Investigating the *in vitro* mode of action of okra (*Abelmoschus esculentus*) as hypocholesterolemic, anti-inflammatory, and antioxidant food

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ABSTRACT

Okra (*Abelmoschus esculentus*) have been introduced as food relatively recently in Europe. It is native to India and one of the most important vegetables in Nigeria. The leaves can be consumed but also the fruit is rich in nutrients and bioactive compounds (i.e., dietary fiber, vitamins, oils, polysaccharides, polyphenols) and several health promoting actions have been ascribed, including a lipid-lowering properties. In this work the effects of fruit and leaf extracts on expression of key mediators of cholesterol metabolism, i.e., the low-density lipoprotein receptor (LDLR) and proprotein convertase subtilisin/kexin type 9 (PCSK9), were investigated in human hepatoma cell line Huh7. Furthermore, effects on proinflammatory cytokines (IL-1β, IL-6 and TNF-α) expressed by THP1-derived macrophages were studied to assess potential anti-inflammatory actions.

Okra fruit extract significantly induced the mRNA and protein levels of the LDLR by 1.4 ± 0.3 and 4.8 ± 1.5-fold, respectively without any significant modification of PCSK9 expression. In addition, fruit extract showed a significant sequestering ability of cholic acid. Leaf butanol extract exerted similar action by inducing the expression of both the LDLR ($+3.1 \pm 1.6$-fold vs control) and PCSK9 ($+1.3 \pm 0.4$-fold vs control). The evaluation of the potential anti-inflammatory effect revealed a significant action of leaf butanol extract with reduced mRNA levels of IL-1β (-28 ± 8 % vs control), IL-6 (-11 ± 1 % vs control) and TNF-α (-43 ± 8 % vs control), while fruit extract did not show any anti-inflammatory activity. Finally, leaf ethyl acetate extract showed a significant antioxidant capacity comparable to ascorbic acid. Taken together, we provided evidence that leaf butanol extract and, more effectively, fruit extract induced the LDLR expression, effect that may explain the previously reported hypocholesterolemic action of okra. In addition, okra's extracts reduced the expression of pro-inflammatory cytokines from THP1-derived macrophages, an effect that may suggest a vascular protective action of okra.

1. Introduction

Okra (*Abelmoscus esculentus*) (Fig. 1) is a tropical vegetable native to India and one of the most important vegetables in Nigeria (Liu et al., 2021). The fruit have nice taste and is rich in polysaccharides, polyphenols, flavonoids, tannins, sterols and triterpenes (Adelakun, Oyelade, Ade-Omowaye, Adeyemi, & Van de Venter, 2009; Arapitsas, 2008; Liao, Dong, Shi, Liu, & Yuan, 2012; Savello, Martin, & Hill, 1980). The entire Okra plant is edible including the immature pods, which are very popular for their taste, and the leaves are commonly used as a green leafy vegetables like dandelion for cooking (Liu et al., 2021; Petropoulos, Fernandes, Barros, & Ferreira, 2018). Due to its composition okra

extracts have been used as additive. For example, okra gum can be used as emulsifier or stabilizer in beverage, okra seed powder can substitute for coffee following baking and grinding to obtain decaffeinated beverage (Ghori, Alba, Smith, Conway, & Kontogiorgos, 2014). Okra fruit composition is valuable being low in calories, rich in proteins, vitamins, minerals, and dietary fibers (Liu et al., 2021). Its high value as food and its potential health promoting properties have been recently reviewed (Petropoulos et al., 2018). Seeds are the part with larger protein content and in okra pods average crude protein value is 10–25 % on dry weight base (Liu et al., 2021; Petropoulos et al., 2018). Okra contains other relevant classes of constituents as polysaccharides tocopherols, fatty acids, chlorophylls, and important fraction is the

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Fig. 1. Pictures of Okra (*Abelmoscus esculentus*) leaf (left) and fruit (right).

mucilage (Liu et al., 2021; Petropoulos et al., 2018). The hydrolysis of *A. esculentus* mucilage yielded rhamnose, galactose, glucose, galacturonic and glucuronic acids but not arabinose (Woolfe, Chaplid, & Otchere, 1977).

Previously published data suggested that *A. esculentus* can elicit a significant hypoglycemic and hypolipidemic action. Consumption of 10 g of okra powder (approximately corresponding to 100 g of fresh okra fruit) blended in 150 g yogurt for 8 weeks resulted beneficial in modulating fasting plasma glucose, homeostatic model of assessment for insulin resistance (HOMA-IR), triglycerides (TG), total cholesterol (TC), and low-density lipoprotein-cholesterol (LDL-C) in type 2 diabetic patients (Moradi et al., 2020).

In the recent years the evaluation of the hypocholesterolemic activity of food derived natural products, such as red yeast rice (RYR) and phytosterols, allowed to develop a rational food-based approach for controlling hypercholesterolemia. The associated cardiovascular diseases (CVD) can be managed and partly controlled with healthy lifestyle, dietary inhabit, and a with complementary use of food supplements or nutraceuticals (Mach et al., 2020). Indeed, there is interest in foods with health promoting properties with hypocholesterolemic and hypoglycemic actions, and promising evidence have been reported on okra extracts (Chukwuma, Islam, & Amonsou, 2018; Fan et al., 2014; Moradi et al., 2020; Uddin Zim, Khatun, Khan, Hossain, & Haque, 2021; Wang, Chen, Ren, & Yang, 2014), but further research is needed to evaluate its composition, its mode of actions and safety profile.

Okra administered to male C57BL/6 mice fed hyperlipidemic diet, was able to induce cholesterol metabolism by CYP7A1 and inhibiting the *de novo* lipogenesis by a downregulation of sterol regulatory element-binding protein 1c (SREBP1c) and fatty-acid synthase (FAS) (Wang et al., 2014). These effects have been attributed to the fiber and polyphenol content of fresh okra pods (Wang et al., 2014). Similar results were confirmed in streptozotocin-treated rats, where the oral administration of okra produced a significant hypolipidemic effect (Erfani Majd, Tabandeh, Shahriari, & Soleimani, 2018). Same authors suggested a potential inhibition of PPAR-α and PPAR-γ in the pancreas (Erfani Majd et al., 2018), and the okra extract utilized in this study contained 141 mg gallic acid/g and 147 mg quercetin/g of dry extract, respectively (Erfani Majd et al., 2018). In an additional study, three-week administration of mucilage and peel-seed suspensions, at a dose of 150 mg/kg and 200 mg/kg in diabetic mice, was shown to significantly reduce TG, TC, LDL-C, and HDL-C (Uddin Zim et al., 2021).

Thus, okra can be evaluated as a health promoting food that might improve the control of cholesterol and glycemia. Up to now limited information is available about its chemical composition especially related to phytoconstituents with possible health promoting effects, furthermore leaves are poorly investigated regarding composition and their possible health beneficial actions. In the present study, we investigated the composition of okra fruit and leaf extracts and the effect on the expression of the two main regulators of cholesterol homeostasis, such as the LDLR and Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) in human hepatoma cell line Huh7. Furthermore, the potential anti-inflammatory effects were evaluated in THP1-derived macrophages, the antioxidant capacity of fruit and leaf extracts as well as the ability of fruit polysaccharide fraction to sequestrate cholic acid were finally assessed using *in vitro* chemical assays.

2. Material and methods

2.1. Fruit and leaf extraction

The Okra *(Abelmoscus esculentus)* plants were grown in Santa Cristina (Treviso, Italy) for this study 20 plants were used, and leaves (60 different leaves, three for each plant) and mature fruits (40 fruits two from each plant), soon after flowering, were collected. The fruits (5 Kg) were washed and then pulps were separated from the seeds, and final material was homogenised with water (500 g in 500 mL). The material was left to swell for 15 min. Then, methanol (500 mL) was added to the extract to precipitate carbohydrates and mucilage fractions. Samples were filtered obtaining a solid that will be indicated as "okra fruit precipitate", this material was freeze dried and stored at −20 °C until analysis. The collected liquids were concentrated to dryness using rotary evaporator under vacuum at 55 °C obtaining a brown powder (2.9 % yield, based on fresh fruit weight) that will be indicated as fruit extract. This extract was very soluble in DMSO > 20 mg/mL. Fresh leaves (350 g in 500 mL) were extracted in ultrasound bath using methanol and the liquid was then evaporated under vacuum yielding an extract that was a green sticky solid.

2.2. Leaf extract fractionation using liquid–liquid partition with ethyl acetate and butanol

Leaf extract was suspended in water and methanol (10:1) and subjected to liquid–liquid partition using solvents with different polarity, namely ethyl acetate and butanol. Organic layers were separated and dried with sodium sulphate and solvents were evaporated under vacuum yielding residue of ethyl acetate and butanol fraction resulting in yields of 0.3 % and 0.6 % respectively. The leaf extract, ethyl acetate leaf extract (EtAC) and butanol leaf extract (ButOH) were soluble in DMSO and were used for *in vitro* assays. Dried extracts were stored at −20 °C until further analysis.

2.3. HPLC-DAD-MS^n analysis

Fruit and leaf dried extracts (10 mg) were solubilised in methanol

(25 mL) using ultrasound bath and analysed using an HPLC-DAD-MS^n system. The HPLC-MS^n system consisted of an Agilent 1260 quaternary pump coupled to 1260 Agilent diode array detector (DAD) pump (Agilent Technologies, Santa Clara, CA, USA) and a Varian MS 500 mass spectrometer (Varian, Santa Clara, CA, USA) equipped with electrospray (ESI) ion. As stationary phase, Eclipse XDB C18 column (150 × 3 mm, 3.5 μm) (Agilent Technologies, Santa Clara, CA, USA), was used. As mobile phase, a mixture of 1 % formic acid in water (A), acetonitrile (B) was used, and gradient was as follows: 0 min, 5 % B; 30 min, 100 % B; 32 min, 100 % B; 32.5 min, 5 % B; 34 min, 5 % B. The flow rate was 0.4 mL/min. Injection volume was 10 μL and the column temperature was set at 30 °C. DAD allowed to collect chromatograms in the λ range of 200–640 nm. MS data were acquired both in positive and negative ion mode, in the *m/z* range 100–2000. Fragmentation pattern of most intense ion species was obtained using the turbo data depending on scanning (TDDS) function of the instrument. MS parameters were as follows: needle voltage, 4.9 kV; shield voltage, 600 V; capillary voltage, 80 V; RF loading, 80 %; nebulizing gas pressure, 1.73 10^5 Pa (nitrogen); drying gas pressure, 1.04 10^5 Pa; drying gas temperature, 300 °C. Identification of compounds was obtained based on comparison with the literature and reference compounds, when available. For compounds quantification, quercetin-O-glucoside, chlorogenic acid, were used. Standard solutions were prepared in the concentration ranges 1–100 μg/mL and calibration curves were built.

2.4. Sugar content

10 mg of okra fruit extract and okra fruit precipitate samples were extracted with 10 mL of water in a heated bath (37 °C) for 30 min. Samples were centrifuged at 13.000 rpm (centrifuge radium 10 cm) for 10 min and supernatant was used for LC analysis. For the analysis of sugar, an HPLC system coupled to evaporative scattering detector (ELSD) was used. The chromatographic system was composed by an Agilent 1100 HPLC pump coupled to a SEDERE Sedex 60 LT ELSD (Sedere, Orleans, France). An Agilent HI-PLEX Ca^{2+} column was used as stationary phase, while water was used as mobile phase, maintained at isocratic conditions for 25 min at a flux of 0.6 mL/min. Stationary phase was maintained at 80 °C. ELSD parameters were as follows: gain, 10 AU; drying gas pressure, 2.3 10^5 Pa; evaporative gas temperature, 60 °C. Sugar in okra samples were quantified using calibration curves of glucose, fructose, and sucrose, built by standard solution obtained in water at concentration ranges 10–100 μg/mL.

2.5. In vitro binding of bile salt assay

The assay for *in vitro* binding of bile salt was performed using a method previously published (Goel et al., 1998) and partly revisited allowing to measure the sequestration capacity of okra fruit precipitate. 50 mM potassium phosphate buffer was prepared adjusting the pH≃6.5 to simulate the intestinal fluid conditions. A stock solution of cholic acid in methanol was prepared at 8.5 mg/mL. 1 mL of this solution was diluted 1:5 with the phosphate buffer to a final volume of 5 mL. Then aliquots of okra precipitate extract (100–400 mg) were added while control solution received the same amount of solvent but without okra precipitate and were transferred into 15 mL falcon tubes. The resulting solutions were vortexed and allowed to stand for 2 h in a thermostatically controlled bath at 37 °C while stirring to simulate body temperature and peristaltic movements. After 2 h, all falcons were centrifuged, the supernatant from each was further diluted 1:50 with buffer solution for the HPLC analysis.

For the analysis of cholic acid, an HPLC system coupled to evaporative scattering detector (ELSD) was used. The chromatographic system was composed by an Agilent 1100 HPLC pump coupled to a SEDERE Sedex 60 LT ELSD. As stationary phase Agilent Eclipse XDB C18 column (150 × 4.6 mm, 3.5 μm), was used. As mobile phase, a mixture of 1 % formic acid in water (A) and acetonitrile (B) was used, and gradient was

as follows: 0 min, 20 % B; 12 min, 100 % B; 16 min, 100 % B; 20 min, 20 % B. The flow rate was 1.0 mL/min. Injection volume was 10 μL and the column temperature was set at 30 °C. ELSD parameters were as follows: gain, 10 AU; drying gas pressure, 2.2 10^5 Pa, evaporative gas temperature, 60 °C. Quantitative analysis of cholic acid was obtained by calibration curves obtained in concentration ranges 10–100 μg/mL. The decrease of the free cholic acid in the solution after adding of okra samples was expressed as % with respect to initial amount.

2.6. Folin-Ciocalteau assay

Total phenolic amount in different extracts was determined by spectrophotometric method using Folin-Ciocalteau reagent, as previously described (Baldan et al., 2017). Results are expressed as mg/g of gallic acid equivalents (GAE).

2.7. DPPH assay

The free radical scavenging ability of extracts against DPPH (1,1-diphenyl-2 picrylhydrazyl) free radical was evaluated using a previously described protocol (Baldan et al., 2017). The results were expressed as IC$_{50}$ (μg/mL).

3. *In vitro* experiments

3.1. Reagents

Eagle's minimum essential medium (MEM), trypsin-EDTA, penicillin, streptomycin, sodium pyruvate, ʟ-glutamine, nonessential amino acid solution, fetal bovine serum (FBS), plates, and Petri dishes were purchased from EuroClone (Pero, Milan, Italy). Okra extracts were dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 100 mg/ml. Simvastatin was obtained by Merck (Darmstadt, Germany) and was dissolved to a stock concentration of 50 mM in 0.1 M NaOH, and the pH was adjusted to 7.2 according to manufacturers. The solution was then sterilized by filtration. Quercetin was purchased from Merck (Darmstadt, Germany) and dissolved in DMSO as a stock solution of 10 mM.

3.2. Cell cultures

Human hepatic cancer cells (Huh7) were cultured in MEM supplemented with 10 % Fetal Bovine Serum (FBS), 1 % ʟ-glutamine 200 mM, 1 % sodium pyruvate 100X, 1 % nonessential amino acids 100X, and 1 % penicillin/streptomycin solution (10.000U/mL and 10 mg/mL, respectively), at 37 °C in a humidified atmosphere of 5 % CO$_2$ and 95 % air. For the experiments, cells were incubated with indicated final concentrations in MEM/10 %FBS. The final concentration of solvent (DMSO) did not exceed 0.5 % v/v, and the same amount was added to all the experimental points in each assay.

THP-1 monocyte cells were cultured in RPMI media supplemented with penicillin (10,000 U/mL), streptomycin (10 mg/mL), nonessential amino acid, 10 % (FCS and β-mercaptoethanol 0.05 mM. To obtain THP-1 macrophages, THP-1 monocytes were seeded in appropriate multiwell plate and treated with Phorbol 12-myristate 13-acetate (PMA; 3.2 × 10^{-7} M) for 72 h. Then, macrophages were treated for 24 h with PCSK9 0.25, 0.5, 1 and 2.5 ug/ml, using TNF-α 10 ng/ml as positive control.

3.3. Cell viability assay of Huh7

Cells were seeded in MEM/10 % FBS in a 96-well tray at the cellular density of 8.000 cells/well. The day after, treatments were added, and after 72 h the cell viability was evaluated by the sulforhodamine assay (SRB), as previously described (Rimoldi et al., 2017).
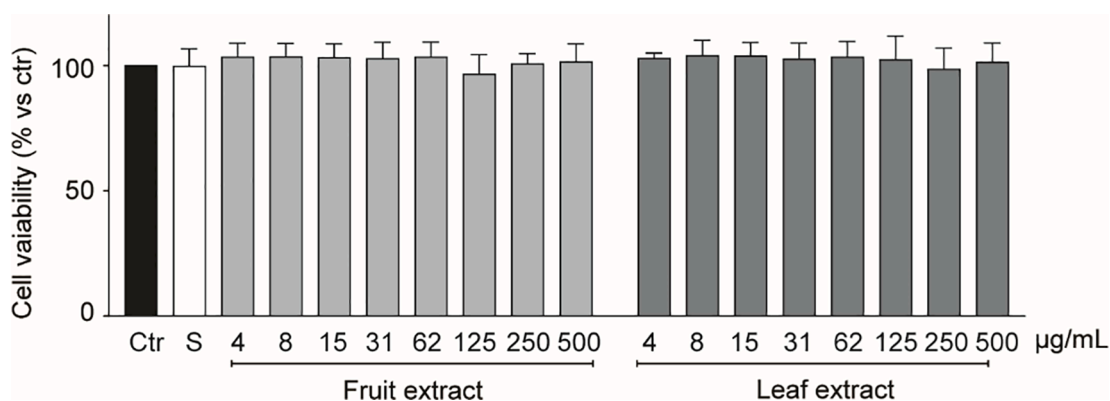
Fig. 2. Cytotoxicity assay of leaf and fruit extracts from okra in Huh7 cell line. Ctr: Control; S: simvastatin (5 μM).

3.4. Western blot analysis

Huh7 cells were seeded in MEM/10 % FBS in a 6-well tray at the cellular density of 300.000 cells/well. The day after, the medium was replaced with the compounds/extracts at the indicated concentrations in DMEM/10 % FBS. After 72 h of incubations, intracellular protein content was extracted in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, and 1 % Nonidet-P40, containing 1 % v/v of protease and phosphatase inhibitor cocktails). The protein content was evaluated using a Bicinchoninic Acid Assay (BCA) assay, using bovine serum albumin (BSA) as standard. Protein samples (25 μg) and a molecular mass marker (Thermo Scientific, Rodano, Italy) were separated using 4 %–20 % SDS-PAGE (Bio-Rad, Segrate, Milan, Italy) under denaturation and reduction conditions. The protein samples were then transferred to a nitrocellulose membrane using the trans-Blot® Turbo™ Transfer System (Bio-Rad, Segrate, Milan, Italy), and nonspecific binding sites were blocked with a 5 % nonfat dried milk tris-buffered tween 20 (TBS-T20) solution, in agitation for 60 min at room temperature. The blots were incubated overnight at 4 °C with a diluted solution (5 % nonfat dried milk) of anti-LDLR (rabbit polyclonal antibody, GeneTex GTX132860; dilution 1:1000), anti-PCSK9 (rabbit polyclonal antibody, GeneTex GTX129859; dilution 1:1000), anti-GAPDH (rabbit polyclonal antibody, GeneTex GTX100118; dilution 1:3000), purchased from GeneTex (Irvine, USA). The membranes were washed with TBS-T20 and exposed for 90 min at room temperature to a diluted solution (5 % nonfat dried milk) of the secondary antibodies (peroxidase-conjugate goat anti-rabbit and anti-mouse, Jackson ImmunoResearch (Cambridgeshire, United Kingdom), dilution 1:5000, cod. 111–036-045 and 115–036-062, respectively).
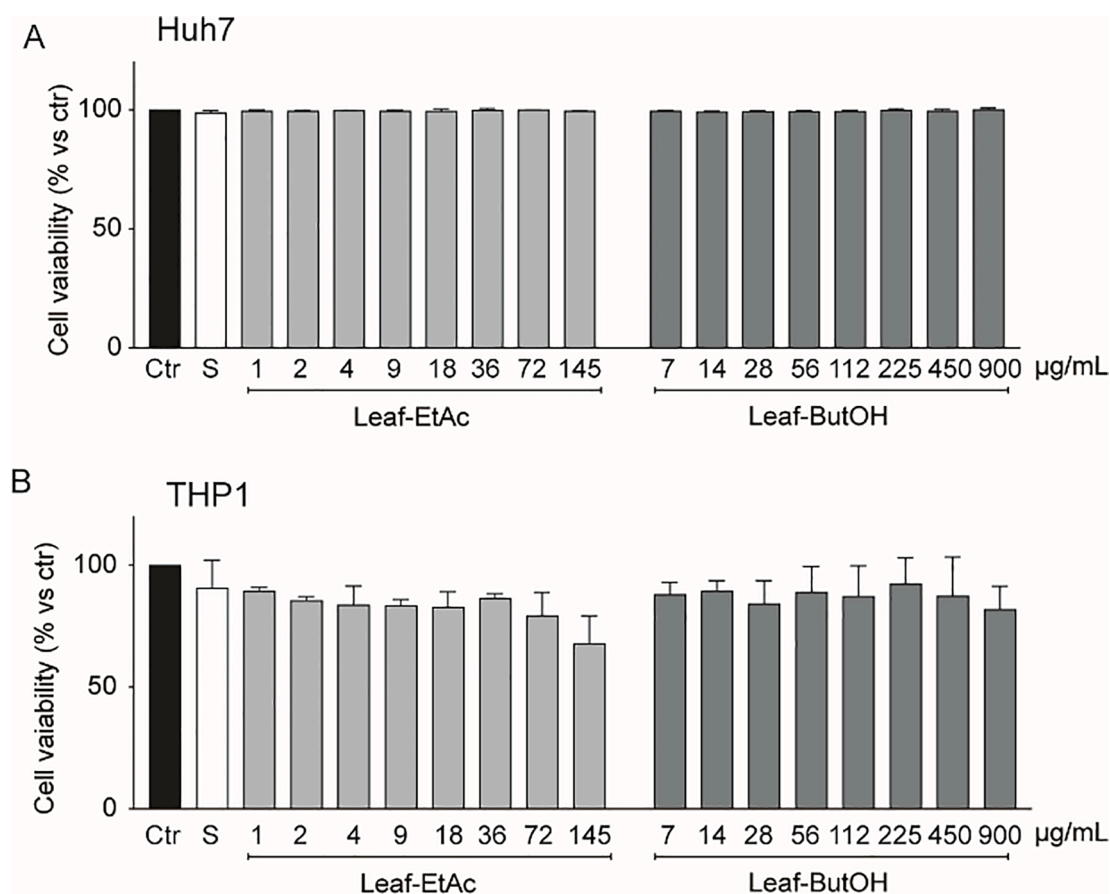

Fig. 3. Cytotoxicity assay of leaf EtAc and ButOH extracts in Huh7, and THP-1 cell line. Ctr: Control; S: simvastatin (5 μM).
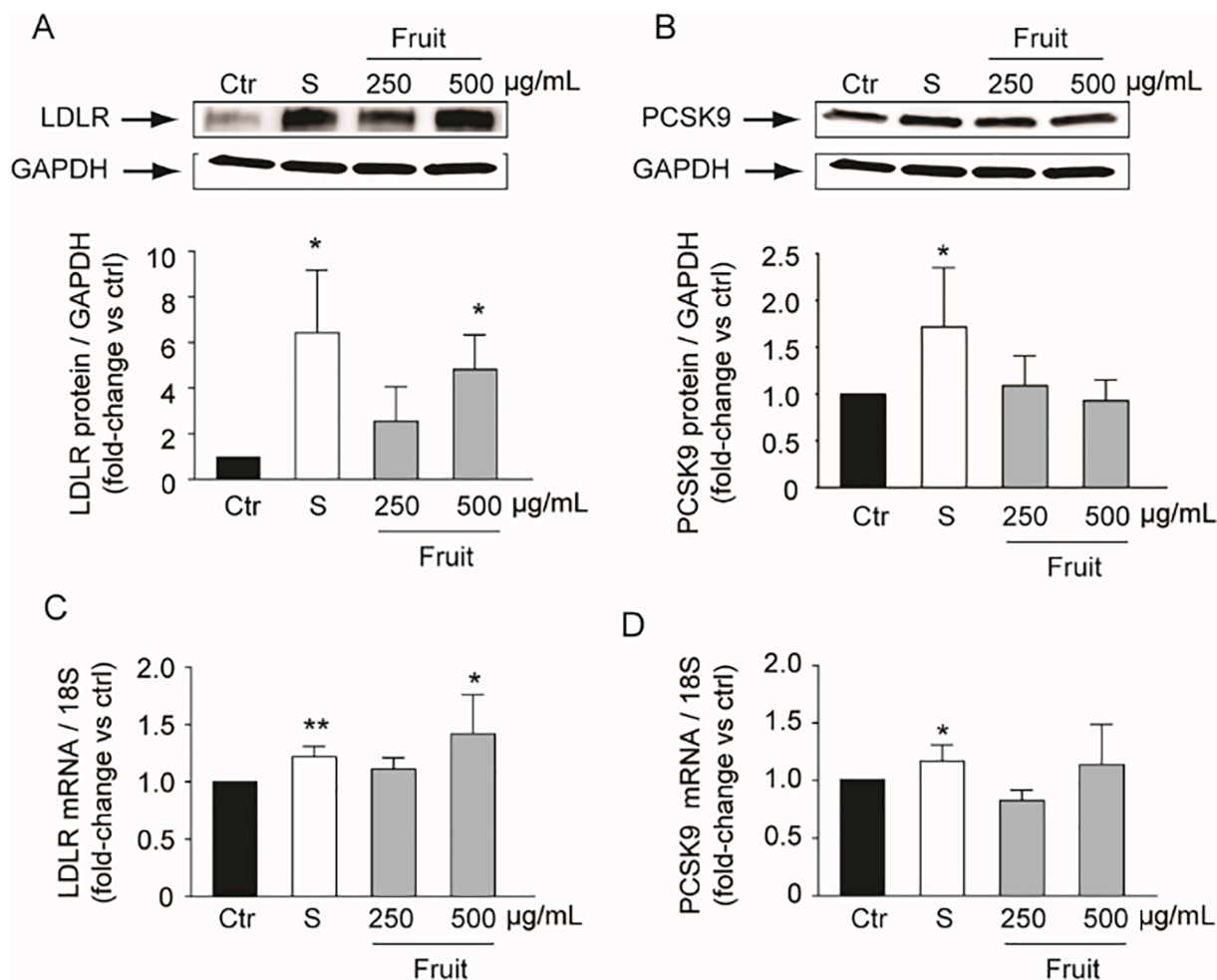
Fig. 4. Effect of fruit extract on LDLR and PCSK9 expression in Huh7 cells. A) and B) LDLR and PCSK9 expression was evaluated by western blot analysis. GAPDH was used as a loading control. Densitometric readings were evaluated using the ImageLab™ software, and the relative intensity of the bands are shown in the histograms. C) and D) LDLR and PCSK9 mRNA expression was evaluated by RT-qPCR analysis and normalized with 18S. *p < 0.01; **p < 0.01 vs Ctr. Ctr: control; S: simvastatin (5 μM).

Immunoreactive bands were detected by exposing the membranes to Clarity™ Western Enhanced ChemiLuminescence (ECL) chemilumines-cent substrates (Bio-Rad, Segrate, Milan, Italy) for 5 min, and images were acquired with an Azure c400 Imaging System (Aurogene, Rome, Italy). The densitometric readings were evaluated using ImageLab™ software (Bio-Rad, Segrate, Milan, Italy).

3.5. Reverse transcription and quantitative PCR (RT-qPCR)

Total RNA was extracted using the iScript™ RT-qPCR Sample Prep reagent (Bio-Rad, Segrate, Milan, Italy), according to the manufacturer's instructions. TranScriba 1step PCR Mix SYBR kit (A&A Biotechnology) was used for qPCR, along with specific primers for 18S (FWD 5′-CGGCTACCACATCCACGGAA-3′, REV 5′-CCTGAATTGTTATTTTTCGT-CACTACC-3′), PCSK9 (FWD 5′-CCTGCGCGTGCTCAACT-3′, REV 5′-GCTGGCTTTTCCGAATAAACTC-3′), LDLR (FWD 5′-TCTATGGAA-GAACTGGCGGC-3′ REV 5′-ACCATCTGTC TCGAGGGGTA-3′), TNF-α (FWD 5′-GGAGGTTGTGAGGCGCTGG-3′, REV 5′- CACGCACCT-CAAAGCTGTTC-3′), IL-6 (FWD 5′-TACATCCTCGACGGCATCTC-3′, REV 5′- AGTGCCTCTTTGCTGCT TTC-3′), IL-1β (FWD 5′-ATGCACCTGTAC-GATCACTG-3′, REV 5′ ACAAAGGACATGGAGAACA-3′). The analyses were performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Segrate, Milan, Italy) with cycling conditions of 50 °C for 10 min, 95 °C for 1 min, and a repetition of 40 cycles at 95 °C for 15 s followed by 30 s at 60 °C. The data were expressed as Ct values and used for relative quantification of targets with ΔΔCt calculations. The ΔΔCt values were determined by multiplying the ratio value between the ef-ficiency of specific primers and housekeeping 18S. The efficiency was calculated as $((10\hat{}(-1/slope)) - 1) \times 100$.

3.6. Statistical analysis

Data are expressed as mean ± standard deviation of at least three independent experiments. Differences between the two groups were analysed via *t*-test analysis (GraphPad). P-values less than 0.05 were considered statistically significant.

4. Results

To investigate the potential hypocholesterolemic effect of okra leaf and fruit extracts, we have first determined the *in vitro* cytotoxicity in hepatocyte Huh7 cell line. The incubation for 72 h with extracts from leaf and fruit with a concentration range from 4 μg/ml up to 500 μg/ml did not significantly affect the cell viability of Huh7 cells (Fig. 2).

Fruit extract was very soluble in DMSO (>20 mg/mL) thus can be used for further investigations. Leaf extract was a green sticky solid partially insoluble in DMSO. For this reason, a liquid–liquid partition was performed, and the ethyl acetate leaf extract (EtAC) and butanol leaf extract (ButOH) obtained were used for *in vitro* assays.

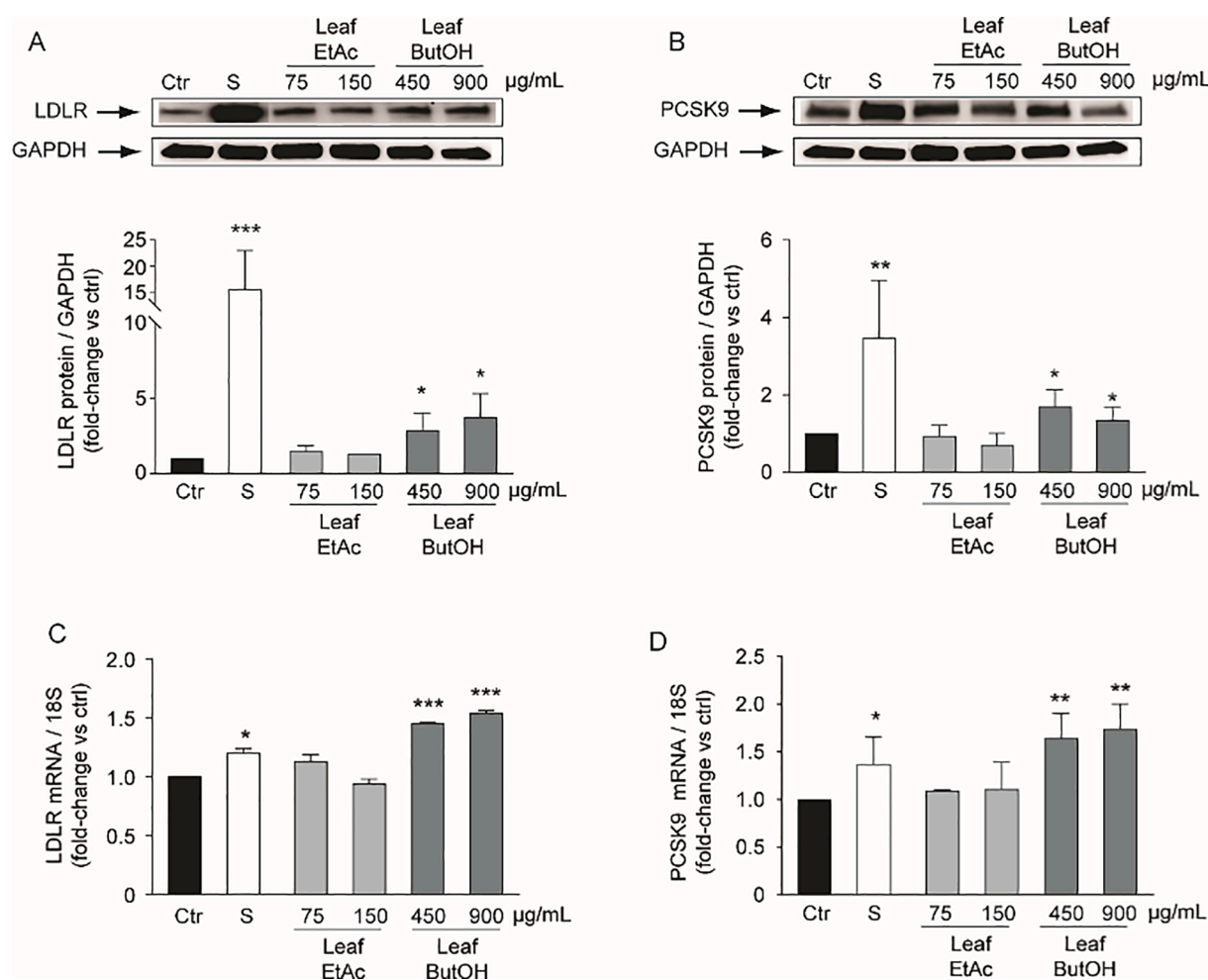EtAc and ButOH fractions of the leaf extract were tested for their

Fig. 5. Effect of leaves EtAc and ButOH extracts on LDLR and PCSK9 expression in Huh7 cell line. A) and B) LDLR and PCSK9 expression was evaluated by western blot analysis. GAPDH was used as a loading control. Densitometric readings were evaluated using the ImageLab™ software, and the relative intensity of the bands are shown in the histograms. C and D) LDLR and PCSK9 mRNA expression was evaluated by RT-qPCR analysis and normalized with 18S. *p < 0.01; **p < 0.01; ***p < 0.001 vs Ctr. Ctr: control; S: simvastatin (5 μM).

potential cell cytotoxicity in Huh7 and THP-1 derived macrophages (Fig. 3). Cells were incubated with leaf ButOH extract at concentration up to 900 μg/mL while leaf EtAc extract up to 145 μg/mL. After 72 h of incubation, we did not find any significant reduction in cell viability of both extracts on Huh7, and THP-1 cell line (Fig. 3).

Thus, all the selected okra extracts were not cytotoxic on Huh7 and THP1 cell lines at the tested concentrations, so we therefore started to assay the effect of fruit extract on the LDLR and PCSK9 expression in Huh7 cells. By western blot analysis we observed that after incubation with 500 μg/mL of fruit extract, Huh7 expressed significantly higher levels of the LDLR ($+4.8 \pm 1.5$-fold vs control), while no changes were observed on PCSK9 (Fig. 3A and B). As expected, the positive control simvastatin, significantly induced both the LDLR and PCSK9 (6.4 ± 2.7-fold and 1.7 ± 0.6 vs control, for the LDLR and PCSK9, respectively).

We then investigated the effect of okra fruit extract at the gene expression level. After 24 h of incubation with 500 μg/mL of fruit extract the mRNA levels of the LDLR increased by 1.4 ± 0.3-fold, while no effect was observed on PCSK9 mRNA expression (Fig. 4C and D). As expected, simvastatin induced the mRNA of both LDLR (1.2 ± 0.1-fold vs control) and PCSK9 (1.3 ± 0.1-fold vs control) (Fig. 4 C and D).

Taken together, from these *in vitro* analyses, it is possible to predict a potential hypocholesterolemic effect of fruit extract mediated by an upregulation of the LDLR in hepatocytes with a different mechanism of action than statins, since okra fruit extract did not alter the expression and protein levels of PCSK9.

In a second set of experiments, we tested the effect of the leaf EtAc and ButOH extracts at non-cytotoxic concentrations on LDLR and PCSK9 expression and compared their activities to the HMG-CoA reductase inhibitor simvastatin. As expected, simvastatin induced both the LDLR and PCSK9 by 15.5 ± 7.5-fold and 3.5 ± 1.5-fold vs control, respectively (Fig. 5A and B). Leaf EtAc extracts did not affect LDLR and PCSK9, while leaf ButOH extract slightly, but significantly, increased both LDLR (3.1 ± 1.6-fold vs control) and PCSK9 (1.3 ± 0.4-fold vs control) at 900 μg/ mL concentration (Fig. 5A and B). Similar effect was observed also at 450 μg/mL (2.5 ± 1.2-fold and 1.7 ± 0.4-fold vs control for LDLR and PCSK9, respectively).

To further investigate the mechanism of action of the leaf extract, we determined the mRNA expression levels of LDLR and PCSK9, both transcriptionally regulated by SREBP pathway. This analysis was conducted by incubating the Huh7 cells for 24 h, and simvastatin was used as positive control. Simvastatin induced the expression of the LDLR by 1.2 ± 0.04-fold and PCSK9 by 1.4 ± 0.3-fold vs control (Fig. 5C and D). As observed by western blot analysis, leaf EtAc extract did not alter the mRNA levels of these two target genes, while leaf ButOH significantly induced the LDLR and PCSK9. Indeed, after the incubation with 900 μg/ mL of leaf ButOH extract the LDLR mRNA increased by 1.5 ± 0.02-fold and PCSK9 mRNA by 1.7 ± 0.3-fold vs control (Fig. 5C and D). Thus, leaf ButOH extract, in our *in vitro* model, acts with a statin-like mechanism, potentially by reducing the intracellular sterols content.

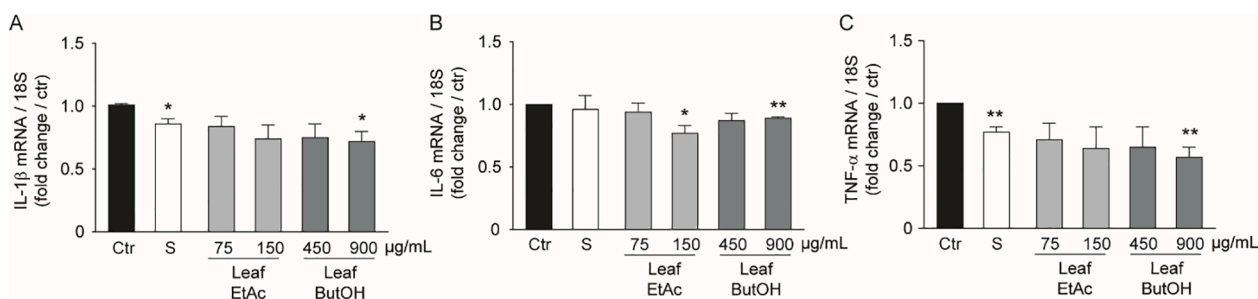Significant activity for a nutraceutical with potential cardiovascular

Fig. 6. Effect of leaf EtAc and ButOH extracts on mRNA expression of proinflammatory cytokines in THP-1 derived macrophages. IL-1β, IL-6 and TNF-α mRNA expression were evaluated by RT-qPCR and normalized with 18S. Ctr: Control; S: simvastatin (5 μM). *p < 0.01; **p < 0.01 vs Ctr.
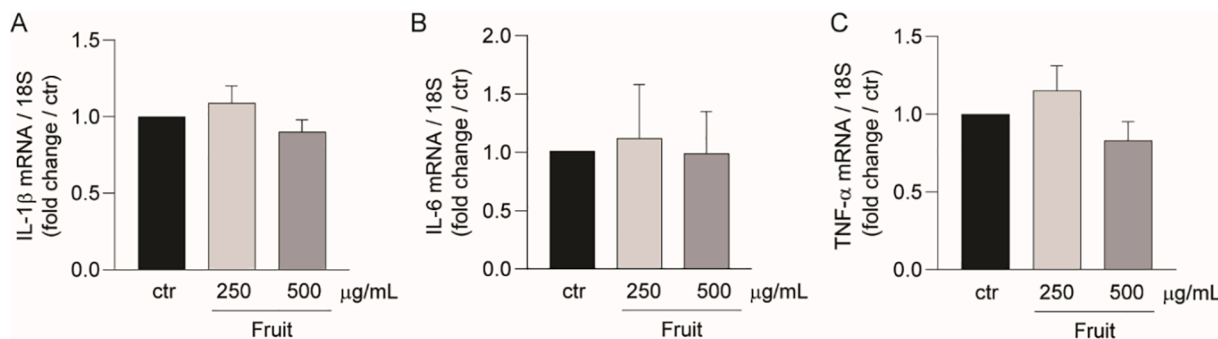

Fig. 7. Effect of fruit extracts on mRNA expression of proinflammatory cytokines in THP-1 derived macrophages. IL-1β, IL-6 and TNF-α mRNA expression was evaluated by RT-qPCR and normalized with 18S. Ctr: Control.

Table 1
Scavenging capacity of okra extracts obtained with DPPH assay, expressed as IC_{50} (μg/mL), reducing capacity of the okra extracts have been evaluated using the Folin Ciocalteau reagent and are expressed as Gallic Acid Equivalents (GAE). * Used as reference compound.

Sample	IC_{50} (μg/ml)	mg/g GAE
Fruit extract	2123.65 ± 20.17	2.58 ± 0.08
Leaf EtAC extract	19.95 ± 1.61	96.02 ± 2.13
Leaf ButOH extract	71.62 ± 2.34	26.45 ± 1.12
Ascorbic acid*	4.30 ± 0.15	–

protection effect can be the modulation of inflammation (Ruscica et al., 2021). We, therefore, conducted a series of experiments with leaf and fruit extracts, in THP-1 derived macrophages, and evaluated the mRNA expression levels of proinflammatory cytokines IL-1β, IL-6 and TNF-α. Simvastatin, significantly reduced IL-1β (−14 ± 4 % vs control) and TNF-α (−23 ± 4 % vs control) mRNA expression (Fig. 6). The leaf EtAc extract at 150 μg/mL reduced the mRNA levels of IL-6 (−23 ± 6 % vs control). Leaf ButOH extract showed an anti-inflammatory effect similar, if not greater, to simvastatin, by reducing the mRNA levels of all three cytokines. The incubation of THP1 macrophages with 900 μg/mL of leaf ButOH extract determined a reduction of mRNA levels of IL-1β (−28 ± 8 % vs control) IL-6 (−11 ± 1 % vs control) and TNF-α (−43 ± 8 % vs control) (Fig. 6).

On the contrary, the incubation of THP-1 cells with 250 and 500 mg/ml of fruit extract did not alter the mRNA expression of pro-inflammatory cytokines (Fig. 7).

The molecular mechanisms that connect the dyslipidemia to CVD are associated with oxidative stress and occurs through multiple processes related with the inflammation and with reactive oxygen species production (Medina-Vera et al., 2021). Many studies provide evidence of an antioxidant effect in dyslipidemias by the consumption of antioxidant foods as berries, garlic, soy and cocoa as examples. Antioxidants are present in many natural foods, and the literature also indicates
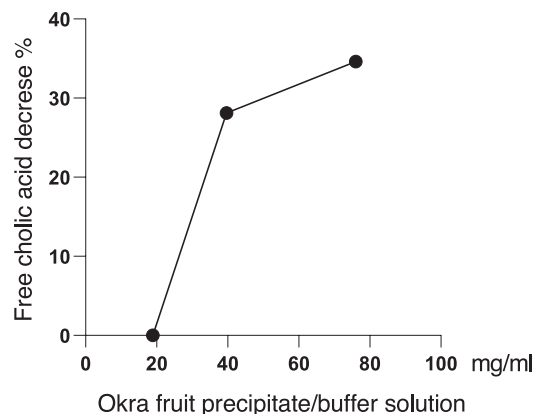

Fig. 8. *In vitro* binding of bile salt assay of okra fruit precipitate expressed as free colic acid decrease percentage.

correlation between food antioxidants and their anti-inflammatory properties supporting a crucial role for prevention of cardiovascular diseases and other degenerative pathologies (Griffiths et al., 2016; Medina-Vera et al., 2021).

Thus, okra extracts were evaluated for their antioxidant properties using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay using ascorbic acid as reference compound. The total reducing power of the samples was measured using the Folin-Ciocalteau (FC) reagent and the results were expressed as Gallic Acid Equivalents (GAE/equivalents). Results are summarized in Table 1, leaf ethyl acetate extract showed a significant antioxidant capacity with IC_{50} comparable to the reference ascorbic acid. Leaf butanol extract presented much lower activity and the fruit extract resulted in negligible effects. The data of the IC_{50} are in agreement with the FC measurements, in fact the leaf EtAC extract that present higher GAE equivalent also resulted the most active in the DPPH assay.

Table 2
HPLC-DAD-Ms[n] characterization of phenols in okra fruit extract, leaf ethyl acetate (EtAC) extract and leaf butanol (ButOH) extract.

Compound	Retention time (min)	[M−H]⁻	Fragmentation	Fruit extract mg/g	Leaf EtAc extract mg/g	Leaf ButOH extract mg/g	Ref.
Phenolic acid derivatives							
Caffeoyl glucaric acid isomer	3.5	371	209 191 173 85	0.440 ± 0.011	–	–	Fernandez-Poyatos, Ruiz-Medina, Zengin, and Llorent-Martinez (2019)
Caffeoyl glucaric acid isomer	5.3	371	209 191 173 85	0.165 ± 0.005	–	–	Fernandez-Poyatos et al. (2019)
Caffeoyl glucaric acid isomer	5.6	371	209 191 173 85	0.146 ± 0.009	–	–	Fernandez-Poyatos et al. (2019)
Caffeoyl glucaric acid isomer	6.4	371	209 191 173 85	0.522 ± 0.055	–	–	Fernandez-Poyatos et al. (2019)
Sinapoyl-hexose	6.8	385	299 223 127	0.219 ± 0.064	–	–	Arapitsas (2008)
Dihydroxybenzoic acid-O-dipentoside	7.4	417	373 285 241 193 152	0.040 ± 0.002	–	0.346 ± 0.028	Beelders, de Beer, Stander, and Joubert (2014) and Tsopmo and Muir (2010)
Sinapoyl-hexose	7.8	385	n.d.	0.104 ± 0.028	–	–	Arapitsas (2008)
Glycosylated flavonoids							
Apigenin pentoside	7.9	401	269 161	0.376 ± 0.016	7.953 ± 0.691	0.474 ± 0.010	Sun, Liu, Yang, Slovin, and Chen (2014)
Quercetin-3-O-gentiobiose	8.7	625	463 301	0.033 ± 0.006	–	–	Apel, Kammerer, Stintzing, and Spring (2017)
Quercetin 3-O-sambubioside	8.9	595	475 445 463 325 300 271 255	0.359 ± 0.065	142.941 ± 1.712	67.909 ± 5.588	Rodriguez-Medina et al. (2009)
Isoquercitrin *	9.6	463	407 301 300 271	0.051 ± 0.004	48.297 ± 0.622	0.796 ± 0.250	*
Luteolin-7-O-glucoside*	10.7	447	285 255	–	2.458 ± 0.060	0.438 ± 0.316	Li et al. (2016)
Isorhamnetin-3-O-glucoside*	10.9	477	314 315 357 285 300 271	–	8.201 ± 0.497	0.395 ± 0.055	Ben Said et al. (2017)
Roseoside	8.3	431 [M+HCOOH]⁻	385 223 205	n.q.	n.q.	n.q.	Spinola, Llorent-Martinez, Gouveia-Figueira, and Castilho (2016)
			Total phenolic acid derivatives	1.637	–	0.346	
			Total glycosylated flavonoids	0.819	209.849	70.012	
			Total phenols	2.456	209.849	70.357	

n.q. not quantified,*identification confirmed by injection of reference compound.

The polysaccharidic fraction contained in the fruit was separated obtaining the "okra fruit precipitate". This material did not show any significant effects on neither PCSK9 and LDLR levels nor on inflammatory cytokines expression. Due to the presence of polysaccharides, we tested the capacity of this material to act as cholic acid sequestrant from a buffer that mimic the intestinal pH. We observed that the okra fruit precipitate can sequestrate 35 % of the cholic acid from the buffered solution at the concentration of 80 mg/mL (Fig. 8). In the same experimental conditions, cholestyramine considered as reference compound presented chelating ability of 95 %, 82 % and 26 % at doses of 20, 10 and 5 mg/mL respectively. A second reference substance, chitosan presented sequestration of 23 % at 60 mg/mL dose. Thus, under our experimental conditions, the okra fruit precipitate showed a sequestering ability similar to chitosan and this property may contribute to the overall hypocholesterolemic effect of this food.

4.1. Chemical analysis of okra

To establish the chemical composition of okra extracts that have shown significant activity in the *in vitro* model related to cholesterol metabolism we have initially performed ¹H NMR and the results showed the presence of different classis of compounds. The leaf samples are shown in Fig. S1 comparing the crude extract and the two fractions, ethyl acetate (EtAc) and buthanol (ButOH). Signals ascribable to flavonoid glycosides are detectable in both extracts (Fig. S2) and results

indicate that these compounds are efficiently extracted in the liquid–liquid partition with organic solvents, on the contrary the citric acid remained in water fraction. The spectra of leaves ButOH and of the leaves EtAc extracts revealed the presence of flavonols, furthermore signals assigned to H-6 and H-8 of flavonol nucleus suggest the presence of one main derivative in the extracts. Presence of resonances of double bond in the range 5,5–5,9 ppm suggests the presence of megastigmane derivatives (Yajima, Oono, Nakagawa, Nukada, & Yabuta, 2009). Fruit NMR showed the presence of sugars as main constituents (Fig. S3).

To obtain detailed quali-quantitative information LC-DAD-MS[n] analysis of extracts were performed. Table 2 shows the different identified phytochemicals and the total amount of phenolic derivatives in each extract. Leaf extracts presented higher quantity of phenols (210 mg/g EtAC and 70 mg/g ButOH) than fruit extract (2.5 mg/g). Glycosylated flavonoids are present in all the extracts, with content of 210 and 70 mg/g for leaf EtAC and ButOH extract respectively. Fruit extract contains 0.8 mg/g of glycosylated flavonoids. Otherwise, fruit extract contains phenolic acid derivatives (total 1.6 mg/g) such as caffeoyl glucaric acid isomers, sinapoyl-hexoside derivatives and dihydroxybenzoic acid-O-dipentoside. Leaf ButOH extract contains dihydroxybenzoic acid-O-dipentoside (0.3 mg/g). Chromatograms obtained from fruit, ethyl acetate and leaves butanol extracts are shown in Fig. 9. Finally, a megastigmane derivative with molecular ion [M−HCOOH]⁻ at *m/z* 431 (Fig. 10) was tentatively assigned to roseoside (Spinola & Castilho, 2016). It was not possible to confirm the assignation and to
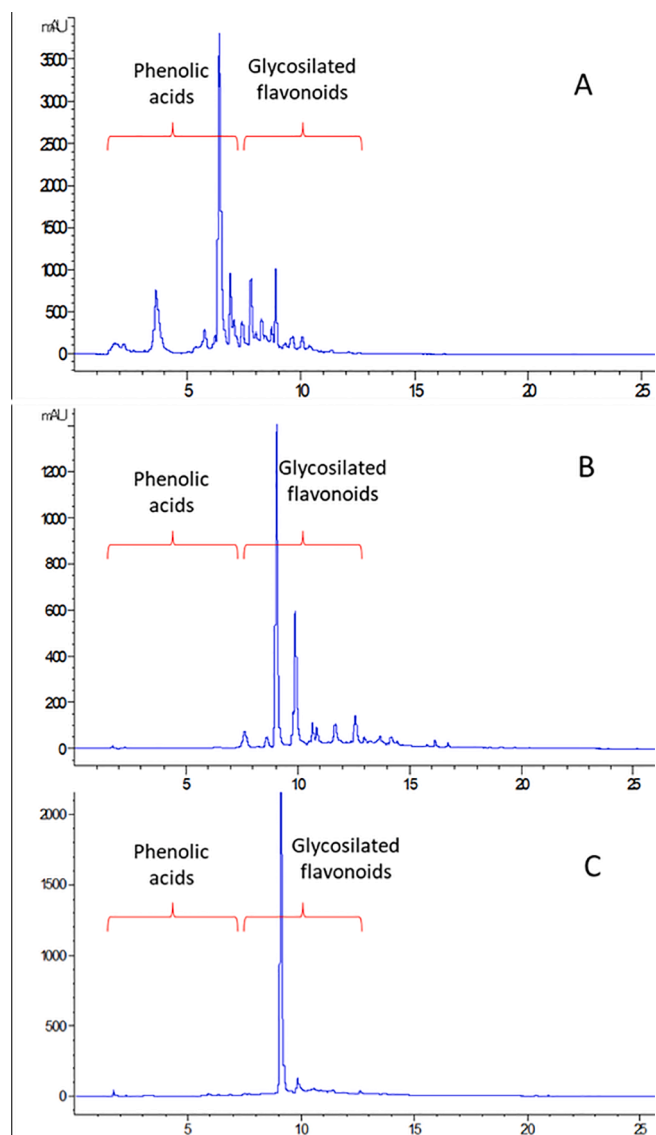
Fig. 9. HPLC-DAD chromatograms of okra fruit extract (A) leaf ethyl acetate (EtAC) extract (B) and leaf butanol (ButOH) extract (C) are reported at 330 nm. Phenolic acid derivatives (3–7 min) and flavonoid glycoside derivatives (7–11 min) are highlighted in red bracket. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quantify of this compound because of the unavailability of a commercial reference standard.

Due to the potential usefulness of both okra plant parts, we decided to evaluate the composition of soluble sugars using HPLC. The results are reported in Table 3 and free sugars were detected in fruit okra extracts but not in leaf extracts, confirming the data obtained with the NMR analysis. Total sugar content was similar for both okra fruit extract and fruit precipitate revealing the presence of sucrose, glucose and fructose in agreement with previous studies.

5. Discussion

Okra is a food that has been recently introduced in Europe and that is known for its valuable taste, nutritional value, and is considered for its health promoting properties. Previous studies have demonstrated an hypocholesterolemic action of okra, especially in diabetic patients (Moradi et al., 2020) and in experimental models of type 1 diabetes (Chukwuma et al., 2018; Erfani Majd et al., 2018; Uddin Zim et al.,

2021; Wang et al., 2014). In the present study, we provided new insights in the basic molecular mechanism of action of this vegetable showing potential usefulness as healthy food or as nutraceutical. We found that okra fruit extract significantly induces the LDLR in Huh7 hepatoma cell line. The basic molecular mechanism that mediates this effect is still unknown, but we compared with simvastatin and resulted to be different. Indeed, while simvastatin, by inhibiting the cholesterol biosynthesis, induces the activation of the SREBP pathway and thus the transcription of both the LDLR and PCSK9, okra's fruit extract resulted more selective on the activation the LDLR transcription without any effect on PCSK9. As a comparison with other natural products that are used as nutraceuticals, berberine has a mutual effect on the LDLR (induction) and PCSK9 (reduction) (Lupo et al., 2019). Berberine, stabilizes the mRNA of the LDLR leading to increased expression of the receptor (Kong et al., 2004), and has a negative effect on the transcription of PCSK9 (Li et al., 2009). Thus, okra's fruit extract act differently from berberine, nevertheless, the effect on LDLR protein expression, observed in Huh7 cell line, was quite remarkable and similar to the reference drug, simvastatin. Thus, the effect of this key player of cholesterol homeostasis could explain the hypocholesterolemic action of okra reported in in vivo experimental models (Erfani Majd et al., 2018; Uddin Zim et al., 2021), and in diabetic patients (Moradi et al., 2020).

Okra fruit extract is rich in hydroxycinnamic acid derivatives, and previous literature revealed the presence of sinapoyl-hexoside in red cabbage young seedlings extracts that were studied for their ability to reduce LDL-C, liver cholesterol, and inflammatory cytokines (Huang et al., 2016). In addition, the okra fruit, that is mostly composed by the polysaccharide fraction, showed a significant ability to sequestrate the cholic acid used as model for bile acids. This effect can be valuable in the overall mechanisms of action of this fruit because can help to increase the fecal excretion of bile acid thus contributing to the overall reduction of cholesterol.

Differently from fruit extract, ButOH leaf extract induced the expression of both LDLR and PCSK9 at protein and mRNA levels. This effect suggests a possible inhibition of cholesterol biosynthesis or reduced intracellular levels of sterols with subsequent induction of the SREBP-mediated transcription of LDLR and PCSK9. The induction of PCSK9 expression may counteract hypocholesterolemic action by inducing the degradation of the LDLR. Nevertheless, under our experimental conditions, we found that leaf ButOH extract significantly induced the LDLR at protein levels, thus suggesting a potential hypocholesterolemic action. Furthermore, the fiber fraction can also contribute to the hypocholesterolemic effects due to sequestering capacity on cholic acid. Antioxidant activity of the leaf's extracts are significant and can at least, in part, explain some of the anti-inflammatory properties observed in the in vitro model.

The most abundant phytochemicals in leaf extracts have been identified as quercetin-3-sambubioside for both extracts while in ethyl acetate we observed significant amount of isoquercitrin. Leaf extracts are rich in glycosidic flavonoids but did not present hydroxycinnamic derivatives. This indicates a different content in secondary metabolites in the two different plant parts and this can explain the different behavior observed in the bioassays for the leaf's extracts compared to fruit. Furthermore, considering the flavonoid glycosides content, we can observe that the leaf EtAc extract in the anti-inflammatory assay's presents similar activity on the expression of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) in THP-1 derived macrophages but at lower dose compared to the ButOH fraction. This may reflect the larger content of quercetin-3-sambubioside and isoquercitrin in EtAc. These two derivatives, in fact have been previously studied for their anti-inflammatory activities (Overman, Chuang, & McIntosh, 2011; Wu, Wu, Huang, Jao, & Yen, 2009). Thus, leaf extracts showed a relevant effect in THP1-derived macrophages, although additional evidence are required to confirm this anti-inflammatory action, i.e., by measuring the high sensitivity C-reactive protein (hsCRP).

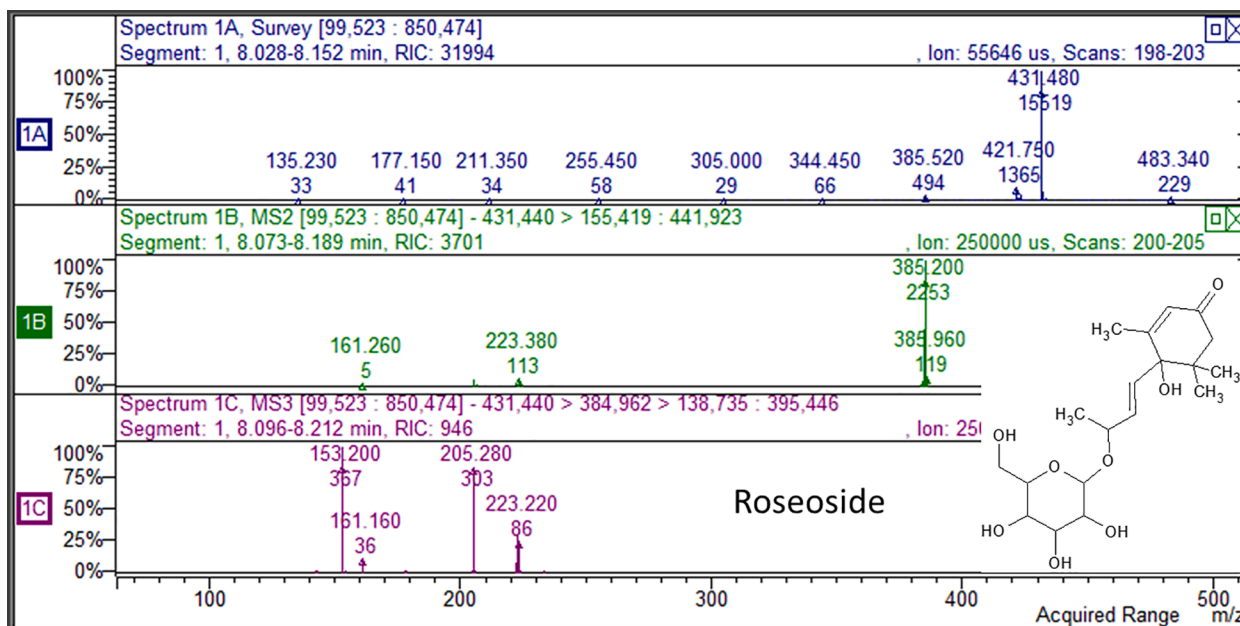DPPH assay and FC were used to estimate the antioxidant activity

Fig. 10. HPLC-MS^n fragmentation pattern of roseoside (molecular ion [M+HCOOH−H]$^-$ at *m/z* 431, [M−H]$^-$ at *m/z* 385).

Table 3
Sugar profile of okra extracts expressed as % DW, <LOQ, below limit of quantification 2 µg/g.

	Sucrose Tr 9.5	Glucose Tr 11.3	Fructose Tr 13.8	Total sugar
leaf ethyl acetate (EtAC) extract	<LOQ	<LOQ	<LOQ	<LOQ
leaf butanol (ButOH) extract	<LOQ	<LOQ	<LOQ	<LOQ
okra fruit extract	28.5 ± 1	22.5 ± 2	32.3 ± 2	83.3
okra fruit precipitate	17.1 ± 2	22.5 ± 2	23.1 ± 1	62.7

and presence of compounds with reducing capacity in the okra extracts. The results showed that leaf EtAc extract presents a scavenging capacity comparable to ascorbic acid, used as a reference substance. Minor antioxidant activity is observed for leaf ButOH extract while fruit extract did not show significant effect. Previous literature reported a relation between free radical scavenging activity and the presence of polyphenols (Di Meo et al., 2013). The antioxidant activity of leaf EtAc extract can be correlated with the presence of glycosidic flavonoids, in particular quercetin 3-sambubioside and isoquercitrin.

6. Conclusions

Consumption of fruit and/or leaf of okra or their extracts may contribute to control cholesterol levels, systemic inflammation, and providing a moderate but significant antioxidant activity.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2022.100126.

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