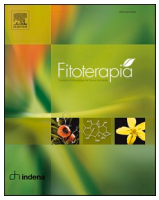
Fitoterapia 172 (2024) 105743

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote



Polyphenol-enriched extracts of *Sarcopoterium spinosum* fruits for counteracting lipid accumulation and oxidative stress in an *in vitro* model of hepatic steatosis

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ARTICLE INFO

Keywords:
Sarcopoterium spinosum fruit extracts
Corilagin
Phenolome profile
Anti-steatotic activity
Antioxidant acitvity

ABSTRACT

Sarcopoterium spinosum (L.) Spach is a Rosaceae shrub employed in the folk medicine in the Eastern Mediterranean basin. The previous few studies have focused on the *S. spinosum* roots, while the fruits have been poorly investigated. The present study aims to assess the biological properties of *S. spinosum* fruits collected in Lebanon and subjected to ethanolic, water or boiling water extraction. The extracts were compared for the phenol and flavonoid contents, and for the *in vitro* radical scavenging ability. The ethanolic extract (SEE) was selected and characterized by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS/MS) showing a phenolome rich in tannins (ellagitannins), flavonoids (quercetin derivatives), and triterpenes. The biological activity of SEE was tested on a cellular model of moderate steatosis consisting of lipid-loaded hepatic cells treated with increasing concentrations of SEE (1–25 μg/mL), or with corilagin or quercetin as comparison. In steatotic hepatocytes the SEE was able (i) to ameliorate the hepatosteatosis; *(ii)* to counteract the excess ROS and lipid peroxidation; *(iii)* to restore the impaired catalase activity. The results indicate that the ethanolic extract from *S. spinosum* fruits is endowed with relevant antisteatotic and antioxidant activities and might find application as nutraceutical product.

1. Introduction

Medicinal plants are a source of bioactive compounds and are largely investigated for functional food applications. Moreover, their study may help to direct and optimize the identification of new drugs or nutraceuticals [1]. The phenolic compounds (PCs) are phytochemicals characterized by the presence of phenolic rings and they are found in most edible plants. Although they are not classified as nutrients, their dietary intake provides health-protective effects. PCs are classified into phenolic acids, flavonoids, anthocyanins, and tannins [2] that are largely studied for their efficacy in preventing many diseases, including diabetes, obesity and cancer [3]. The interest for plant PCs depend on their wide availability, variety, and negligible side effects [4].

Lebanon notably boasts one of the highest densities of floral diversity in the Mediterranean basin as it includes various climates allowing the growth of many endemic valuable medicinal plants [5]. Rosaceae is one

Abbreviation list: ABTS, 2–2′-azino-bis(3-ethylbenzo-thiazoline-6-sulphonate); APAP, *N*-acetyl-para-aminophenol; BSA, Bovine serum albumin; CAT, Catalase enzyme; Cg, Corilagin; DCF, 2′-7′dichlorofluorescein; DCF-DA, 2′-7′dichlorofluorescin diacetate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; GAE, Gallic Acid Equivalents; LD, Lipid droplet; MDA, Malondialdehyde; NAFLD, Non-alcoholic fatty liver disease; OP, steatotic cells; PCs, phenolic compounds; QE, Quercetin equivalent; Qu, Quercetin; ROS, Reactive oxygen species; SBWE, Boiling water extract; SEE, Ethanol extract; SWE, Water extract; TA, Tormentic acid; TBA, Thiobarbituric acid; TBARS, Thiobarbituric acid reactive substances; TEAC, Trolox equivalent antioxidant activity; TG, Triglyceride; TPC, Total phenol content.

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https://doi.org/10.1016/j.fitote.2023.105743
Received 13 June 2023; Received in revised form 6 November 2023; Accepted 7 November 2023
Available online 10 November 2023

of the most important plant families worldwide, with >3000 members [6] which includes many plant fruits, such as apples, strawberries and almonds. *Sarcopoterium spinosum* (L.) Spach is an Eastern Mediterranean shrub being mentioned as a medicinal plant in a large number of ethnobotanical surveys [7]. Most studies on *S. spinosum* have been performed on the roots which are described to play anti-inflammatory [8], antidiabetic [9], and insulin-sensitizing [10] activities. However, in Lebanon, the folk medicine preferentially uses *S. spinosum* fruits for treating diabetes and for weight loss [5], also because fruits are extremely rich in phenolic compounds. This is the reason of our study on the extracts from *S. spinosum* fruits.

Non-alcoholic fatty liver disease (NAFLD) has emerged as a leading cause of chronic liver disease worldwide, with an estimated global prevalence of 25% in the adult population [11]. NAFLD describes hepatic fat deposition as part of the metabolic syndrome. NAFLD spectrum ranges from simple hepatic steatosis which may progress to non-alcoholic steatohepatitis with inflammation and hepatocellular ballooning, up to liver fibrosis, cirrhosis, and hepatocellular carcinoma [11]. Obesity, type 2 diabetes and dyslipidemia are leading risk factors for NAFLD [12]. To date, no effective pharmacological treatment is available for NAFLD, and the most effective approach is the weight loss through a balanced diet and appropriate physical activity. Therefore, the use of natural compounds for treating NAFLD and other metabolic disorders is currently gaining interest [13].

In the present study, we explored the alleged beneficial properties of the *S. spinosum* fruits from South Lebanon plants in terms of antisteatotic and antioxidant effects. After a preliminary characterization of three different extracts, we focused on the ethanolic extract to assess the phenolome, the lipid-lowering, antioxidant and cytoprotective activities using a cellular model of hepatic steatosis. The effects of the *S. spinosum* ethanolic extract were compared to those of corilagin (Cg) and quercetin (Qu) representing the most abundant and more promising PCs in the extract. Our findings seem to indicate that the ethanol extract showing significant *in vitro* antisteatotic and antioxidant effects could find nutraceutical applications for treatment of moderate hepatic steatosis.

2. Materials and methods

2.1. Chemicals

Reagents were purchased from Sigma-Aldrich (Italy) unless otherwise specified.

2.2. Plant materials and extract preparation

Sarcopoterium spinosum (L.) Spach fruits (Fig. 1) were collected from the wild in the Haddatha in South Lebanon, (Latitude: 33° 09′ 60.00“ N, Longitude: 35° 22’ 59.99”), during the August 2020. They were identified by Prof. George Tohme, a taxonomist president of Lebanese CNRS, and classified according to his book (Illustrated Flora of Lebanon, 2014) based on morphological and binocular analyses. A voucher specimen (R5.36) was deposited in the Lebanon National Herbarium at the Lebanese University, Faculty of Sciences. The fruits were dried at room temperature in the shade for three weeks. Then, 50 g of ground material were extracted with 1 L of ethanol (99%) for 3 h with agitation at room temperature. After ethanol evaporation at 42 °C in a rotary evaporator (Heidolph Instruments, Schwabach, Germany) we collected the dried pellet representing the SEE. Two different aqueous extracts were prepared by incubating 50 g of ground materials with either 1 L of distilled water at room temperature for 3 h with agitation (SWE) or with 1 L of distilled boiling water (100 °C) for 3 h with agitation (SBWE). Then, SWE and SBWE separetly were freeze-dried in an Alpha 1–4 LD plus lyophilizer (CHRIST, Osterode am Harz, Germany) to obtain the water-soluble extracts.

2.3. Phytochemical profiling of the extracts

Total phenol content (TPC) was quantified according to Folin–Ciocalteu method [14]. Briefly, 100 μL of sample (1 mg/mL) were mixed with 0.5 mL of 10% Folin–Ciocalteu reagent and incubated in the dark for 5 min. After addition of 1.4 mL of 10% Na_2CO_3 the samples were incubated for 30 min at room temperature. Absorbance (λ_{760}) was read with a UV-VIS spectrophotometer (Model U-2900, Hitachi High

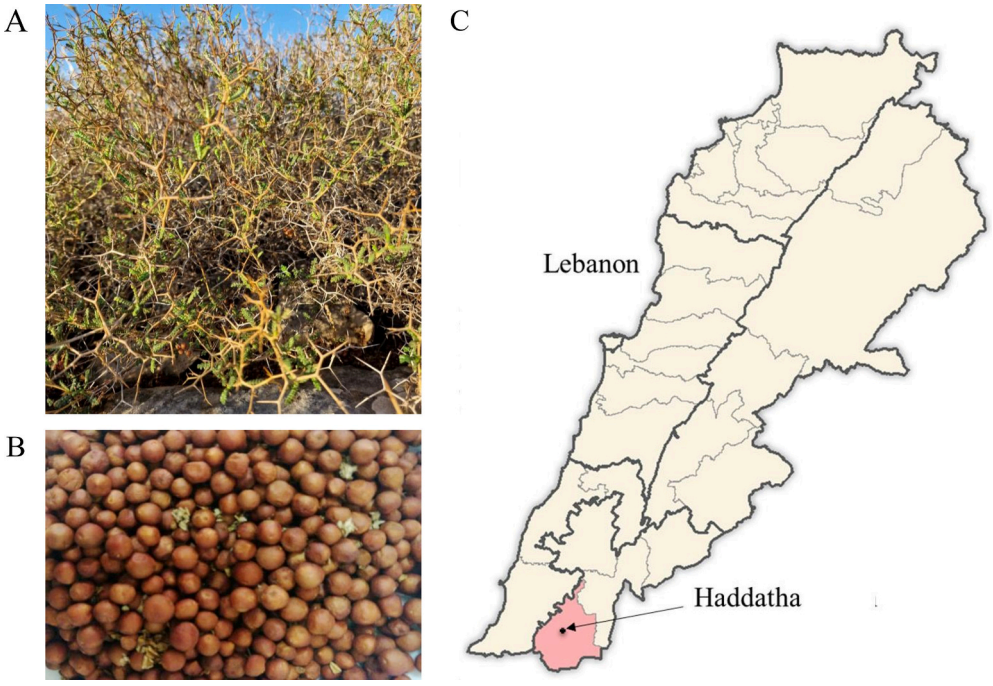

Fig. 1. Characteristics and distibution of Sarcopterium spinosum shrub.
S. spinosum plant in its natural environment: the shrub (A); the fruits (B); the region of the fruit collection: Haddatha, South Lebanon (C).

Technologies, Japan) against a blank. Gallic acid (0–100 μg/mL) was used as reference and results were expressed in mg Gallic Acid Equivalents (GAE) per gram dry extract.

Total flovonoid content (TFC) was quantified according to a standard method [15]. Briefly, 0.3 mL of sample (1 mg/mL) were mixed with 1.2 mL distilled water and 90 μL 5% $NaNO_2$. After incubation for 5 min in the dark at room temperature, 90 μL of 10% $AlCl_3$ were added. Then, 0.6 mL NaOH (1 M) and 0.72 mL distilled water were added and after 25 min the absorbance (λ_{510}) was read with a UV-VIS spectrophotometer against a blank. Quercetin dissolved in ethanol (5–100 μg/mL) was used as reference and results were expressed as mg Quercetin equivalent (QE) per g dry extract.

2.4. Protein quantification

The protein content of each sample was quantified by Bradford assay using bovine serum albumin (BSA) as a standard [16].

2.5. Antioxidant activity determination

The ABTS assay was performed according to R. Re [17]. Briefly, 200 μL of ABTS solution were added to 50 μL of sample (1–5000 μg/mL) and incubated at room temperature for 20 min in the dark. Absorbance (λ_{734}) was read against equal amount of ABTS solution using a UV-VIS spectrophotometer microplate reader (Model FLUOstar Optima, BMG Labtech). Trolox was used as a reference and reults were estimated as Trolox equivalent antioxidant activity (TEAC) (μg TE/mg dry extract).

The DPPH assay was performed according to W. Brand-Williams [18]. Briefly, 50 μL aliquot of sample (0–2 mg/mL) was mixed to 200 μL of DPPH solution (0.1 mM in methanol). After incubation in darkness for 30 min at room temperature, the absorbance (λ_{490}) was measured using a UV–VIS microplate reader against DPPH solution as a blank. Trolox was used as a reference and results were expressed as TEAC (μg TE/mg dry extract).

The FRAP assay was performed according to I.F.F. Benzie [19]. Briefly, 25 μL of the sample (0–2 mg/mL) were mixed with 175 μL of FRAP working solution (300 mM acetate buffer (pH 3.6), 20 mM ferric chloride) and 10 mM TPTZ (2,4,6- tri (2-pyridyl)—*S*-triazine) made up in 40 mM HCl. The three solutions were mixed (10:1:1 ratio, v:v:v). The mixture was incubated at 37 °C in the dark for 30 min and then the absorbance (λ_{593}) was read using a UV–VIS microplate reader against FRAP solution as a blank. Trolox was used as reference and results were expressed as TEAC (μg TE/mg dry extract).

2.6. HPLC-MS/MS analysis

The ethanolic exctract was analyzed by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS and MS/MS) using an Agilent 1100 HPLC-MSD Ion Trap XCT system, equipped with an electrospray ion source (HPLC-ESI-MS) (Agilent Technologies, Santa Clara, CA, USA). For HPLC-MS/MS analyses the ethanol extract was analyzed both as crude extract and after fractionation to improve the compound identification according to P. F. P. dos Santos [20]. Briefly, 1.7 g of the SEE was re-suspended in 200 mL water:methanol mixture (H_2O-MeOH, 8:2, *v*/v) and partitioned with 100 mL of hexane to obtain the free hexane-soluble extract (fSEE). The remained H_2O-MeOH solution was dried in rotary evaporator. The component separation of the SEE and fSEE was performed on a Jupiter C18 column 1 mm × 150 mm with 3.5 μm particle size (Phenomenex, USA). For eluents: water (eluent A) and MeOH (eluent B) were used, both added with 0.1% formic acid. The gradient applied was: 15% eluent B for 5 min, linear to 100% eluent B in 35 min, and finally, hold at 100% eluent B for another 5 min. The flow rate was set to 50 μL/min with a column temperature of 30 °C. The injection volume was 8 μL. Ions were detected in the positive and negative ion mode, in the 100–1000 *m/z* range, and ion charged control with a target ion value of 100,000 and an accumulation time of 300 ms. A capillary voltage of 3800 V, nebulizer pressure of 20 psi, drying gas of 8 L/min, dry temperature of 360 °C, and 3 rolling averages (averages: 5) were the parameters set for the MS detection. MS/MS analysis was conducted using an amplitude optimized time by time for each compound. Once the total ion current was obtained, the *m/z* signals with a significant signal-to-noise ratio (5:1) were extracted. Both full-scan and tandem spectra were acquired.

2.7. Cell culture and treatments

Rat hepatoma FaO cells (European Collection of Authenticated Cell Cultures-ECACC, Salisbury, UK) were cultured in Coon's modified Ham's F12 medium with low glucose (1.8 g/L), supplemented with 2 mM Glutamine and 10% Fetal Bovine Serum (Euroclone, Italy) at 37 °C in a humidified atmosphere with 5% CO_2. Stock solutions of SEE, corilagin (Cg) (MedChemExpress, USA) and quercetin (Qu) were prepared in DMSO with final concentration of 5 mg/mL. For treatments, cells were plated in cell culture dishes and grown for 24 h in high-glucose (4.5 g/L) medium with 0.25% BSA until reaching 80% confluence. Then, cells were exposed for 3 h to a mixture of oleate/ palmitate at a final concentration of 0.75 mM (2:1 M ratio) in order to mimic *in vitro* the effect of high-fat diet [21]. After exposure to oleate/palmitate mix, medium was changed and the steatotic cells were incubated for 24 h with the extract at different concentrations (1, 10, and 25 μg/mL). For comparison, steatotic cells were treated with either 10 μg/mL corilagin (16 μM) or 10 μg/mL quercetin (33 μM). Each experiment was performed at least in quadruplicate. The results represent the average of at least four independent experiments in triplicate. The potential cytotoxicity of the extracts, was evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test [22].

2.8. Quantification of the intracellular triglyceride content

At the end of each treatment, cells were scraped and lysed by passing cell suspension through a 25 gauge needle. Lipids were extracted using the chloroform/methanol method [23]. The TG content was quantified by adding 500 μL of Triglycerides liquid kit reagent (Sentinel Diagnostics, Italy) for 15 min at 37 °C. Absorbance (λ_{546}) was read with a UV-VIS spectrophotometer against a blank. Glycerol (0.007–0.2 μg/mL) was used as reference. The TG content was normalized to protein content and expressed as percent TG content relative to control.

2.9. Optical microscopy and morphometric analyses

Cells grown on coverslips were fixed with 4% paraformaldehyde and visualized both with absorption and fluorescence microscopy [24]. For absorption microscopy, Oil-RedO (ORO) staining was employed, and cells were stained with 0.3% ORO solution. Images are acquired by Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany). For fluorescence microscopy, LDs were stained with 1 μg/mL BODIPY 493/503 (Molecular Probes, Life Technologies, Italy), and DNA with 2 μg/mL Hoechst 33342 (Thermofiher Scientific, Italy). Images were acquired at 100× magnification by A1R Nikon inverted CONFOCAL microscope. All images were processed by using ImageJ software for quantification of lipid droplet size and number [25].

2.10. Quantification of ROS production

The production of ROS was quantified *in situ* using the oxidation of the cell-permeant 2′-7'dichlorofluorescin diacetate (DCF-DA, Fluka, Germany) to 2′-7'dichlorofluorescein (DCF) [26]. Cells were grown in plates, scarped and collected by centrifugation. Then, cells were loaded with 1 μM DCF-DA in PBS prepared from stock 10 mM stock prepared in DMSO for 30 min at 37 °C in the dark. Then, cells were centrifuged and resuspended in 2 mL PBS. The fluorescence was read with a LS50B

fluorimeter (Perkin Elmer, USA) at 25 °C using a water-thermostated cuvette holder (λex = 495 nm; λem = 525 nm). Results were normalized to protein content and expressed as percent flourescence intensity relative to control. The results represent the average of at least three independent experiments in triplicate.

2.11. Determination of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) assay allowed spectrophotometrically to determine lipid peroxidation which is based on the reaction of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA) [27]. Briefly, 250 μL of cell suspension was incubated for 45 min at 95 °C with 500 μL of TBA solution (0.375% TBA, 15% trichloroacetic acid, 0.25 N HCl). Then, 750 μL of N-butanol was added, centrifuged and the absorbance of the organic phase (upper phase)was measured at 532 nm in a UV-VIS spectrophotometer. Results were normalized to protein content and expressed as percent MDA levels relative to control

2.12. Determination of catalase activity

Catalase (CAT, EC 1.11.16) activity was evaluated in the 12,000 ×g supernatant of cell lysates following the consumption of hydrogen peroxide (H_2O_2) at 25 °C [28]. Briefly, 10 μL of the supernatant was added to 487 μL of phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, PH 7). Instantly after 3 μL of H_2O_2 (30%) was added to the sample the acquistion started for 3 min (acqusition was recorded every 15 s). The absorbance was read at 240 nm with a UV-VIS spectrophotometer at 25 °C using a Peltier-thermostated cuvette holder. Enzyme activity was calculated as μmoles of decomposed H_2O_2 per min/mg protein, presented as percentage values to controls and normalized for total proteins.

2.13. Statistical analysis

Data are means ± S.D. of at least three independent experiments. The differences among the groups were compared using a one-way analysis of variance (ANOVA) followed by Tukey's post-test (version 8.0, GraphPad Software, Inc., USA).

3. Results

3.1. Chemical characterization of three different S. spinosum extracts

S. spinosum fruits collected in South Lebanon (Fig. 1) to prepare the ethanol (SEE), water (SWE) and boiling water (SBWE) extracts. The three extracts were characterized for their phenol and flavonoid contents, and the radical scavenging activity. The extracts showed similar total phenol content (TPC) and total flavonoid content (TFC). In details, TPC ranged from 147.85 to 162.22 mgGAE/g dry extract, and TFC from 38.81 to 45.39 mgQE/g dry extract (Table 1).

The *in vitro* radical scavenging potential was tested using three different assays (Table 1). The ABTS assay gave the highest radical scavenging activity for SEE, with a TEAC (654.2 μg TE/mg) higher than SWE (420.94 μg TE/mg) and SBWE (373.80 μg TE/mg). Moreover, the radical scavenging activity of SEE was further assessed by the DPPH assay (549.5 μg TE/mg), the standard assay for hydrophobic compounds, whereas the radical scavenging activity of the aqueous extracts SWE and SBWE were assessed by the FRAP assay, which is appreciated for its rapid performance and high accuracy and showed similar activities for the two extrcats (312.3 ± 20 *vs* 312.0 ± 12 μg TE/mg, respectively). Based on these results we selected the ethanolic extract for further investigations.

Table 1
Phytochemical content and antioxidant ability of S. spinosum fruit extracts.

S. spinosum fruit	TPC mgGAE/g	TFC mg QE/g	ABTS μg TEAC/mg	DPPH μg TEAC/mg	FRAP μg TEAC/mg
Water (SWE)	158.44 (±33.1)	45.39 (±5.85)	420.94 (±37)	–	312.3 (±20)
Boiling Water (SBWE)	162.22 (±29.3)	41.33 (±4.24)	373.80 (±25)	–	312.0 (±12)
Ethanol (SEE)	147.85 (±36.7)	38.81 (±2.98)	654.2 (±65)	549.5 (±31)	–

Three extracts were prepared using different extraction solvents: water (SWE), boiling water (SBWE) and ethanol (SEE). Total phenolic content (TPC), total flavonoid content (TFC) radical scavenging ability (ABTS and DPPH) and ferric reducing antioxidant power (FRAP) were reported. ABTS, DPPH and FRAP were expressed as Trolox equivalent antioxidant capacity TEAC (μg TE/mg dry extract). Values are the mean ± standard deviation (S.D.) from at least three independent experiments in triplicate ($n = 3$).

3.2. HPLC-MS/MS analysis of the ethanolic S. spinosum extract

The phenolome of SEE was analyzed by HPLC-MS/MS in negative ionization mode. Fruits are rich in carbohydrates and fats that may form insoluble complexes with the PCs thus interfering with HPLC detection. Therefore, we preliminarily treated the crude extract by water/methanol mixture and hexane fractionation to eliminate most of the apolar molecules. By HPLC-MS/MS analysis we detected 24 peaks (Fig. 2A). By comparison with isolated standards and data in literature, at now, we could identify 17 major compounds (Table 2). Ellagitannins was the most abundant group of PCs in SEE (approximately 50.8% of the total extract). The ellagitannins identified in SEE were the following: the casuarictin isomer, the corilagin, the pedunculagin and the castalagin/vescalagin. A second abundant group of PCs was that of flavonoids, with the quercetin glucuronide and the quercetin-diglucoside being the main ones (about 7.5% of the total extract). Also triterpenes were present in high quantity in the SEE (about 11.9%). The main triterpenes identified were the tormentic acid its derivatives: 23-hydroxytormentic acid ester glucoside [M + HCOO]-, oxidized 23-hydroxytormentic acid ester glucoside [M + HCOO]-, gallic acid ester triterpenoid glycoside, oxidized 23-hydroxytormentic acid ester glucoside [M + HCOO]- isomer, di-oxidized 23-hydroxytormentic acid ester glucoside [M + HCOO], oxidized tormentic derivative, 23-hydroxytormentic acid. We wish to note that seven peaks could not be identified yet, due to lack of similarities for negative ionization segments and retention time in literature libraries.

3.3. The ethanolic S. spinosum extract counteracts lipid accumulation in hepatocytes

The biological effects of SEE were assessed on lipid-loaded FaO cells representing a reliable *in vitro* model for hepatic steatosis. First, by MTT assay we excluded any significant cytotoxicity of SEE at all the tested concentrations (1, 10, 25 μg/mL) (Fig. 2B). Then, the intracellular TG accumulation was quantified spectrophotometrically showing an increase (+158%, $p \leq 0.0001$) in the steatotic FaO cells (OP) compared to controls. The incubation of the steatotic cells with SEE for 24 h resulted in a significant decrease in the TG content at all the concentrations with maximal efficacy at the intermediate concentration (SEE 10 μg/mL) (−139% *vs* OP ($p \leq 0.0001$) (Fig. 3A).

For a deeper analysis, the morphometry of the cytosolic lipid droplets (LDs) was assessed using both absorption microscopy after ORO staining (Fig. 3B), and fluorescence microscopy after BODIPY 493/503 staining (Fig. 3C). While few and small LDs were detected in the control heaptocytes (about 9 LDs/cell, and 1.45 μm^2 average area), in the steatotic haptocytes (OP) we observed a significant ($p \leq 0.0001$) increase in both

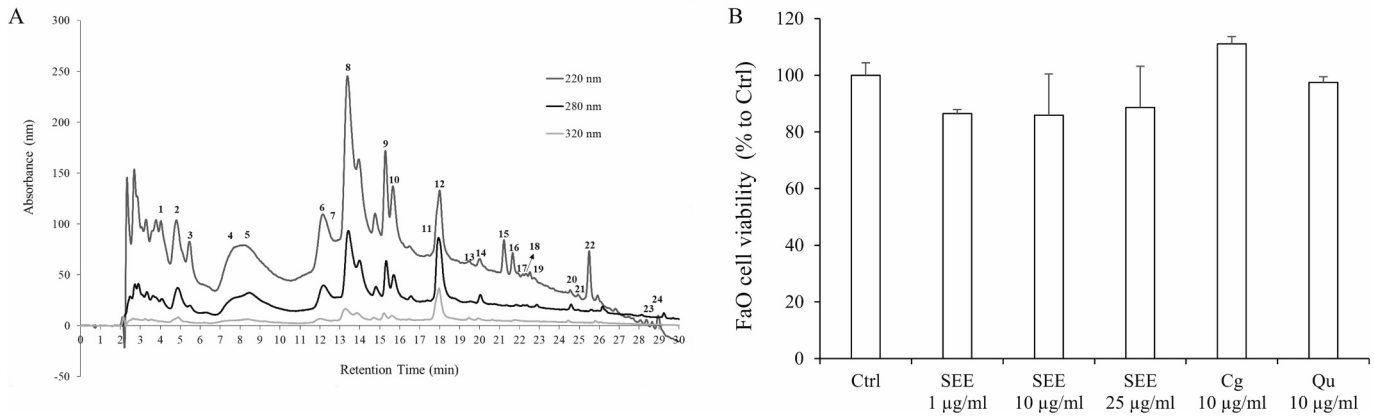

Fig. 2. HPLC-MS/MS analysis and cytotoxicity of the *S. spinosum* ethanolic extract.
The HPLC chromotograms (A) of the ethanolic extract of *S. spinosum* fruits recorded at two wavelengths (220 nm and 280 nm).
The identified peaks: 3 = Corilagin; 4 = Pedunculagin; 6 = Castalagin/Vescalagin; 8 = Casuarictin isomer; 9 = Casuarictin isomer; 11 = Quercetin derivative/ Quercetin -diglucoside; 12 = Quercetin glucuronide; 15 = 23-hydroxytormentic acid ester glucoside [M + HCOO]-; 16 = oxidized 23-hydroxytormentic acid ester glucoside [M + HCOO]-; 17 = Gallic acid ester triterpenoid glycoside; 18 = oxidized 23-hydroxytormentic acid ester glucoside [M + HCOO]- isomer; 19 = Di-oxidized 23-hydroxytormentic acid ester glucoside [M + HCOO]-; 20 = Tormentic acid derivative; 21 = oxidized tormentic acid derivative; 22 = 23-hydroxytormentic acid; 23 = Tormentic acid; 24 = oxidized tormentic acid. (B) cell viability after 24 h incubation of FaO cells with SEE different concentrations, Cg and Qu. Data are the mean ± S.D. of three independent experiments.

Table 2
Major components identified in *S. spinosum* fruit ethanol extract by using HPLC-MS/MS in the negative ionization mode.

#	RT (min)	[M-H]-	MS/MS fragments	Tentatively identified compound	Class
1	5.6	633.1	301/463	Corilagin	Ellagitannins
2	7.5	783.2	633/301	Pedunculagin	Ellagitannins
3	12.2	935	633/301 633/301/	Castalagin/Vescalagin	Ellagitannins
4	13.5	935	897 633/301/	Casuarictin isomer	Ellagitannins
5	15.4	935	897/783	Casuarictin isomer	Ellagitannins
6	15.8	625.3	301	Quercetin derivative/ Quercetin -di-glucoside	Flavonoids
7	17.9	477.1	301	Quercetin glucuronide	Flavonoids
8	21.4	711.3	503/665	23-hydroxytormentic acid ester glucoside [M + HCOO]$^-$	Triterpenoids
9	21.8	709.3	501/663	Oxidized 23-hydroxytormentic acid ester glucoside [M + HCOO]$^-$	Triterpenoids
10	22.4	817.4	655/697	Gallic acid ester triterpenoid glycoside	Triterpenoids
11	22.6	709.3	501/663	Oxidized 23-hydroxytormentic acid ester glucoside [M + HCOO]$^-$ isomer	Triterpenoids
12	22.8	707.3	499/661	Di-oxidized 23-hydroxytormentic acid ester glucoside [M + HCOO]$^-$	Triterpenoids
13	24.6	695.3	487/647	Tormentic acid derivative	Triterpenoids
14	25.0	693.3	485/645	Oxidized Tormentic acid derivative	Triterpenoids
15	25.6	503.2	485/471/ 453/441	23-hydroxytormentic acid	Triterpenoids
16	28.4	487.3	468/425/ 443/407	Tormentic acid	Triterpenoids
17	28.8	485.3	466/423/ 441/405	Oxidized tormentic acid	Triterpenoids

the number (18 LDs/cell) and the average size (3.22 µm^2, +83.48% *vs* control) of LDs (Fig. 3D and Fig. 3E,respectively). The exposure of the steatotic cells to SEE led to a slight reduction in the LD number, but a large reduction in the LD size. The maximal effect was observed at the intermediate concentration of SEE (10 µg/mL) which reduced both the LD number (to about 15 LDs/cell) and the average size to 1.98 µm^2 (−85.57%; $p \leq 0.0001$) compared to OP.

3.4. The ethanolic *S. spinosum* extract ameliorates oxidative stress in hepatocytes.

The ectopic TG accumulation in hepatocytes typically leads to excess ROS production that, in part, is counteracted by antioxidant enzymes, such as catalase, and in part triggers lipid peroxidation reactions leading to oxidative stress [29]. We quantified the intracellular ROS production, mainly hydrogen peroxide (H_2O_2), by *in situ* fluorometric analysis of DCF-stained cells (Fig. 4A). The DCF signal increased in steatotic hepatocytes (OP) compared to control (+31%; $p \leq 0.05$), and it was reduced after treatment with all the concentrations of SEE. The maximal efficacy was observed at the intermediate concentration (SEE 10 µg/mL) leading to a reduction of −48% *vs* OP ($p \leq 0.001$).

Regarding the antioxidant enzymes, we observed an impairment in the catalase activity in steatotic hepatocytes (OP) compared to control (−28.05%; $p \leq 0.0001$) (Fig. 4B). The catalase activity showed a recovery after treatment with the SEE both at the lower and intermediate concentrations (+26.26% and + 22.65% *vs* OP, respectively; $p \leq 0.001$ and $p \leq 0.01$).

Then we assessd the lipid peroxidation processes being triggered by the excess ROS. We quantified the MDA level by the TBARS assay (Fig. 4C) and we observed an increase in steatotic hepatocytes (OP) compared to control (+98%; $p \leq 0.0001$), and a significant decrease upon exposure to all the SEE concentrations. Also in this case the maximal efficacy was at the intermediate concentration (10 µg/mL) leading to MDA reduction of −121% compared to OP ($p \leq 0.0001$).

3.4. Biological activity of the single agents corilagin and quercetin

HPLC-MS/MS analysis showed that SEE is extremely rich in Cg and Qu, an ellegitannin and a flavonoid, respectively (Fig. 5). Therefore, we assessed the antisteatotic and antioxidant effects of these two PCs as single agents as comparison with the SEE. Also in this case any cytotoxicity of the compounds at the tested concentration was excluded by MTT assay (Fig. 2B). We selected the concentration of 10 µg/mL for the single agents because it resulted the most effective for all the studies on SEE.

When steatotic hepatocytes were incubated for 24 h with either Cg or Qu (10 µg/mL) we observed a significant decrease in the intracellular TG content for both compounds, but the effect was larger for Cg

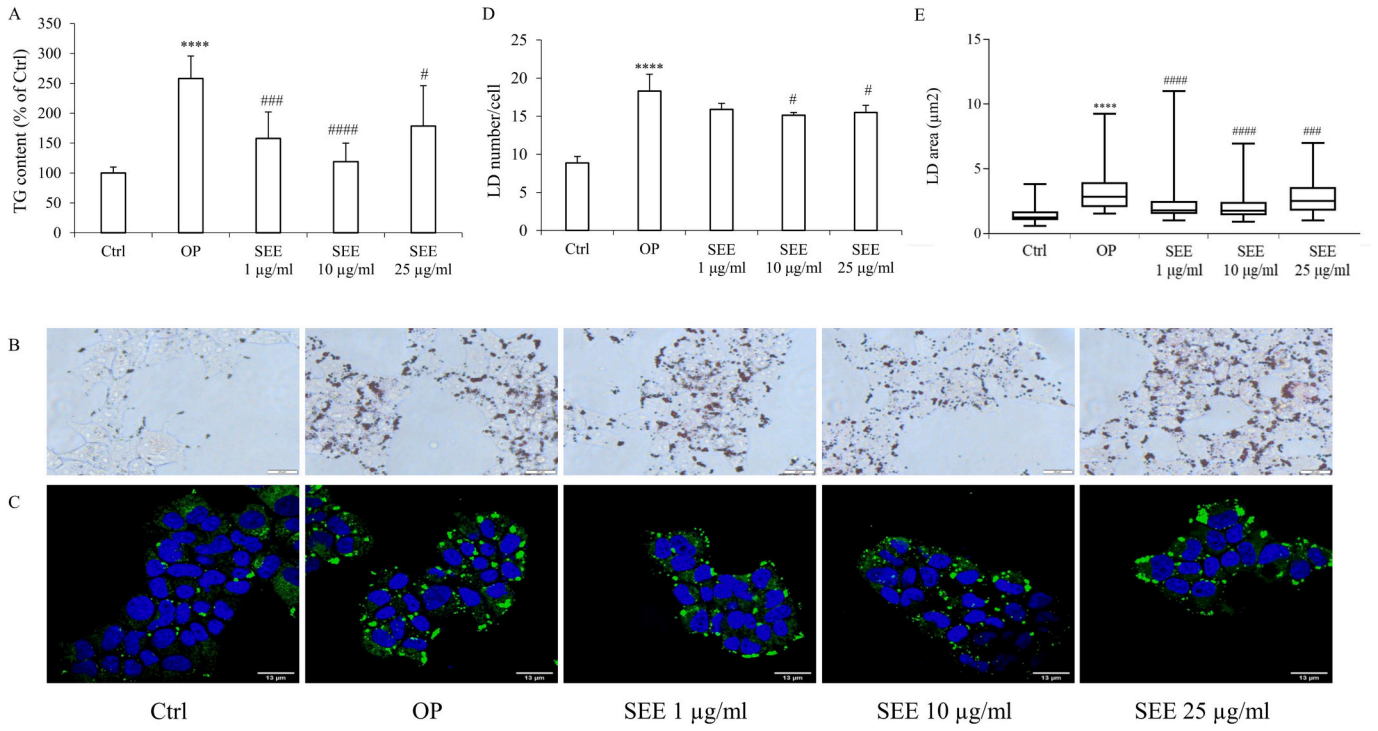

Fig. 3. Antisteatotic activity of the *S. spinosum* fruit ethanolic extract.
FaO cells were incubated in the absence (Ctrl) or the presence of oleate/palmitate (OP); then the OP cells were treated with different concentrations of *S. spinosum* ethanolic extract for 24 h. (A) The TG content expressed as percent relative to control after normalization for protein content. Representative images of FaO cells (B) after ORO staining and acquisition by Leica DMRB light microscope, and (C) after Hoechst/BODIPY 493/503 staining and acquisition by A1R Nikon inverted CONFOCAL microscope. Magnification 100×. ImageJ software was used for the image analysis to detect: (D) the number of LDs/cell and (E) the average size of LDs. Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups was assessed by ANOVA followed by Tukey's test. Symbols: Ctrl *vs* OP cells ****$p \leq 0.0001$; OP *vs* all treatments #$p \leq 0.05$, ###$p \leq 0.001$, #### $p \leq 0.0001$.

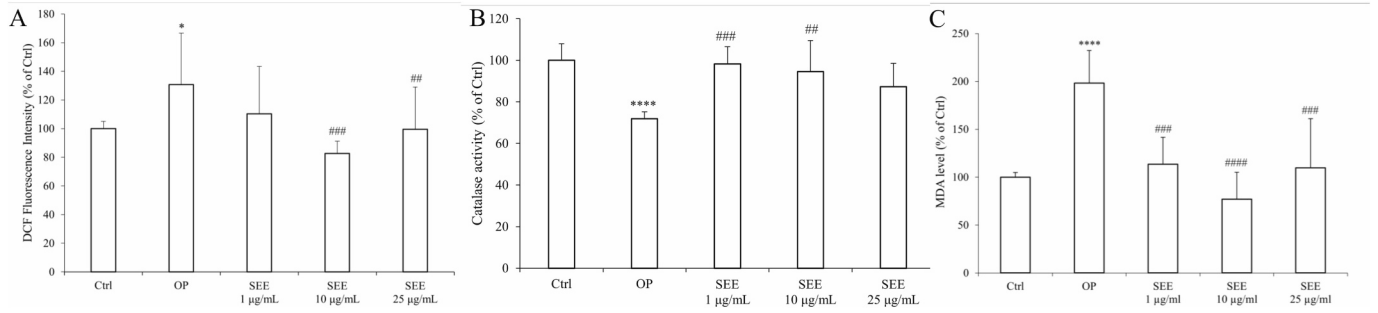

Fig. 4. Antioxidant activity of the *S. spinosum* fruit ethanolic extract.
FaO cells were treated as described above. (A) The intracellular ROS level was quantified by spectrofluorometric assay on DCF-stained cells and expressed as percent relative to control after normalization for total proteins. (B) The catalase activity was evaluated by spectrophotometric assay and expressed as percent relative to control after normalization for total proteins. (C) The intracellular level of MDA (pmol MDA/mL × mg of sample protein) was quantified by TBARS assay and expressed as percent relative to control after normalization for total proteins. ANOVA followed by Turkey's test was used to assess the statistical significance between groups. Symbols: Control *vs* OP, *p ≤ 0.05, ****p ≤ 0.0001; OP *vs* all treatments, ##$p \leq 0.01$, ###p ≤ 0.001, #### p ≤ 0.0001.

(−103.19%; $p \leq 0.01$) than for Qu (−67.91%; $p \leq 0.05$) (Fig. 5A) A significant reduction in both the number (Fig. 5C) and the size (Fig. 5D) of LDs was observed in ORO stained cells (Fig. 5B), confirming that Cg was more effective than Qu also in reducing the LD number (fom 9 to about 7 LDs/cell) and the LD size (form 0.7 µm^2 to 0.48 µm^2) with respect to OP.

The oxidative stress was quantified by DCF staining (Fig. 5E), and the TBARS assay (Fig. 5F). The DCF fluorescence intensity was reduced after treatment with both compounds, and the maximal efficacy was observed for Qu that led to a reduction of −31% compared to OP ($p \leq 0.0001$) (Fig, 5E). Aso the MDA level was significantly reduced by both compounds (Fig. 5F), but in this case the maximal efficacy was observed for Cg leading to a reduction of −143% compared to OP ($p \leq 0.001$).

4. Discussion

The present study reports the first characterization of the phenolome of an ethanolic extract from *S. spinosum* fruits, whereas the previous studies focused on the roots. Moroever, our findings showed for the first time the direct biological effect of *S. spinosum* fruits on hepatic cells, focusing on the lipid lowering and antioxidant activity.

In the Beduin traditional medicine, *S. spinosum* shrub is employed as antidiabetic [30] and anti-inflammatory [8] agent. The few scientific publications on *S. spinosum* focused almost exclusively on roots, which

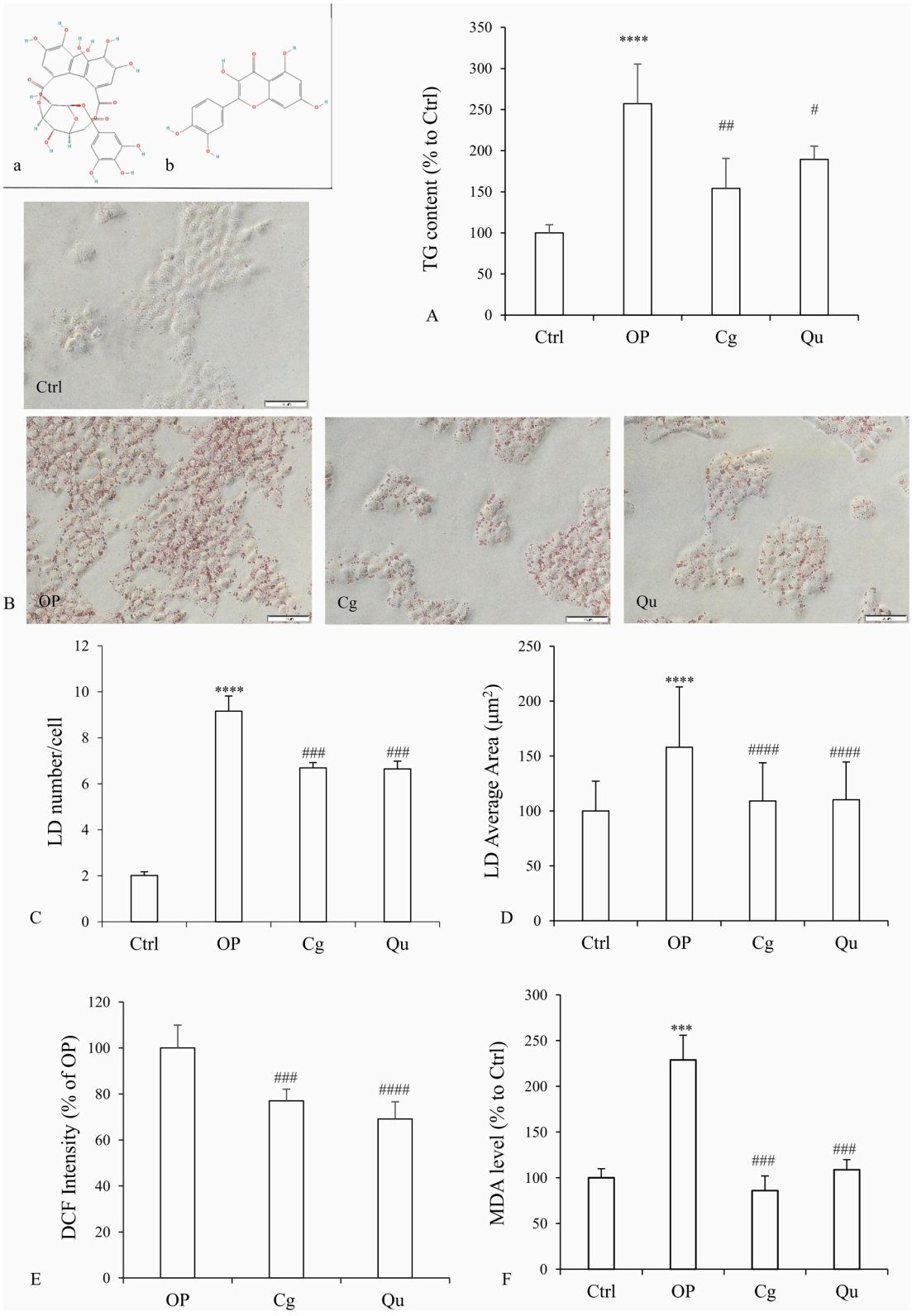

Fig. 5. Antisteatotic and antioxidant activity of Corilagin and Quercetin.
In the inset, the chemical formula of (a) corilagin and (b) quercitin (National Center for Biotechnology Information (2023). https://pubchem.ncbi.nlm.nih.gov/). The FaO cells treated as described above were analyzed for the bioligcal activity. (A) The TG content was quantified by spectrophotometric assay. (B) Representative images of ORO stained cells were captured by Leica DMRB light microscope and analyzed by ImageJ software to detect: (D) the number of LDs/cell and (E) the average size of LDs. (F) The ROS level was quantified by spectrofluorometric assay on DCF-stained cells. (G) The intracellular level of MDA (pmol MDA/mL × mg of sample protein) was quantified by TBARS assay. Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups was assessed by ANOVA followed by Tukey's test. Symbols: Ctrl *vs* OP cells ***p ≤ 0.001, ****p ≤ 0.0001; OP *vs* all treatments #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001, #### p ≤ 0.0001.

seemed to improve the insulin sensitivity and type 2 diabetes, prevented the development of hepatic steatosis in mouse models [9,10,31]. By contrast, the beneficial potential of fruits has not been elucidated yet, although edible fruits are among the best sources of dietary polyphenols [32]. At now, there is only one paper comparing the effects of fruits, leaves and roots of *S. spinosum* in adipocytes [33]. We wish to emhaisize that Lebanese people employ *S. spinosum* fruits more than roots for sustainibility reason, becuase leaves and stems were typically used for fire and cooking in the old times, and also because the preservation of the roots ensures the regrowth of the plant.

Typically fruits may be consumed after processing into functional products, including extracts, in order to increase the polyphenol content and improve the biological effects. Our study investigated *S. spinosum* fruits collected in South Lebanon and subjected to solvent extraction using either ethanol or water, at both room and boiling temperature, to mimic the traditional way of use. We did not observe significant differences among the extracts for their phenolic and flavonoid content, but the ethanolic extract showed the highest radical scavenging ability *in vitro*. Although usually the phenolic content of an extract directly correlates with the radical scavenging potential [34], our findings showed the best radical scavenger activity for the ethanolic extract without a significantly higher phenolic content. This can depend on the different pool of polyphenols contained in the different extracts, and on the level and type of their hydroxylation [35].

Once selected the *S. spinosum* ethanolic extract for further investigations, we characterized its phytochemical profile by HPLC-MS/MS. This represents the first characterization of the phenolome of *S. spinosum* fruits. The extract results extremely rich in polyphenols, and the most abundant group are the ellagitannins. Ellagitannins are the hydrolyzable subgroup of tannins that are known for their antioxidant and anti-inflammatory activities [36]. In our extract, the main ellagitannins identified are the following: corilagin known for its antidiabetic, antihyperlipidemic and antioxidant properties [37], casuaricitin being endowed with antioxidant and antiviral potential [38], pedunculagin with anti-inflammatory effects [39], and castalagin/vescalagin with antibacterial activity [40]. Also an appreciable quantity of flavonoids was detected in SEE, in particular two quercetin derivatives which were previously detected in *S. spinosum* [7]. Of note, the quercetin is well known for its lipid lowering and antioxidant effects [41]. Moreover, the SEE is also rich in triterpenes such as the tormentic acid and its derivatives; these bioactive compounds had been detected also in *S. spinosum* root extracts [42], and are known for their hypolipidemic and hepatoprotective effects [43]. In conclusion, the phenolome of the *S. spinosum* fruit extract shows large similarity with those of other Rosaceae [44].

Recently, western diet and sedentary lifestyle habits increased the prevalence of hepatic steatosis and NAFLD worldwide. A relaible cellular model of hepatic steatosis had been developed and widely employed by our group [23,24,45], and adopted for the present study. Our findings show that the ethanolic extract from Lebaneese *S. spinosum* fruits is able to counteract *in vitro* the moderate hepatic steatosis resulting from exposure of hepatic cells to excess of exogenous fatty acids. The lipid-lowering activity of the extract was sustained by its action on lipid droplets whose number and size were increased markedly in steatotic cells compared to controls, and decreased significanlty upon incubation with the extract. Interestingly, our results on a cellular model of hepatic steatosis are in line with previous studies showing the biological activity of an aqueous extract of *S. spinosum* roots on both a mouse model of fatty liver disease [46], and on diabetic mice [31]. Of note, while in mice with fatty liver, the root extract was able to improve insulin resistance and to prevent hepatic steatosis [46], in diabetic mice the extract improved glucose tolerance and insulin sensitivity, but did not reduce hepatic steatosis. The novelty of our approach is the demonstration that a polyphenols-enriched ethanolic extract prepared from *S. spinosum* fruits exterts a potent and direct action on steatotic hepatic cells thus indicating that the biological activity of the extract does not depend on the secondary metabolites derived from digestion and biotransformation preocesses.

As well known, excess hepatic fat depot leads to overproduction of free radicals and oxidant species resulting in a vicious circle leading to oxidative stress and inflammation. Indeed, both oxidative stress and the consequent lipid peroxidation are elevated in NAFLD patients [47]. The oxidative stress condition was observed also in our cellular model of moderate steatosis, where both the ROS levels and the lipid peroxidation increased markedly in steatotic hepatocytes. The *in vitro* free radical scavanger ability of SSE was confirmed also *in situ* at the cellular level thus sustaining the anti-oxidant potential of the extract. Indeed, in the steatotic hepatocytes, the fat-induced oxidative stress was counteracted by SEE which led to a significant reduction in both ROS and lipid peroxidation levels. Interestingly, catalase, the main antioxidant enzyme which decomposes the excess of H_2O_2, resulted impaired in the steatotic FaO cells, and this may be a sign of an unbalance in the anti-oxidant system due to the excees fat depot. The catalase activity was restored by the treatment with SEE. Of note, the published results on catalase activity are conflicting with some studies reporting a decrease [48] and others reporting an increase [49] depending on the models of NAFLD. Of note, our findings about the anti-oxidant and hepatoprotective ability of SEE well parallel a previous study reporting the hepatoprotective activity of a water/methanol extract from *S. spinosum* roots being able to reduce the oxidative stress in a model of hepatic damage in CCl4-insulted rats [50].

Based on the phenolome profile recorded by HPLC-MS/MS analysis and on the literature data, we focused on the ellegitannins (corilagin) and the flavonoids (quercetin) as the possible central players of the beneficial activity of the *S. spinosum* extract. Indeed, both quercetin [51] and corilagin [37] are well known for their beneficial effects. We assessed the hepatoprotective efficacy of both these PCs as single agents and compared them with the SEE. Both Cg and Qu played antisteatotic activity, but Cg was more effective than Qu in reducing the intracellular TG accumulation and in protecting the cell membrane from the lipid peroxidation. Interestingly, Qu was more effective in reducing the ROS production. Indeed, different polyphenols can influence different number of signaling pathways that have an impact on various cellular and tissue-level processes [52]. However, we want to note that the *S. spinosum* extract was more effective than both Cg and Qu in counteracting fat accuumulation and ROS production. Maybe this effects depends on synergistic or additive effects along the pool fo PCs being present in the crude plant extract [53].

In conclusion, a lot of epidemiological data provide evidence for the health benefits of diets rich in herbs, fruits and vegetables, mainly due to their bioactive constituents, including polyphenols endowed with well-documented potential to ameliorate the interconnected pathological processes of inflammation and oxidative stress [54]. The present study demonstrates the hepatoprotective effects of an ethanolic extract from *S. spinosum* fruits being rich in PCs and suggests that the ellagitannins and flavonoids that are abundant in the SEE may sustain the beneficial activity. These findings unveil the potential of this shrub to be employed as functional food or nutraceutical supplementary to counteract/improve the metabolic disorders associated to overnutrition, oveweight and NAFLD.

Credit author statement

All authors contributed to this work significantly. H. Zbeeb carried on cell cultures and treatments, spectrophotometric experiments, optical microscopy and participated in writing the original draft of the manuscript; G. Lupidi and M. Khalil perfomed the chemical characterization of the extracts and revised the manuscript. H. Khalifeh followed the fruit collection and prepared the extracts. L. Zeaiter carried on the fluorescence microscopy experiments; F. Baldini performed the fluorometric analyses and contributed to optical microscopy experiments. A. Salis and G. Damonte carried on the HPLC and mass spectroscopy analyses. L.

Vergani conceived and designed the study, analyzed, and elaborated data, and wrote the manuscript.

Funding

This work was supported by University of Genoa, Italy (FRA 2021 and 2022).

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.

Acknowledgements

We would like to thank Prof. Alberto Diaspro and the Nikon Imaging Center (Istituto Italiano di Tecnologia) for fluorescence microscopy acquisition.

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