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-1\(\beta\), IL-6 and TNF-\(\alpha\)) 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 ± 1.6-fold vs control) and PCSK9 (+1.3 ± 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-1\(\beta\) (−28 ± 8 % vs control), IL-6 (−11 ± 1 % vs control) and TNF-\(\alpha\) (−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, okr’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 (*Abelmoschus 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, Oye-lade, Ade-Omonye, Adefemii, & Van de Venter, 2009; Araptitas, 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, Fernandez, 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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Okra administered to male CS7BL/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; Moradi et al., 2020; Uddin Zim et al., 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 (Esfani Majd, Tabandeh, Shahriari, & Soleimani, 2018). Same authors suggested a potential inhibition of PPAR-α and PPAR-γ in the pancreas (Esfani Majd et al., 2018), and the okra extract utilized in this study contained 141 mg/kg of hyperlipidemic 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 hypcholesterolemic 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.

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

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 ultrason 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<sup>a</sup> analysis

Fruit and leaf dried extracts (10 mg) were solubilised in methanol.
(25 mL) using ultrasound bath and analysed using an HPLC-DAD-MS system. The HPLC-MS 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 electro spray (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 (TDMS) function of the instrument. MS parameters were as follows: needle voltage, 4.9 kV; shield voltage, 600 V; capillary voltage, 2000 V; drying gas pressure, 2.3 × 10⁵ 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 Ca²⁺ 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 × 10⁵ 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 to 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. DAD parameters were as follows: gain, 10 AU; drying gas pressure, 2.2 × 10⁵ Pa, evaporative gas temperature, 60 °C. Quantiative 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 IC₅₀ (µg/mL).

3. In vitro experiments

3.1. Reagents

Eagle’s minimum essential medium (MEM), trypsin-EDTA, penicillin, streptomycin, sodium pyruvate, L-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 dissolved at a 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 % L-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 % CO₂ 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 % (PCS 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 µg/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).
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. 2. Cytotoxicity assay of leaf and fruit extracts from okra in Huh7 cell line. Ctr: Control; S: simvastatin (5 µM).

Fig. 3. Cytotoxicity assay of leaf EtAc and ButOH extracts in Huh7, and THP-1 cell line. Ctr: Control; S: simvastatin (5 µM).
Immunoreactive bands were detected by exposing the membranes to Clarity™ Western Enhanced Chemiluminescence (ECL) chemiluminescent 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′-CCTGAATTGTTATTTTTCGTCACTACC-3′), PCSK9 (FWD 5′-CCTGCGCGTGCTCAACT-3′, REV 5′-GCTGGCTTTTCCGAATAAACTC-3′), LDLR (FWD 5′-TCTATGGAACTGGCGGC-3′, REV 5′-ACCATCTGTCGAGGGGTA-3′), TNF-α (FWD 5′-GGAGGTTGTGAGGCGCTGG-3′, REV 5′-CACGCACCTCAAAGCTGTTC-3′), IL-6 (FWD 5′-TACATCCTCGACGGCATCTC-3′, REV 5′-AGTGCCCTCTTGTCTGC-3′), IL-1β (FWD 5′-ATGCACCTGTACGATCACTG-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 efficiency of specific primers and housekeeping 18S. The efficiency was calculated as ((10^{-1/slope}) - 1) × 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...
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 ± 1.5-fold vs control), while no changes were observed on PCSK9 (Fig. 3 A and B). As expected, 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, Huh7 expressed significantly higher levels of the LDLR (+4.8 ± 1.5-fold vs control), while no changes were observed on PCSK9 (Fig. 3 A 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).

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. 5 A 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. 5 A 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
The 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 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 reagent and the results were expressed as Gallic Acid Equivalents (GAE). Results are summarized in Table 1, leaf ethyl acetate extract showed a significant antioxidant capacity with IC50 comparable to the reference ascorbic acid.

**Table 1**

| Sample          | IC50 (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 | –        |

* Used as reference compound.

**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.

**Fig. 8.** In vitro binding of bile salt assay of okra fruit precipitate expressed as free colic acid decrease percentage.
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 presents 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 $^1$H NMR and the results showed the presence of different classes of compounds. The leaf samples are shown in Fig. S1 comparing the crude extract and the two fractions, ethyl acetate (EtAc) and butanol (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 fruit extract contain 0.8 mg/g of glycosylated flavonoids. Otherwise, fruit extract contains 0.8 mg/g of glycosylated flavonoids. Leaf ButOH extract contains dihydroxybenzoic acid-O-dipentoside. Leaf EtAc extract revealed the presence of flavonols, furthermore signals assigned to H-6 and H-8 of flavonol nucleus suggest the presence of an isomer.

Table 2

| 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 Llorente-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      | –                      | –                       | Arapitas (2008)              |
| Dihydroxybenzenic acid-O-dipentose | 7.4            | 417       | 373 285 241 193 152 | 0.104 ± 0.028      | 0.346 ± 0.028          | –                       | Apel, Kammerer, Stintzing, and Spring (2017) |
| Sinapoyl-hexose                | 7.8                  | 385       | n.d.                | 0.104 ± 0.028      | –                      | –                       | Arapitas (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-sambubiose       | 8.9                  | 595       | 475 445 463 325 300 | 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 ± 0.060         | 0.796 ± 0.055           | *                           |
| Luteolin 7-O-glucoside*        | 10.7                 | 447       | 285 255             | –                  | 2.458 ± 0.060          | 0.438 ± 0.031           | Li et al. (2016)            |
| Isoharmethin 3-O-glucoside*    | 10.9                 | 477       | 314 315 357 285 300 | 8.201 ± 0.049      | 209.849 ± 0.055        | 70.012 ± 0.028          | Ben Said et al. (2017)      |
| Roseoside                      | 8.3                  | 431       | 385 223 205         | 0.104 ± 0.028      | 0.346 ± 0.028          | 0.016 ± 0.009           | Spinoila, Llorente-Martinez, Gouveia-Figueira, and Castilho (2016) |
| Total phenolic acid derivatives|                      |           |                     | 1.637 ± 0.016      | –                      | 0.346 ± 0.016           |                             |
| Total glycosylated flavonoids  |                      |           |                     | 0.819              | 209.849 ± 0.016        | 70.012 ± 0.028          |                             |
| Total phenols                  |                      |           |                     | 2.456              | 209.849 ± 0.016        | 70.357 ± 0.028          |                             |

n.q. not quantified, *identification confirmed by injection of reference compound.
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 extracts but not in leaf extracts, confirming the data obtained with the reference standard.

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
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 polyantioxidant activity is observed for leaf ButOH extract while fruit extract comparable to ascorbic acid, used as a reference substance. Minor providing a moderate but significant antioxidant activity.

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.

Table 3

| Sugar profile of okra extracts expressed as % DW, <LOQ, below limit of quantification 2 µg/g. | Sucrose | Glucose | Fructose | 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 |

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

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