Tunis). All mice were acclimatized for a week adaptation period before the *in vivo* experiments and were accommodated in a controlled animal room under constant temperature (22 ± 2 °C) as well as the relative humidity of (50–60%), and lighting for 12 h–12 h alternately (lights on at 07:00 h and off at 19:00 h). All the mice were housed in polypropylene cages (22.5 × 37.5 cm) with bedding and a wide mesh wire barrier placed on the bottom to prevent coprophagia.

2.6.2. Evaluation of acute toxicity

For this study, healthy mice (n = 10) were on a water diet for 3–4 h and were randomly classified into eight groups. The mice in the control group received a dose of physiological saline via oral gavage (10 ml/kg, *b.w.*) whereas the treated groups received acutely hydromethanolic extract of VVL at the dose levels of 7.5, 15, 30, 60, 120, 240 and 480 mg/kg b.*w.* After the acute phase, animals were observed individually at 2-h intervals during the first 24-h for toxic signs and symptoms of hyperactivity, changes in behavior (alertness, mood and motor activity), seizures, sedation, fur erection, urination, mucous membranes, respiration, autonomous parameters (eyes, salivation and diarrhea), neurological changes and mortality. Thereafter, mice were kept under observation once daily for 14 extra days.

2.6.3. Hepatoprotective activity

For the evaluation of the protective role of hydromethanolic extracts of VVL from wild and cultivated accessions on the CCl_4-induced hepatotoxicity in animals, ninety mice were equally allocated into nine groups of ten animals each (n = 10).

Group I – Normal control group: treated with vehicle solution (NaCl, 0.9% at a flow rate of 10 ml/kg, *b.w.*) once a daily for seven days. On the seventh day, 1 h after the last administration, they received an equivalent volume of sunflower oil intraperitoneally (at a rate of 10 mL/kg, *b.w.*).

Group II – CCl_4-treated group (model control group): mice were administered by oral route with physiological saline (NaCl, 0.9% at a flow rate of 10 ml/kg, *b.w.*) once a day for seven days. One hour after the last dosing, animals were injected intraperitoneally

with CCl_4 (12%, v/v, dissolved in sunflower oil, 10 mL/kg, *b.w.*).

Group III – Silymarin-treated group (Standard group): mice were given daily with the drug silymarin (reference drug) by oral route once daily for 7 days at 100 mg/kg, *b.w.*, dissolved in physiological saline and then challenged acutely by intraperitoneal injection with CCl_4 (12%, v/v, dissolved in sunflower oil, 10 mL/kg, *b.w.*) on the 7th day, 1-h after the final administration of silymarin.

Group IV, V and VI: served as extract pretreated groups and were given three increased doses (7.5, 15 and 30 mg/kg) of hydromethanolic extracts of VVL of wild ecotype ("Nefza-I") gavaged once daily for seven consecutive days. On the 7th day, 1-h post-dosing, animals were injected by *i.p.* route with CCl_4 (at a rate of 10 mL/kg, *b.w.*).

Group VII, VIII and IX: served as extract pretreated groups and were given three increased doses (7.5, 15 and 30 mg/kg) of hydromethanolic extracts of VVL of cultivated variety ("Marsaoui") delivered by gavage once daily for seven consecutive days. On the 7th day, 1 h after the last dosing, animals were injected by *i.p.* route with CCl_4 (at a rate of 10 mL/kg, *b.w.)*.

In the final step of the experiment, all mice were starved for 12 h after treatment while given enough water to drink. Sacrifiation, have been done 48-h after the completion of the experiment. Blood samples were withdrawn in Eppendorf tubes, then serum was collected by centrifuging at 3000×*g* for 15 min at 4 °C, therefore stored at −20 °C for subsequent biochemical measurements. The liver tissues were excised and then aliquoted into 3 different pieces to be used as follows: the first aliquot was quickly washed with 0.9% NaCl and kept at −80 °C until analysis, the second aliquot was adequately preserved for various biochemical analyses, and the third aliquot constitutes a representative section of the liver immediately fixed in 10% buffered formalin for standards histology processing and H&E staining for histopathological evaluation.

2.6.4. Biochemical measurements of serum enzymes

Biochemical markers of liver function, *i.e.*, the level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma-glutamyl transferase (GGT) were analyzed in serum of all the studied groups. The concentrations of different serum markers were measured spectrophotometrically by colorimetric method, following the manufacturer's instructions mentioned on commercially available enzymatic kits (Biomaghreb, Ariana Tunis, Tunisia).

2.6.5. Biochemical estimation of hepatic markers

Liver tissues were homogenized in ice-cold phosphate-buffered saline, pH 7.4 using an ultra-Turrax. The whole homogenates were centrifuged at 9000×*g* for 15 min at 4 °C and the cytosolic supernatants were collected for the estimation of the enzyme marker and antioxidant parameters determination. Total protein content was measured by Biuret colorimetric method [26]. Levels of the end-product of lipid peroxidation (malondialdehyde, MDA) were determined according to the method described by Buege and Aust [27]. SOD and CAT activities were measured by following the process developed by Misra and Fridovich [28], and Aebi [29], respectively. Reduced glutathione (GSH) content was quantified spectrophotometrically using Ellman's reagent (DTNB) through the methodology of Moron et al. [30].

2.6.6. Histopathological examination

All steps were conducted at Anatomy and Pathological Cytology Department, Farhat Hached University Hospital, Sousse University. Isolated liver tissues were fixed in 10% neutral formalin, which was dehydrated, embedded in paraffin blocks and cut into 5 μm-thick sections using a rotary microtome. Thereafter, fixed tissue sections were stained with hematoxylin and eosin (H&E) according to standard techniques. Finally, stained slides were viewed under a light microscope (Olympus, Japan) at a magnification of 200 × and photographed for evaluation and documentation of pathological changes.

2.7. Statistical analysis

Values are expressed as means ± standard deviation (SD) and the analysis was performed using Graphpad Prism (version 5.02) for windows and Graphpad InStat (version 3.00) for McIntosh (GraphPad Software, San Diego California USA). All the assays were repeated at least three times. Student's *t*-test was used to assess statistical differences between the two experimental groups. Additionally, to compare more than two groups were tested by one-way analysis of variance (ANOVA) followed by Tukey post hoc multiple comparisons analysis. The inhibitory concentration of 50% (IC_{50}) was determined by a nonlinear regression curve using the GraphPad Prism® version 5.0 statistical software package (from GraphPad Software). Antioxidant activities are expressed as IC_{50} and its 95% CI (95% confidence interval). IC_{50} represented the geometric mean of three independent experiments. Differences were considered as statistically significant when probability values (p) are less than 0.05.

3. Results

3.1. LC-PDA-ESI/MS analyses of VVLE

The characterization of the phenolic profile of the hydromethanolic extracts of VVL was carried out by the LC-ESI/MS technique using the negative mode. Thus, the main molecules detected with their quantification (retention times and *m/z* ratios) are summarized in Table 1. Four phenolic compounds were identified in both tested extracts of VVL using a UV detector. After analysis, LC-ESI/MS similar profiles were observed among the spontaneous ecotype and the cultivated variety. The major extracted-ion chromatograms of identified compounds in VVLE constitute a rich source of bioactive compounds, belonging to the flavonol family namely the glycosides of flavonoids are displayed as shown in Fig. 1a and b. All the samples revealed the presence of four quercetin glycosides among

them are quercetin-7-*O*-glucuronide (peak 1), quercetin-3-*O*-glucoside, quercetin-3-*O*- glucuronide (peak 3) and rutin. Peaks 1 and 3 showed the same $[M-H]^-$ ions at *m/z* 477 and based on their different retention times, these peaks were tentatively identified as quercetin-7-*O*-glucuronide and quercetin-3-*O*-glucuronide, respectively. Based on these results, we find that the predominant flavonol presented in high amounts in both tested extracts is quercetin-3-*O*-glucuronide. Indeed, we first noted that the concentration of quercetin-3-*O*-glucuronide (peak 3, $t_R = 14$ min) and quercetin-7-*O*-glucuronide (peak 1, $t_R = 14.7$ min) which was considerably higher in the leaf extract of wild ecotype compared with "Marsaoui" cultivated variety (23.32 ± 1.06 vs. 10.24 ± 0.12 mg/g DM, $p < 0.05$ and 2.92 ± 0.08 vs. 0.45 ± 0.10 mg/g DM, $p < 0.01$ by Student *t*-test). Additionally, the signal recorded at *m/z* 463 close to this of quercetin-3-*O*-glucoside ($C_{21}H_{20}O_{12}$) identified at 14.11 min with high composition for the extract of spontaneous ecotype "Nefza-I" than in those of cultivated variety "Marsaoui" (2.83 ± 0.03 vs. 1.48 ± 0.07 mg/g DM, $p < 0.01$). Peak 4, which presented a $[M-H]^-$ at *m/z* ratio 609 and at 13.4 min retention time by matching with their authentic standards, was identified and characterized as rutin (quercetin-3-*O*-rutinoside).

3.2. In vitro antioxidant activity

3.2.1. Total antioxidant capacity (TAC) by phosphomolybdenum test

The TAC of the two *Vitis vinifera* accessions differs significantly between genotypes (Table 2). The best TAC of about 507.12 mg EAA/g DM (95% confidence interval (CI): 507.83–511.34) is measured for the hydromethanolic leaf extract of the wild ecotype "Nefza I", while the cultivated variety "Marsaoui" shows the lowest antioxidant capacity 322.54 mg EAA/g DM (95% CI: 308.10–336.04).

3.2.2. DPPH radical scavenging assay

The results of the DPPH scavenging assay of leaf taxa are tabulated in Table 2. The IC_{50} of DPPH radical scavenging activity was significantly ($p < 0.001$) varied between the accessions and it was 50 μg/mL (95% CI: 47.15–61.90) for the hydromethanolic extract of leaf of "Nefza-I" ecotype and 370 μg/mL (95% CI: 352.15–389.06) for that of hydromethanolic extract of "Marsaoui" variety.

3.2.3. Reducing power

According to the results from Table 2, ferric reducing antioxidant power significantly varied between the genotype ($p < 0.01$). In addition, the results revealed that the wild ecotype was found to be the most active one in terms of reducing ability and consequently with the lowest IC_{50} value of 163.94 μg/mL (95% CI: 157.33–170.55). On the other hand, Marsaoui cultivated variety exhibited low efficiency with higher a IC_{50} value of reducing power assay (368.09 μg/mL, 95% CI: 353.51–382.68).

3.2.4. Ferrous ion chelating activity assay

Analysis of the ferrous chelating capacity of grapevine leaf extracts was revealed in Table 2. Again, the results followed a similar trend where the "Nefza-I" ecotype showed greater iron chelating activity compared with the "Marsaoui" variety (the values of the IC_{50} were respectively 18.55 μg/mL (95% CI: 16.90–20.36) and 42.80 μg/mL (95% CI: 40.94–44.75), $p < 0.01$).

3.3. Acute toxicity studies

During the observation period, the acute toxicity study indicated that hydromethanolic leaves extracts were found safe at examined doses of 75–480 mg/kg of mice. Significant variation in the behavior was not observed for 2 week study. Thus, the LD_{50} value of grapevine leaves extract was estimated to be more than 480 mg/kg *b.w.* To our knowledge, there are no published scientific work has so far been reported on the deleterious effects of *V. vinifera* leaves. Based on the results of the acute toxicity test, reasonable doses of 7.5, 15 and 30 mg/kg b.*w.* of VVLE which were below the 480 mg/kg b.*w.* were selected for the hepatoprotective activity test.

3.4. Protective effects of hydromethanolic extracts of VVL against CCl_4-induced liver injury in mice

As depicted in Table 3, AST, ALT, ALP, LDH and GGT levels in the serum of the CCl_4 treatment group were significantly higher than that in the normal control group ($p < 0.001$). Meanwhile, seven-day pre-treatment with hydromethanolic extracts of VVL to CCl_4-induced animals showed a significant ($p < 0.001$) reduction in all the liver marker enzyme concentrations in a dose-dependent manner

Table 1
Identification and content of quantified compounds in the hydromethanolic extracts of *V. vinifera* L. leaves using LC-UV-ESI/MS.

Peak	t_R (min)	m/z	Identity	Molecular family	Concentrations (mg/g DM)	
					accessions	
					"Nefza-I"	"Marsaoui"
1	14.7	477	Quercetin-7-*O*-glucuronide	Flavonoid	2.92 ± 0.08***	0,45 ± 0,10
2	14.11	463	Quercetin-3-*O*-glucoside	Flavonoid	2.83 ± 0.03***	1,48 ± 0,07
3	14	477	Quercetin-3-*O*-glucuronide	Flavonoid	23.33 ± 1.06***	10,24 ± 1,24
4	13.4	609	Rutin	Flavonoid	1.33 ± 0.15***	0,095 ± 0,02

DM = dry matter; t_R = retention time; *m/z* = mass/charge. Values of phenolic compounds are represented as means ± SD of three measurements and are statistically compared by unpaired two-tailed Student's *t*-test. ***$p < 0.001$ significantly different from "Marsaoui" variety.

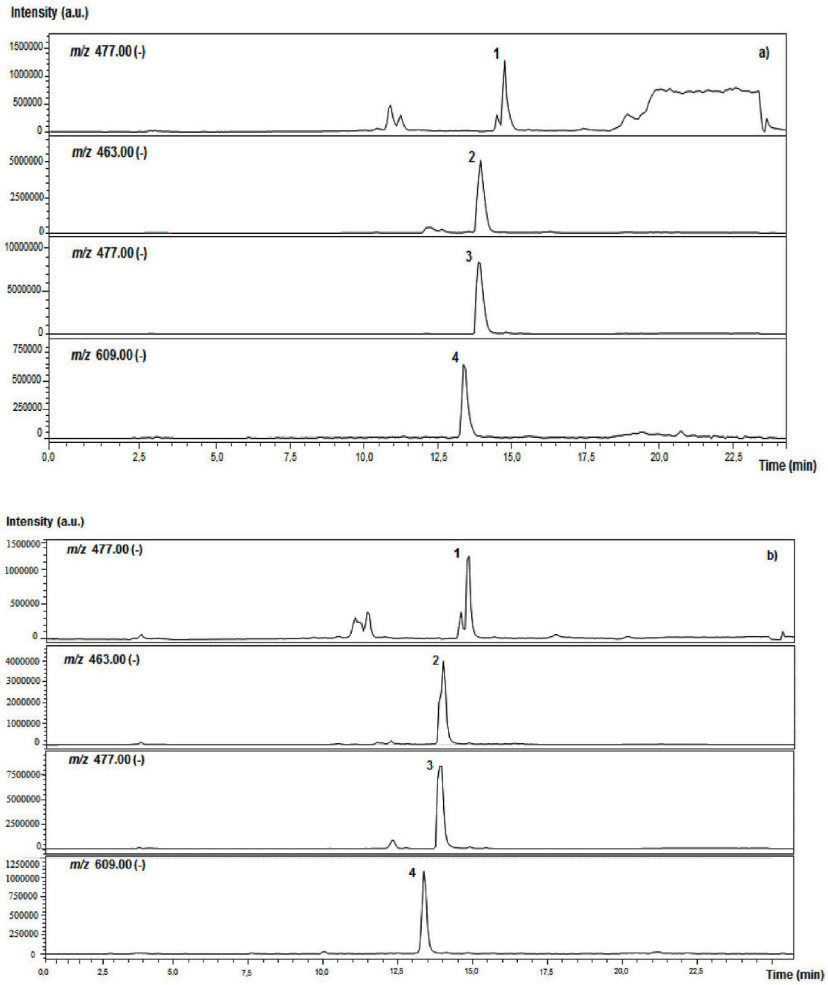

Fig. 1. LC-ESI/MS extracted-ion chromatograms of hydromethanolic extracts of *V. vinifera* leaves L. of wild ecotype "Nefza I"(a) and cultivated "Marsaoui" variety (b). Peak identifications were confirmed by retention times (t_R) and *m/z* ratios, as deduced from standard compounds. (1) quercetin-7-*O*-glucuronide; (2) quercetin-3-*O*-glucoside; (3) quercetin-3-*O*-glucuronide; (4) rutin.

Table 2
Antioxidant and free radical scavenging potential of VVLE *in vitro*.

Samples	Extraction yield (%)	Total antioxidant capacity (mg AAE/g DM)	DPPH IC_{50} (μg/mL) (95% CI)	Reducing power IC_{50}(μg/mL) (95% CI)	Iron chelating activity IC_{50} (μg/mL) (95% CI)
"Nefza-I"	14.30** (13.34–15.26)	507,12** (502,83–511,83)	50,32*** (47,15–61,90)	163,94**§ (157,33–170,55)	18,55**§ (16,90-20,36)
"Marsaoui"	10.50 ± 0.81 (9.69–11.31)	322,54 (308,10–336,04)	370,79§§§ (352,15–389,06)	368,09§§§ (353,51–382,68)	42,80§§ (40,94-44,75)
Ascorbique acide	–	–	32,49*** (28,32–36,92)	68,05*** (63,60–72,50)	6,66** (5,34-7,98)

VVLE = *V. vinifera* leaves extracts, DM = dry matter, AAE = ascorbic acid equivalent. Values were given as the mean of three parallel measurements (n = 3) ± SD. Data related for total antioxidant capacity are statistically compared by unpaired two-tailed Student's *t*-test. Data of IC_{50} are expressed as geometric mean and its 95% CI of three independent experiments. Comparison among the means of IC_{50} was analyzed by one way ANOVA followed by Tukey's multiple comparisons tests. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ significantly different from Marsaoui variety. $^{\S}p < 0.05$; $^{\S\S}p < 0.01$ and $^{\S\S\S}p < 0.001$ significantly different from standard (ascorbique acide).

irrespective of accession used. Interestingly, the effects of the groups pretreated with the wild "Nefza-I" ecotype extract were the most noticeable ($p < 0.05$, $p < 0.01$ and $p < 0.001$). "Nefza-I" ecotype extract (15–30 mg/kg) decreased the levels of AST, ALT, ALP, LDH and GGT in serum to 47.95 and 42.54; 50.50 and 43.08; 34.83 and 29.33; 714.15 and 652.54; 3.88 and 3.14, respectively, which did not show significant differences compared to those of normal control group. Moreover, only wild "Nefza-I" ecotype extract high dose (30

Table 3
Effects of pre-treatment with VVLE and silymarin on levels of serum biochemical indicators quantified in CCl_4-induced hepatotoxicity in mice.

Experimental groups	ALT (U/L)	AST (U/L)	Parameters ALP (U/L)	LDH (U/L)	GGT (U/L)
Normal control group	33.73 ± 5.70	42.72 ± 4.05	29.02 ± 2.99	632.75 ± 27.01	2.91 ± 0.57
CCl_4 group	130.50 ± 11.69***	113.51 ± 9.95***	91.89 ± 9.47***	1673.86 ± 48.76***	8.79 ± 0.51***
Silymarin (100 mg/kg)	50.16 ± 9.29*, ###	52.85 ± 6.94###	34.65 ± 5.43###	733.17 ± 58.76###	3.66 ± 0.52###
VVLE ("Nefza-I")					
7.5 (mg/kg)	75.25 ± 10.28***, ###	76.83 ± 7.08***, ###, **γ**	48.40 ± 5.91***, ###, **α**	1058.19 ± 93.89***, ###, **β**	6.05 ± 0.47***, ###, **β**
15 (mg/kg)	47.95 ± 6.08###, **β**	50.50 ± 3.89###, **β**	34.83 ± 5.34###, **α**	714.15 ± 59.94###, **α**	3.88 ± 0.73###, **γ**
30 (mg/kg)	42.54 ± 6.63###, **α**	43.08 ± 6.62###, **α**	29.33 ± 2.86###	652.54 ± 32.44###, **α**	3.14 ± 0.51###, **γ**
VVLE ("Marsaoui")					
7.5 (mg/kg)	87.30 ± 9.02***, ###	96.25 ± 5.61***, ###	62.65.± 8.08***, ###	1258.50 ± 86.80***, ###	7.17 ± 0.6***, ###
15 (mg/kg)	64.05 ± 8.36***, ###	60.08 ± 5.26**, ###	44.91 ± 7.12**, ###	804.55 ± 48.0**, ###	5.90 ± 0.66***, ###
30 (mg/kg)	52.73 ± 3.04**, ###	52.96 ± 4.19###	32.65 ± 5.43###	721.22 ± 50.88###	4.33 ± 0.54*, ###

CCl_4 = carbon tetrachloride, VVLE "Nefza-I" = *V. vinifera* leaves extracts from wild ecotype ("Nefza-I"), VVLE "Marsaoui" = *V. vinifera* leaves extracts from cultivated variety ("Marsaoui"), AST = aspartate transaminase, ALT = alanine transaminase, ALP = alkaline phosphatase, LDH = lactate dehydrogenase, GGT = gamma-glutamyl transpeptidase. Outcomes were depicted as means ± S.D per group of triplicate measurements, (n = 10). Data were subjected to one way ANOVA followed by Tukey's multiple comparisons tests.*$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ significantly different from normal control group. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ significantly different from CCl_4 group. $^{\alpha}p < 0.05$; $^{\beta}p < 0.01$ and $^{\gamma}p < 0.001$ significantly different from "Marsaoui" variety.

mg/kg) resulted in significantly lower ALT, AST, ALP, LDH, GGT serum levels than silymarin group values ($p < 0.05$).

3.5. Effects of hydromethanolic extracts of VVL in CCl_4-induced oxidative stress markers

Acute CCl_4-treated mice led to a significant increase of 56% in MDA concentrations ($p < 0.001$) compared to the normal control group (Fig. 2a). Compared with the CCl_4 group, pre-administration with various doses of VVLE of two accessions (7.5–30 mg/kg) significantly restored the antioxidant status of the oxidatively stressed mice, regardless of grapevine accession pre-treatment ($p < 0.001$). This effect appeared to be dose-dependent. Similarly, silymarin significantly reduced MDA level by 50% as compared to the CCl_4 treated group ($p < 0.001$). On the other hand, we could observe that pre-treatment with wild ecotype "Nefza-I" at 30 mg/kg resulted in a better reduction in the level of MDA than silymarin (0.44 ± 0.04 vs. 0.54 ± 0.03 nmol/mg protein, $p < 0.001$).

The levels of both enzymatic and non-enzymatic antioxidants (SOD, CAT, and GSH) were distinctly decreased in liver homogenate of CCl_4-induced mice when compared to the normal control group by 66.6, 56 and 58%, respectively ($p < 0.001$). In contrast, pre-treatment with VVLE significantly refurbished the levels of these enzymes in a dose-dependent manner as compared to the CCl_4-treated group (Fig. 2b–d). In the same manner, daily pre-treatment mice with silymarin (100 mg/kg), significantly ($p < 0.001$) reinstated the reduced SOD and CAT activities, which restored the GSH content comparable to that of the normal control group values. In addition, compared with the "Marsaoui" cultivar extract, the "Nefza-I" ecotype extract (irrespective of pre-treatment dose) more significantly enhanced the activities of SOD, CAT and GSH content ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively).

3.6. Effects of hydromethanolic extracts of VVL on histoarchitecture of liver

To explore the hepatoprotective effect of hydromethanolic extracts of VVL, H&E staining of liver tissues of control and treated mice was shown in Fig. 3. In the normal control group, liver tissue displayed normal architecture with liver lobules structure clearly visible revealing a vibrant central vein, a well-preserved cytoplasm and neat nucleus and distinct sinusoidal spaces (Fig. 3a). Conversely, in all animals treated with CCl_4, multiple histopathological changes manifested by severe hepatocellular degeneration and necrosis around the central vein, a dense inflammatory infiltrate in peri-portal and intra-lobular zones, and the destruction of the normal architecture of hepatic cells, the hepatic lobules were not clear, disrupted central vein, some hepatic sinusoids disappeared, the arrangement of hepatocytes was irregular as shown in Fig. 3b. The markers of pathological changes in the liver, for instance, necrosis, infiltration of inflammatory cells, and disappearance of the liver structure, were effectively ameliorated in the animal group receiving the reference drug i.e. silymarin (100 mg/kg), while a few dispersed cytoplasmic vacuolations still existing (Fig. 3c). However, compared with the CCl_4 group, pre-treatment of mice with VVLE markedly alleviated CCl_4-induced histopathological changes in the liver in a dose-dependent fashion (Fig. 3d–i). In the groups pretreated with a low dose (7.5 mg/kg) of VVLE of both accession, the liver sections presented histological changes, such as inflammatory infiltrate in peri-portal and intra-lobular zones (Fig. 3d and g).

4. Discussion

Studies highlighting the potential protective activities of herbal natural products and their bioactive compounds against hepatic injuries and their potent anti-inflammatory, and antioxidative properties, have attracted prominent attention [8]. This current study was designed to identify and quantify the phenolic compounds of hydromethanolic extracts of VVL, and to investigate their potential

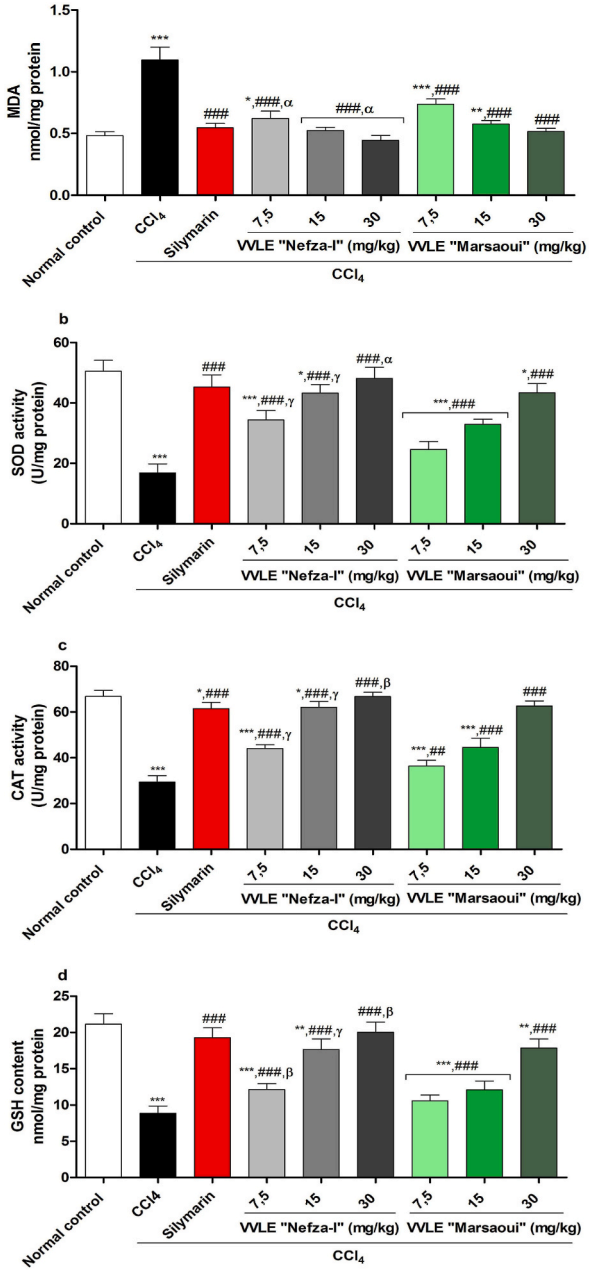

Fig. 2. Effects of pre-treatment with VVLE and silymarin on the antioxidant capacity of liver tissue in CCl_4-induced acute toxicity in mice. (a) MDA; (b) SOD; (c) CAT; (d) GSH. Outcomes were depicted as means ± S.D per group of triplicate measurements, (n = 10). Data were subjected to one way ANOVA followed by Tukey's multiple comparisons tests. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ significantly different from normal control group. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ significantly different from CCl_4 group. $^{\alpha}p < 0.05$; $^{\beta}p < 0.01$ and $^{\gamma}p < 0.001$ significantly different from "Marsaoui" variety.

hepatoprotective effects against acute experimental liver injury caused by CCl_4 in mice. Although the phenolic composition in leaves of some Tunisian autochthonous grapevines has been studied [12,13] and the incidence of bioactive phytochemicals was reported in the literature [31].

In the present study, the most common flavonols detected were quercetin-7-*O*-glucuronide, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, and rutin as reported previously [13,32]. A similar type of result was reported in a previous study in which the flavonols were the most abundant group, with quercetin-3-glucuronide (34.9 and 19.1 mg/g for Ahmar-Bou-Amar and Le Tizourine Bou Afraraet, respectively) and quercetin-3-glucoside (12.8 and 9.83 mg/g for Ahmar-Bou-Amar and Le Tizourine Bou Afraraet leaves extracts, respectively) standing out in both varieties from Algeria [33]. The two species of wild and cultivated autochthonous

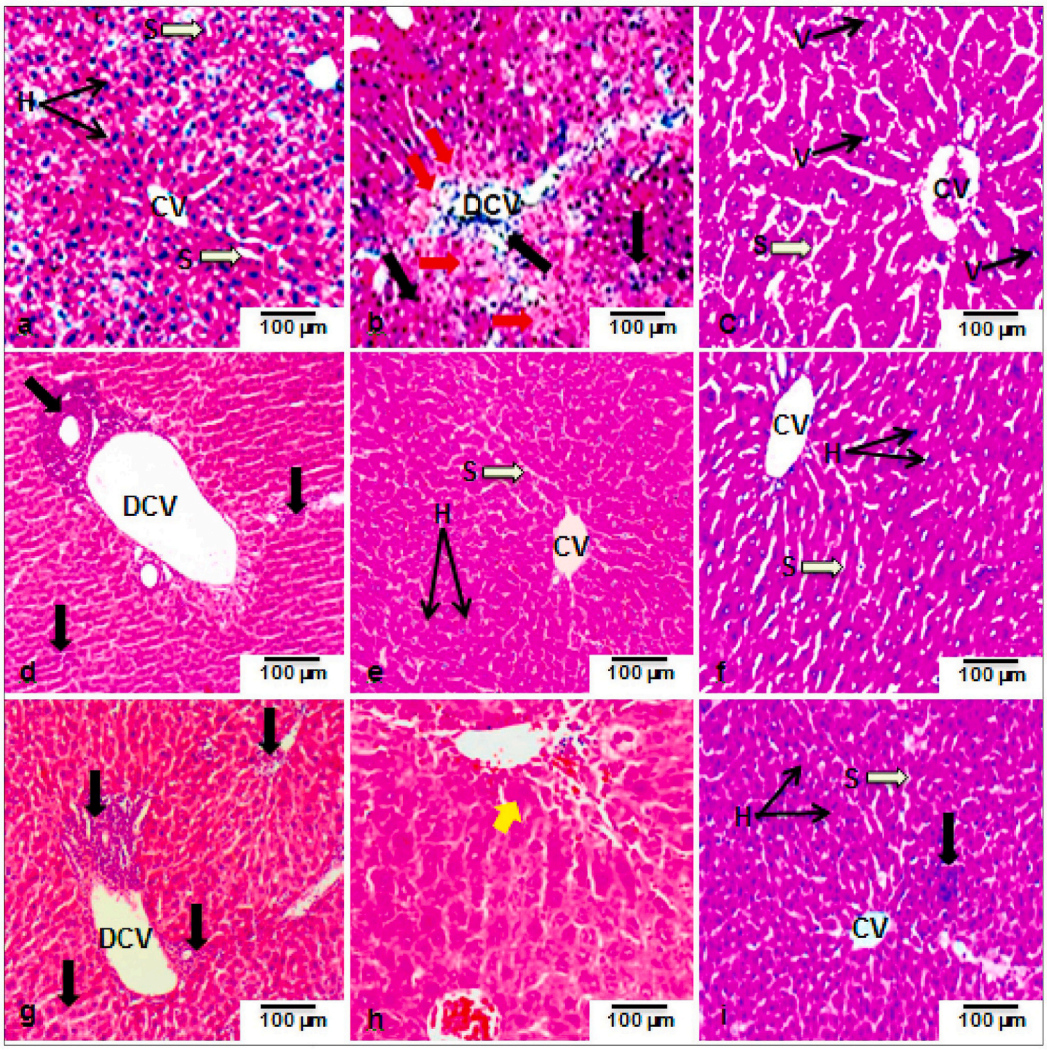

Fig. 3. Histologic evaluation of liver in the different experimental groups (H&E 200 ×), scale bar = 100 µm. (**a**): Normal control group showing normal cytoarchitecture of liver; (**b**): CCl_4-treated group showing loss of the normal liver architecture, disrupted hepatocytes, a dense inflammatory infiltrate in peri-portal and intra-lobular zones (black arrows) and necrotic areas (red arrows); (**c**): Silymarin treated group (100 mg/kg) protected hepatocytes from CCl_4-induced injuries, even though dispersed cytoplasmic vacuolations were still visible; (**d**, **g**): 7.5 mg/kg wild ecotype ("Nefza-I") and 7.5 mg/kg cultivated variety ("Marsaoui") showing dilated and damaged of central vein, inflammatory infiltrate in peri-portal and intra-lobular zones (black arrows); (**e, f**): 15 and 30 mg/kg wild ecotype ("Nefza-I") well protected the liver against CCl_4 injuries; (**h, i**): 15 and 30 mg/kg cultivated variety ("Marsaoui") although protected, still showed necrotic changes (yellow arrow) and slight inflammatory infiltrates (black arrow). CV: central vein; H: hepatocytes; DCV: damged central vein; S: sinusoid; (V) vacuolization.

grapevine contained similar flavonol constituents mainly quercetin glycosides. However, the amounts of these compounds differed among the both accessions, which were particularly abundant only in wild ecotype "Nefza-I" leaves grapevine ($p < 0.001$). Pariyar and co-workers revealed that the flavonoids in VVL identified by HPLC were flavonol derivatives *viz*. quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, which was the predominant ingredient of hydroethanolic extract of *Vitis labruscana* leaf harvested in North Jeolla province, South Korea [34], thereby consolidating our findings. Nevertheless, a similar pattern was observed in VVLE by HPLC technique, which was rich in flavonol compounds being quercetin-3-*O*-galactoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside and rutin [35,36]. Furthermore, earlier study conducted by Fernandes et al. revealed that glycosylated quercetin represent the most abundant active ingredients of different varieties of VVL from Portugal [37]. Also, the work of Fernandes et al. identified a coelution of the 2 peaks corresponding to quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside detected by HPLC-DAD recorded at 320 nm [37]. A similar observation was made in the work of Tacchini et al. which showed a coelution of 2 molecules detected at *m/z* 477 and 463 that corresponds to quercetin-3-*O*-β glucuronide and quercetin-3-*O*-β-glucopyranoside in the hydroethanolic extract of VVL of Lambrusco variety collected in Italy [38].

Usually, the antioxidant phytocompounds of VVL mainly flavonoid glycosides are the key to the antioxidant property. Herein, it has

been shown that hydromethanolic extracts of VVL have excellent antiradical and antioxidant capacity via various *in vitro* antioxidant assays based on the reduction process of samples (total antioxidant method (phosphomolybdenum), ferric reducing antioxidant power) and free radical scavenging activity of compounds (DPPH radical scavenging assay and chelation power on ferrous ions). In terms of total antioxidant capacity, findings indicated the highest activity was exhibited by leaves of wild ecotype "Nefza I". Previous literature study reported that aqueous extracts of VVL showed significant variability in the total antioxidant capacity of 20 Portuguese VVL varieties that ranged from 174 to 550 mg EAG/g DM [37], which are in agreement with our study reports. Indeed, our extracts showed higher total antioxidant capacity than the methanolic and aqueous extracts of VVL collected from the Mediterranean region of Turkey (47.22 and 33.17 mg EAA/g DM, respectively) [39]. In the DPPH free radical scavenging activity test, leaf extracts of "Nefza-I" exhibited similar $DPPH^{\bullet}$ radical scavenging activity to that found for VVLE of two varieties collected in northern Serbia with IC_{50} values were found to be in the order of 47.2 and 53.6 μg/mL respectively [40]. Katalinić et al. found DPPH radical scavenging capacity in VVL collected from Croatia with IC_{50} values of 61.69 and 70.32 μg/mL, respectively [41]. Our results are in agreement with those described by Fernandes and his co-researchers, who compared the DPPH radical scavenging abilities of VVLE of 20 Portuguese varieties and found IC_{50} values ranging from 96 to 191 μg/mL [37]. Recently, Pintać et al. found scavenging activity with IC_{50} values ranging from 130 to 382 μg/mL for methanolic extracts of VVL of eight varieties originating from Serbia [14]. In addition, our results showed that the radical scavenging activity of VVLE (wild and cultivated) was higher than that of the methanolic extracts of six Iranian native cultivars of VVL which have IC_{50} values between 3.16 and 9.41 mg/ml [42]. Also, Trindade et al. showed that the aqueous extract of *Vitis labrusca* leaves collected in northeastern Brazil exhibits antioxidant activity with an IC_{50} of 32.85 mg/mL [36]. Given Deng et al. the ethanolic extract from the aerial part of *Vitis thunbergii* collected from Taichung County, Taiwan showed an IC_{50} value around of 192.69 μg/ml [43]. Recently, Loizzo et al. showed that the hydroalcoholic extracts of VVL from six varieties native to southern Italy exhibited $DPPH^{\bullet}$ radical scavenging activity with IC_{50} values ranging from 7.19 to 77.88 μg/mL [11]. Opposed to our results, VVL from southern Tunisia studied by Aouey and co-workers, the ethanolic extract exhibited IC_{50} value of an 11.18 μg/mL [12]. The reducing power is associated with the ability of leaves extracts to reduce the ferric iron to ferrous (from Fe^{3+} to Fe^{2+}) from the reduction of the Fe^{3+}/ferricyanide complex and serves as an important indicator of antioxidant activity [44]. The observed ferric reducing antioxidant power shows that VVLE is efficient in reducing ferricyanide (Fe^{3+}) to ferrous a state (Fe^{2+}). It was close to that of aqueous extracts of VVL reported by Fernandes et al. [37] (the EC_{50} values ranging from 148 to 780 μg/mL) and higher than those of aqueous extracts of VVL indicated by Lima et al. [15] and Barreales et al. [45] (the EC_{50} values ranging from 371 μg/mL to 534 μg/mL and 397 μg/mL to 580 μg/mL, respectively). Among the transition metals ions such as iron are regarded as the most powerful lipid oxidation pro-oxidant, and are involved in the formation of ROS, such as hydroxyl radical ($^{\bullet}OH$) leading to increased lipid peroxidation via the Fenton reaction [46]. Regarding the Fe^{2+}-chelating ability assay, the same trend was observed, with the leaves extracts of wild "Nefza-I" grapevine ecotype expressing a better ferrous chelating ability compared to the leaves extracts of "Marsaoui" cultivar grapevine variety ($p < 0.01$), which might be attributed to the content of active compounds namely flavonol derivatives presented by this extract. This is in agreement with the finding of Mosbah et al. who demonstrated that the chelating ability of Tunisian *Feijoa sellowiana* leaves extract is mostly due to the presence of phenolic compounds [47]. Thus, the metal chelating capacity of the VVLE could be able to chelate Fe^{2+} and are clearly in agreement with the reduction of the MDA marker that was induced by Fe^{2+} overload. Many studies have indicated that bioactive antioxidant compounds can form a complex with transition metal ions and thereby stabilizing the oxidized form of the metal ion, rendering them unable to participate in metal-catalyzed hydroperoxide decomposition reactions or Fenton reactions [48,49]. The antioxidant activity of both accessions through the various methods may be related to their content of flavonol glycosides like quercetin-7-*O*-glucuronide, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide and rutin. The study performed by Anđelković et al. showed that VVL of the Vranac variety from Serbia had better antioxidant activity than Merlot, mainly because of the pronounced content of flavonol glycosides [40]. Also, a recent study of VVL from Serbia demonstrated that flavonol glycosides might be the main carriers of antioxidant activity [14]. In this way, our findings are generally in line with the published data. Overall, the VVL, especially the wild ecotype "Nefza-I" were more effective electron or hydrogen donators with strong antioxidant activities by their redox properties which act as radical scavenging, singlet oxygen quencher, reducing power and metal chelating potential. Hence the current results show that regardless of the assay being used, significant differences were obtained between the two autochthonous grapevine accessions. Altogether these data clearly show that VVLE from wild ecotype "Nefza-I" appear to be richer in flavonol (and in particular quercetin glycosides) that contribute to its strong antioxidant capacity. Previous literature showed that the chemical constituents of VVL depends on many factors including agro-climatic conditions, cultivation practices and degree of maturation, soil type, harvesting period and the plant genotype [11,15]. Notably, we herein found that genotype seems to be the most important factor influencing the phenolic content of these underused bioresources. Hence, genotype explains the variability in antioxidant capacities verified in VVLE from autochthonous Tunisian accessions. These *in vitro* results could make VVL an excellent candidate for prevention against the *in vivo* CCl_4-induced hepatotoxicity in mice. In our model, oral CCl_4-administration induced significant hepatopathy evidenced by elevated serum liver function parameters, including a noteworthy increase in ALT, AST, ALP, LDH and GGT activities in the serum by several times compared with those in the normal control group ($p < 0.001$). These signs provided solid evidence that when CCl_4 enters the body could cause liver damage, resulting in hepatic dysfunction, loss and rupture of hepatocytes' membrane functional integrity, thereby leading to accelerating the leakage of these biomarkers into the systemic circulation, which might be directly resulted from the induction of ROS by the action of CCl_4 administration [4]. Simultaneously, CCl_4 can lead to a disturbance in protein metabolism, demonstrating inflammatory reactions, necrosis, and apoptosis [8,50]. Our outcome corroborates previous reports in which CCl_4 increased the levels of serum biomarker enzymes in the blood of animals [1,8]. Importantly, serum biomarker enzyme activities were up-regulated in a dose-dependent manner after seven-day pre-treatment with hydromethanolic extracts of VVL irrespective of accession. This suggests that VVLE possesses hepatoprotective potentials, which ameliorated liver dysfunction and restored cell membrane function against CCl_4-injured hepatotoxicity in mice.

Therefore, the strongest effect was achieved with the extract of leaves of wild ecotype "Nefza-I", which was the closest to that of the normal control group, indicating that wild "Nefza-I" ecotype extract possessed a potent effect to reduce these biochemical alterations induced by CCl_4 than this of the "Marsaoui" cultivated variety. Previous studies have also confirmed the hepatoprotective effect of ethanolic, hydroalcoholic, and methanolic extracts of VVL against CCl_4 and ethanol-induced liver damage [16,51,52]. It has been well described that CCl_4, a typical environmental pollutant, can stimulate the generation of ROS and upregulate free radicals triggering oxidative stress and causing the destruction of cellular structures and development of acute liver injury [50,53]. In good line with previous results, herein, CCl_4 intoxication induced liver oxidative damage as evidenced by a significant elevation of MDA contents (end product of membrane lipid peroxidation), accompanied by diminished activities of the antioxidant enzymes (SOD and CAT) and GSH level in liver tissues. Orhan et al. [16], Pari and Suresh [51], and Amen et al. [52] used animal experiments to confirm that the ethanolic, hydroalcoholic, and methanolic extracts of VVL offered protection from acute liver injury in mice induced by ethanol and CCl_4, and the indexes of liver lipid peroxidation (MDA, SOD, CAT, and GSH) were close to those of the normal control group, and were better than those of the silymarin group. Experimental results of our study show that pre-treatment with hydromethanolic extracts of VVL of autochthonous accessions markedly prevented these changes in liver tissues in a dose-dependent manner. This finding can be explained by the elimination of MDA amount by both enzymatic and non-enzymatic antioxidants (SOD, CAT, and GSH) from pre-treatment of VVL in CCl_4-induced mice. All of these data suggest that VVLE could attenuate oxidative damage, via its capacity for free radical scavenging and antioxidation, which may be one of the pivotal mechanisms of VVL to protect the liver and justify its potential as an antioxidant agent against liver injury. Moreover, the leaves of wild ecotype "Nefza-I" was the most optimal, which indicates that wild ecotype "Nefza-I" extract effectively increased the antioxidant capacity in the liver tissue of mice with liver injury at all doses confused, reducing the oxidation of CCl_4 in the body by inhibiting liver damage and thus plays a hepatoprotective role. Based on the LC-ESI/MS data, VVLE contained four major flavonol glycosides (quercetin-7-*O*-glucuronide, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide and rutin), they can counteract the deleterious effects of CCl_4-induced acute liver injury in mice. These antioxidant phytoconstituents might be the cause of VVLE protective traits. Coincidentally with the results of this research, several previous studies have shown that flavonoid and their glycosides from VVL and those from other plants species exerted a protective effect on liver damage produced by hepatotoxic agents, via suppressing oxidative stress [52,54–56]. Similarly, these findings were consistent with the results of experimental studies of other researchers who showed that querectin glycosides such as quercetin-3-*O*-glucoside, quercetin-7-*O*-glucuronide has hepatoprotective activity against acetaminophen and lipopolysaccharide/D-galactosamine (LPS/D-GalN)-induced hepatocellular oxidative stress [57,58]. Furthermore, the hepatoprotective activities of quercetin-3-*O*-glucuronide were previously reported [59,60]. Finally, rutin and quercetin successfully ameliorated CCl_4-induced hepatotoxicity not only alleviating nitrosative stress but also downregulating the expression of cyclooxygenase (COX)-2 and nuclear factor kappa B (NF-κB), tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) in the liver tissue of mice and exerting an anti-fibrotic effect [61].

5. Conclusion

Taken together, the chemical profile showed that leaves of both considered accessions were found to be a rich source of flavonol glycosides mainly quercetin-3-*O*-glucuronide. Autochthonous wild "Nefza-I" ecotype yielded the highest amounts of most of the active molecules. Furthermore, our results indicated that the VVLE of wild autochthonous ecotype "Nefza-I" expressed considerable antioxidant activity compared with the "Marsaoui" cultivated variety based on multiple *in vitro* antioxidant assays. The biochemical and histopathological results clarified the role of VVLE in improving CCl_4-induced hepatotoxicity via suppressing oxidative stress. These results suggest that VVLE contain potential bioactive ingredients; quercetin-7-*O*-glucuronide, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide and rutin (quercetin-3-*O*-rutinoside) and other antioxidant constituents which may be responsible for protection against hepatic dysfunction induced by CCl_4. Therefore, our experimental results give evidence of the beneficial use of VVLE as a promising raw material for the development of hepatoprotective functional food. All these findings provide a theoretical foundation for better understanding the role of each component of this vital by-product to prevent liver injury caused by chemicals.

Author contribution statement

Nabil Saadaoui: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ahmed Mathlouthi: Performed the experiments.

Ali Zaiter, Safia EL-Bok, Moncef Mokni, Mounira Harbi, Néziha Ghanem-Boughanmi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Amadou Dicko: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Mossadok Ben Attia: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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