Food & Function

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2025, **16**, 7823

Effects of 'Mapo' tangelo (*Citrus* × *tangelo*) extracts, essential oil, and isolated compounds on LDL receptor and PCSK9 expression in the human hepatocarcinoma cell line Huh7

Irene Ferrarese,[a] Maria Giovanna Lupo,[b] Ilaria Rossi,[a] Giorgia Marodin,[a] Haixia Chen,[c] Stefania Sut,[a] Nicola Ferri[b] and Stefano Dall'Acqua*[a]

Peel (MPLE), pulp extract (MPPE) and essential oil (MPO) of *Citrus* × *tangelo* were studied for their chemical composition and for their ability to act on the expression of the low-density lipoprotein (LDL) receptor and proprotein convertase subtilisin/kexin 9 (PCSK9), two key players in cholesterol metabolism, in the human hepatocarcinoma cell line Huh7. MPPE demonstrated the ability to induce the LDL receptor and to reduce PCSK9. Chemical characterization of the extracts was conducted *via* LC-DAD-MS for MPLE and MPPE and *via* GC-MS for MPO. The main compounds isolated from MPLE and MPPE were ferulic acid, narirutin, 3′-methoxy-narirutin, nobiletin, 3-methoxynobiletin and tangeretin. MPPE significantly induced the LDL receptor (+1.43 ± 0.49-fold *vs.* basal) and suppressed PCSK9 levels (−64% ± 24% *vs.* basal). Among the different isolated compounds, ferulic acid showed the most interesting modulation of both the LDL receptor (+1.26 ± 0.14-fold *vs.* basal) and PCSK9 (−59% ± 14% *vs.* basal), showing potential cholesterol-lowering properties.

Received 17th April 2025,
Accepted 12th June 2025

DOI: 10.1039/d5fo01511a

rsc.li/food-function

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide, imposing a substantial health and economic burden. The first line of intervention is to recommend a healthy diet that is low in saturated fats, with a focus on whole-grain products, vegetables, fruit, and fish.[1] This is also suggested to prevent metabolic dysfunction-associated fatty liver disease.[2] Hyperlipidaemia, a well-established, modifiable CVD risk factor, is an independent predictor for developing cardiovascular events. Treatment of hyperlipidaemia has been central to targeting the increase in both the prevalence and mortality of CVD, which accounts for >4 million global deaths. Nevertheless, a majority of the European and US population does not meet the low-density lipoprotein cholesterol (LDL-C) target, although many classes of lipid-lowering drugs are available. Current European guidelines recommend the use of functional foods enriched with phytosterols and red yeast rice nutraceuticals for controlling LDL-C levels.[1] However, the European Food Safety Authority (EFSA) recently reduced the maximal dosage of monacolin K, the active compound in red yeast rice, to 2.9 mg per day due to safety concerns, thus reducing its activity. For these reasons, the identification of novel nutraceuticals with lipid-lowering properties represents an unmet clinical need that may help to control mild hypercholesterolemia for primary prevention in the general population. Here, we investigated the potential lipid-lowering effect of 'Mapo', as a starting point for the development of a new nutraceutical product.

'Mapo' tangelo (*Citrus* × *tangelo*) is an Italian citrus cultivar developed in 1950 by crossing the 'Avana' mandarin (*Citrus deliciosa* Ten.) and the 'Duncan' grapefruit (*Citrus paradisi* Macf.).[3–5] The tangelo hybrid exists in a large number of cultivars,[6] and they are tangerine-grapefruit or pummelo hybrids (*C. reticulata* × *C. paradisi* or *C. reticulata* × *C. grandis*).[7] Citrus fruit peels generally represent 40%–50% of the total fruit mass, but this part is not edible and is considered waste. Peels are a source of health-enhancing compounds such as phenolic compounds and terpenoids. Most common are hydroxycinnamic derivatives, such as caffeic, *p*-coumaric, ferulic, and sinapic acids, the flavanones, such as naringin and hesperidin, and the polymethoxylated flavones, such as nobiletin and tangeretin.[8] Compared with the edible fruit part, the peels are rich in many volatile terpenoids as well as in non-terpenoid

[a]*Department of Pharmaceutical and Pharmacological Sciences, University of Padova, 35131 Padova, Italy. E-mail: stefano.dallacqua@unipd.it*
[b]*Department of Medicine, University of Padova, 35128 Padova, Italy*
[c]*Tianjin Key Laboratory for Modern Drug Delivery and High-Efficiency, School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, 300072, China*

derivatives such as polymethoxyflavones that are detected in relevant amounts.[8–10] Among the flavonoids, flavanones are the most represented in citrus tangelo, and the glycosyl derivatives such as naringenin, narirutin, eriocitrin, neoeriocitrin, poncirin, hesperidin, neohesperidin, and didymin are generally more prevalent than the aglycones (commonly the hesperetin, eriodictyol, and naringenin).[11] Several *C*-glycosyl flavones and *O*-triglycosyl flavanone were detected in tangelo juice.[9] Among the polymethoxyflavones, nobiletin, tangeretin, and sinensetin are the most abundant in citrus peels.[10] Tangelos are tangerine-grapefruit or pummelo hybrids[7] and, unlike grapefruit, these varieties contain traces or non-detectable quantities of furanocoumarins. In this respect, the consumption of these varieties in conjunction with therapy with drugs metabolized by the intestinal CYP3A4 enzyme does not cause significant interaction problems.[6]

Recent studies investigated the pharmacological activity and mechanism of action of polymethoxyflavones for their potential therapeutic use, also taking into consideration their higher bioavailability compared to other classes of flavonoids.[9] In this contest, several preclinical studies have demonstrated that citrus flavonoids can exert beneficial effects in the prevention and treatment of cardiovascular diseases (CVDs).[12] In particular, citrus flavonoids (naringin, naringenin, quercetin, hesperidin, hesperetin, and polymethoxyflavones) and citrus extracts have shown a hypolipidemic effect through several biochemical pathways.[12]

To date, the evidence related to the phytochemical characterization and the therapeutic effects of tangelos is relatively poor. Peterson *et al.* stated that in Honeyball, K-Early, Minneola, and Seminole tangelo varieties, flavanones are the predominant compounds, with a concentration of 30 mg per 100 g juice.[7] Other authors reported that hesperidin (21.1 mg L^{-1}), narirutin (6.3 mg L^{-1}), and vicenin-2 (3.9 mg L^{-1}) are the most abundant flavanones in tangelo juice.[11] Other authors reported that Seminole tangelo juice differs from orange juice in the higher content of heptamethoxyflavone and tangeretin.[13] Even if the peels and pulp of citrus tangelos have been chemically characterized,[6,7,11,14] literature concerning the Italian cultivar 'Mapo' is lacking; only three studies have reported exclusively a characterization of the volatile constituents.[3,4,15]

In the present study, we have investigated the effects of 'Mapo' tangelo peel extract (MPLE), pulp extract (MPPE), and essential oil (MPO) on the expression of low-density lipoprotein (LDL) receptor and proprotein convertase subtilisin/kexin 9 (PCSK9), two key players in cholesterol metabolism.[16] Detailed chemical analysis allowed the identification of the main constituents of the different extracts. The main constituents of the extracts, ferulic acid, narirutin, 3′-methoxy-narirutin, nobiletin, 3-methoxynobiletin, and tangeretin, were isolated. Finally, to assess the role of the most abundant constituents among the isolated compounds and the most abundant constituents of the essential oil, D-limonene and γ-terpinene were tested for their ability to modulate the expression of the LDL receptor and PCSK9.

2. Materials and methods

2.1. Fruit samples

The fruits of *Citrus* × *tangelo* ('Mapo' tangelo) were supplied by a local market in Padova (Italy). The oblate fruits were medium-sized, between 7–7.5 cm wide and 6.5–7 cm high.

2.2. MAE extraction and GC-MS characterization of Mapo peel essential oil (MPO)

2.2.1 Microwave-assisted extraction (MAE). A Microwave-Assisted Extraction (MAE) system was used to obtain Mapo essential oil (MPO). A conical flask containing *C.* tangelo peels (200 g) and 50 ml of deionized water was placed in a Milestone Ethos X (Milestone s.r.l., Bergamo, Italy). Experiments were conducted in two parts using a microwave. The MAE extraction parameters were microwave power (500 W first extraction and 800 W second extraction) and extraction time (30 minutes first extraction and 15 minutes second extraction). The oil was recovered by adding diethyl ether and was then stored at 5 °C until use. The extraction yield was calculated in terms of % (w/w) of essential oil extracted per gram of fresh peels and was 0.024% (w/w).

2.2.2 Gas chromatography-mass spectrometric (GC–MS) analysis. For the analysis, a Varian 3900 gas chromatograph equipped with an autosampler and coupled to a Varian Saturn 2100T Ion-trap (MS/MS) was used. As the stationary phase, an Agilent HP-INNOVAX column (30 m × 0.250 mm × 0.25 μm) was used, setting the following oven temperature gradient: 0–3 min, 50 °C, then to 210 °C at 3.5 °C min^{-1}. Total run time: 50 min. The flow rate of the carrier gas (helium) was 1.0 mL min^{-1}. A splitless injection was used, and 1 μL of solution was injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 45–650. The essential oil sample was diluted in diethyl ether at a concentration of 10 microliters of MPO diluted in 25 mL of solvent; nonanol was used as an internal standard. Compounds were identified according to the following criteria: at first, a comparison of the experimental MS spectra with data from mass spectral libraries (NIST 12 database, own libraries) and literature;[17] calculation of the Kovat's retention index using a standard *n*-alkane calibration mixture (C8–C40) dissolved in hexane; when available, a reference solution of standard compounds was used to confirm the identity of the compound.

For quantification purposes, nonanol was used as an internal standard, and mixtures of *n*-nonanol and D-limonene, germacrene D, and sclareol were obtained. Semi-quantification of the compounds was conducted using D-limonene for the monoterpene, germacrene D for the sesquiterpenoids and sclareol for the diterpenoid. Compound quantities are indicated as % based on the oil weight.

2.3. Mapo peels and pulp extraction

Dried Mapo peels obtained from the first extraction using MAE technology were macerated using water and 20% ethanol (800 ml). The mixture was filtered, and the supernatant was concentrated to dryness using a rotary evaporator (55 °C); 10 g

of Mapo peel extract (MPLE) was obtained. The Mapo pulps (200 g) were hand-squeezed, and the obtained juice was frozen and freeze-dried using a Lyovapor L200 (BUCHI Ibérica S.L.U., 08960 Sant Just Desvern, Barcelona, Spain); 40 g of Mapo pulp extract (MPPE) was obtained. MPLE and MPPE were stored at −20 °C.

2.4. Isolation and structural elucidation of Mapo peel extract (MPLE) constituents

MPLE was dissolved in ethanol and loaded on a Sephadex column (4 × 70 cm). Elution of the Sephadex column with methanol 0.5 ml min^{-1} yielded 144 fractions, which were grouped into 10 fractions due to TLC behaviour (1–10). Further steps of separations were conducted by preparative TLC (Merck, Darmstadt, Germany) using $CHCl_3$–MeOH–H_2O, 10 : 5 : 1 as a solvent mixture. Purified fractions were evaporated to dryness under vacuum in a rotary evaporator at 55 °C. The weights of the obtained fractions are as follows: A0 54.4 mg, A 211.3 mg, B 1555 mg, C 171.8 mg, D 152.3 mg, E 35.4 mg, F 31.9 mg, G 33.2, H 27.4 mg, I 21.3 mg. Pure compounds were separated from the fractions using a semipreparative HPLC. The LC system consisted of an Agilent 1260 quaternary pump coupled to a diode array detector (DAD) (Agilent Technologies, Santa Clara, CA, USA). As a stationary phase, a Zorbax SB C-18 column (particle size 15 μm, 21.2 × 150 mm) (Agilent Technologies, Santa Clara, CA, USA) was used. A mixture of 0.1% formic acid in water (A), and methanol (B) was used as the mobile phase, and the gradient was as follows: 0 min, 80% A; 15 min, 20% B.

Structure elucidation was achieved *via* 1D and 2D NMR experiments such as HSQC-DEPT, HMBC, COSY, and NOESY. The isolated compounds were ferulic acid (11.50 mg), narirutin (136.12 mg), homoeriodichtyol-7-*O*-neoesperidoside (7.47 mg), nobiletin (1.22 mg), 3-methoxynobiletin (2.21 mg), and tangeretin 0.82 mg.

2.5. LC-DAD-MS^n analysis of MPLE and MPPE

MPLE and MPPE extracts (50 mg) were solubilised in deionised water with 50% methanol (25 mL) using an ultrasound bath and analysed using an LC-DAD-MS^n system. The system consisted of an Agilent 1260 quaternary pump coupled to a 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 ionization (ESI). As the stationary phase, a Zorbax SB C18 column (250 × 4.6 mm, 5 μm) (Agilent Technologies, Santa Clara, CA, USA) was used. As the mobile phase, a mixture of 0.1% formic acid in water (A), acetonitrile (B) and methanol (C) was used, and the gradient was as follows: 0 min, 90% A, 7.5% B, 2.5% C; 20 min, 80% B, 20% C; 22 min, 80% B, 20% C; 23 min, 90% A, 7.5% B, 2.5% C. The flow rate was 0.75 mL min^{-1}. The injection volume was 10 μL and the column temperature was set at 30 °C. DAD allowed the collection of chromatograms in the λ range of 200–640 nm. MS data were acquired in both positive and negative ion modes, in the m/z range 100–2000. Fragmentation patterns of the most intense ion species were obtained using the turbo data, depending on the scanning (TDDS) function of the instrument. Identification of compounds was based on comparison with the literature and reference compounds, when available. For compound quantification, rutin, naringenin, catechin, sinensetin, and ferulic acid were used. Standard solutions were prepared in the concentration ranges 1–100 μg mL^{-1}, and calibration curves were built and used for quantification purposes. Quantitative data are the mean of three repeated analyses.

2.6. MPLE, MPPE, MPO, and isolated compounds solubilization and treatment

MPLE, MPPE, and MPO extracts were dissolved in DMSO to a stock concentration of 20 mg ml^{-1} and were used at 100 μg ml^{-1} in culture medium; the isolated compounds were dissolved in DMSO to get a stock of 20 mM and diluted to the indicated final concentrations in culture medium for the treatments. Simvastatin was dissolved in physiologic solution to a 50 mM stock concentration and used at 5 μM as previously reported.[18] If not used, all the stock solutions were stored at −20 °C.

2.7. *In vitro* assays

2.7.1. 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). Mapo extracts and isolated compounds were dissolved in dimethyl sulfoxide (DMSO) as stock solutions of 100 mg ml^{-1} and 80 mM. Simvastatin was obtained from Merck (Darmstadt, Germany) and was dissolved to a stock concentration of 5 mM in 0.1 M NaOH, and the pH was adjusted to 7.2 according to the manufacturer's instructions. The solution was then sterilized by filtration. The Huh7 cell line was purchased from Tebubio SRL, Milan, Italy (code product 300156).

2.7.2. 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 100×, 1% non-essential amino acids 100×, and 1% penicillin/streptomycin solution (10.000 U mL^{-1} and 10 mg mL^{-1}, respectively), at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For the experiments, cells were incubated with the 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.

2.7.3. Sulphorhodamine B cell viability assay. The cell viability of the extract and the isolated compounds was assessed by sulphorhodamine (SRB) assay according to the protocol established by Skehan *et al.*[19] Briefly, 8 × 10^3 cells per well were seeded in a 96-well tray in 100 μL per well of complete medium. The following day, the old media were replaced by fresh media, 10% FBS containing treatments after a wash with sterile PBS. Controls were supplied with the appropriate % of

Table 1 Characterisation of the volatile constituents of Mapo peel essential oil (MPO). Calculated retention indices are reported for each compound

Peak number	Retention time (minutes)	Compound	%	RI
1	5.4	β-Pinene[a]	0.26	1111.1
2	5.7	Sabinene	0.12	1122.2
3	7.2	α-Terpinene[a]	1.08	1177.8
4	8.2	D-Limonene[a]	60.12	1212.1
5	9.6	γ-Terpinene	4.20	1254.5
6	9.8	β-Ocimene	0.07	1260.6
7	10.3	*m*-Cymene[a]	0.85	1275.8
8	10.6	Terpinolene	0.23	1284.8
9	11.0	Octanal	0.34	1297.0
10	16.0	*p*-Cymenene	0.04	1436.1
11	16.9	δ-Elemene	0.27	1464.7
12	17.6	α-Copaene[a]	0.20	1485.3
13	19.5	α-Gurjunene	0.04	1541.2
14	20.0	β-Guaiene, *trans*-	0.39	1555.9
15	20.1	Linalool[a]	1.18	1558.8
16	21.1	β-Elemene[a]	2.16	1588.2
17	21.5	β-Gurjunene	0.04	1600.0
18	21.7	Terpinen-4-ol[a]	0.10	1606.1
19	22.5	α-Himalachene	0.03	1630.3
20	22.7	*p*-Mentha-2,8-dien-1-ol	0.04	1636.4
21	23.5	α-Humulene[a]	0.36	1660.6
22	23.8	β-Farnesene	0.20	1669.7
23	24.2	γ-Muurolene	0.24	1681.8
24	24.3	Cedrene	0.08	1684.8
25	24.8	Germacrene D[a]	0.61	1700.0
26	24.9	γ-Himachalene	0.74	1703.0
27	25.4	α-Muurolene	0.33	1718.8
28	25.6	Valencene	0.24	1725.0
29	26.0	α-Farnesene	0.13	1737.5
30	26.5	δ-Cadinene	3.72	1753.1
31	26.8	Unidentified	0.10	1762.5
32	27.1	Unidentified	0.24	1771.9
33	27.4	Perilla aldehyde	0.43	1781.3
34	28.5	Cuparene	0.11	1816.7
35	28.7	Calamenene	0.16	1823.3
36	29.7	Unidentified	0.14	1856.7
37	30.0	Unidentified	0.11	1866.7
38	31.2	α-Calacorene	0.21	1906.7
39	33.1	Caryophyllene oxide	0.49	1970.0
40	34.8	Gleenol	0.14	2026.7
41	35.2	Cubenol	0.16	2040.0
42	35.3	Unidentified	0.20	2043.3
43	35.5	Cubenol, 1,10-di-*epi*-	0.80	2050.0
44	37.2	Unidentified	0.22	2032.0
45	37.5	Unidentified	0.32	2048.0
46	37.7	Globulol	0.49	2052.0
47	37.9	Iso-pimar-9(11),15-diene	0.33	2060.0
48	38.5	Cembrene	3.92	2200.0
49	38.9	α-Cadinol-*epi*	0.48	2210.0
50	39.3	α-Muurolol	2.42	2220.0
51	39.7	Cadalene	0.10	2230.0
52	40.1	α-Cadinol[a]	1.59	2240.0
53	40.3	Unidentified	1.47	2245.0
54	40.8	Unidentified	0.92	2257.5
55	41.3	Kaur-15-ene, (5α,9α,10β)-	0.89	2270.0
56	41.7	Unidentified	0.77	2280.0
57	42.5	Manoyl oxide	1.61	2300.0
58	42.8	Sclareol[a]	2.77	Not calculated

[a] Indicates confirmation of identification by standard injection.

DMSO to be comparable with compounds and Mapo extract treatments. After 72 h of incubation, the SRB assay was performed and absorbances measured at 570 nm with a Victor Nivo multiplate reader by PerkinElmer.

2.7.4. Western blotting for intracellular PCSK9 and LDL receptor detection. Non-toxic compounds and extract concentrations (100 μM or 25 μM and 100 μg ml^{-1} or 50 μg ml^{-1}, respectively) were used for the western blotting analysis. Here, 5×10^5 cells per well were seeded in 60 mm cell culture dishes in 3 mL per dish of complete medium. The following day, the old media were replaced by fresh media, 10% FBS containing treatments after a wash with sterile PBS. Simvastatin 5 μM was used as a positive control as a PCSK9 and LDL receptor inducer. Untreated controls and Simvastatin controls were supplied with the appropriate % of DMSO to be comparable with compounds and Mapo extract treatments. After incubation, the cell monolayer was washed twice with cold PBS, then lysed on ice for 30 min with a home-made mild NP-40 lysis buffer (prepared according to the Abcam recipe). Protein quantification in samples was carried out with bicinchoninic acid assay (SERVA), and samples for the electrophoretic run were equalized to the same concentration by dilution with an appropriate amount of lysis buffer and the addition of a homemade Laemmli loading buffer (prepared according to the Abcam recipe). Protein denaturation was then enhanced by 5 min at 95 °C. A total amount of ≥20 μg of protein per samples were loaded into the SDS-PAGE wells and left to separate under denaturing conditions (Bio-Rad apparatus). Proteins were then transferred semi-dry into a nitrocellulose membrane (Bio-Rad apparatus), upon which membranes were blocked with a 5% non-fat skim milk solution in TBS-Tween 20 1× (hereafter named blocking solution) for 1 h at room temperature. This was followed by overnight incubation with primary antibodies with PCSK9, LDL receptor, or GAPDH as the loading control. On the following day, the membranes were washed three times (15 min each) with TBS-Tween 20 1× (TBST20 1×) and then incubated with HRP-conjugated secondary antibodies for 90 min at room temperature, followed by three further washes with TBST20, 1 × 15 min each, and ECL testing in the c100 Azure system by Aurogene. The following primary antibodies were utilized: anti-LDL receptor (Millipore, Darmstat, Germany; mouse monoclonal antibody, clone 2H7.1; dilution 1 : 1000); anti-PCSK9 (Abcam, cod. ab181142; dilution 1 : 1000), anti-GAPDH (GeneTex, cod. GTX100118; dilution 1 : 5000), secondary anti-mouse antibody was from Jackson ImmunoResearch (cod. 115-036-062; dilution 1 : 5000), and anti-rabbit antibody was from Jackson ImmunoResearch (cod. 113-036-045, dilution 1 : 5000).

2.7.5. 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. The TranScriba 1-step PCR Mix SYBR kit (A&A Biotechnology) was used for qPCR, along with specific primers for 18S (FWD 5′-CGGCTACCACATC CACGGAA-3′, REV 5′-CCTGAATTGTTATTTTTCGTCACTACC-3′) and LDLR (FWD 5′ TCTATGGAAGAACTGGCGGC-3′ REV

5′-ACCATCTGTC TCGAGGGGTA-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 the relative quantification of targets with ΔΔCt calculations. The ΔΔCt values were determined by multiplying the ratio value between the efficiency of specific primers and the 18S housekeeping gene. The efficiency was calculated as $((10^{(-1 \text{ per slope})}) - 1) \times 100$.

2.8. Statistical analysis

Statistical analysis was performed using the Prism statistical analysis package, Version 8.2.1 (GraphPad Software, San Diego, CA, USA). When possible, *p*-values were determined by Student's *t*-test. Otherwise, differences between treatment groups were evaluated by one-way ANOVA. The probability value of $p < 0.05$ was considered statistically significant. The experiments were performed in triplicate, and the data were expressed as mean ± standard deviation (SD).

3. Results

3.1. Chemical characterization of Mapo essential oil (MPO)

In the MPO, 58 different volatile compounds (Table 1) were identified based on the calculation of the Kovats index and comparison of MS data, as well as confirmation by standard injection. As expected, D-limonene (60.1%) and γ-terpinene (4.2%) were the most abundant monoterpenoids, while δ-cadinene (3.7%) and β-elemene (2.2%) were the most represented sesquiterpenoids. The most abundant diterpenes present in our sample of MPO were cembrene (3.9%), sclareol (2.8%), and α-muurolol (2.4%). The qualitative–quantitative data are in good agreement with previously published analytical measurements on the Mapo essential oil (Fig. 1).[3]

3.2. Chemical characterization of Mapo peel extract (MPLE) and Mapo pulp extract (MPPE)

A total of twenty-seven constituents were identified in the two extracts by LC-DAD-MS^n analysis.

Chromatograms showing the negative and positive ion mode BPI are presented in Fig. 2.

Phytochemical data revealed the presence of different derivatives belonging to the classes of limonoids, hydroxycinnamic acids, flavonol O and C glycosides, flavanone glycosides, and derivatives bearing the 3-hydroxy-3-methyl-glutaryl (HMG) moiety. More lipophilic compounds, such as polymethoxyflavones, were detected only in peels. Naringenin-7-*O*-glucoside-*O*-HMG and 3-methoxynobiletin were detected for the first time in the tangelo species. The data of the analysed samples of the two extracts revealed several *O*-glycoside flavonoids that have been detected mainly in pulps, such as taxifolin and eriodyctiol, and luteolin glycosides, while diosmetin di-*C*-glucoside is detected mostly in peels. The phytochemical composition data agree with those in the previously published paper.[11]

Considering the quantitative analysis, we observed that in the analysed samples the most abundant compounds in MPLE are the hydroxycinnamic derivative, ferulic acid (24 mg g^{-1}), the flavanone homoeriodichtiyol-7-*O*-neoesperidoside (29 mg g^{-1}), naringenin 7-*O*-rutinoside (26 mg g^{-1}), and the methoxyflavonoids 3-methoxynobiletin (29 mg g^{-1}), sinensetin (26 mg g^{-1}) and tangeretin (25 mg g^{-1}). MPPE, on the other hand, contains a higher amount of ferulic acid (63 mg g^{-1}) and homoeriodichtyol-7-*O*-neoesperidoside (39 mg g^{-1}), while methoxyflavone is almost absent. The quantitative data are summarised in Table 2.

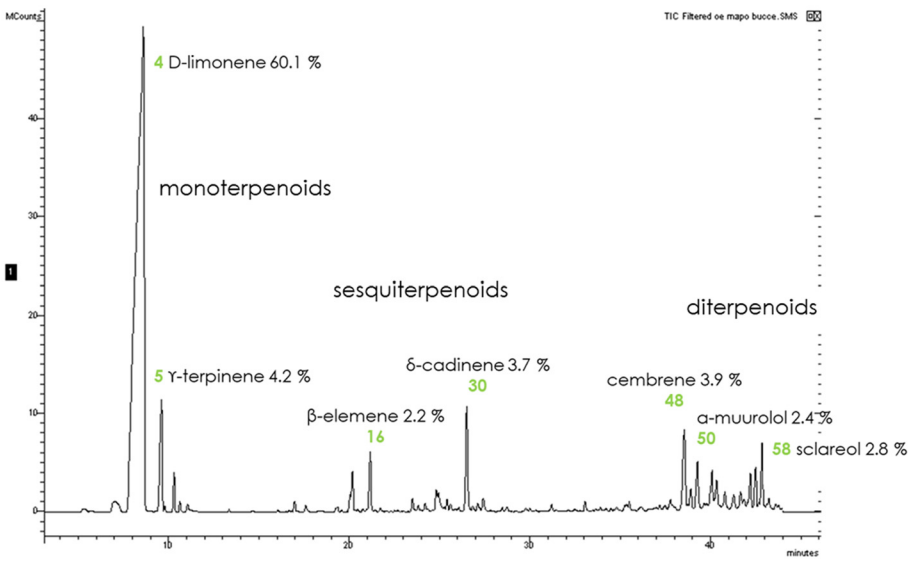

Fig. 1 GC-MS chromatogram of Mapo peel essential oil (MPO).

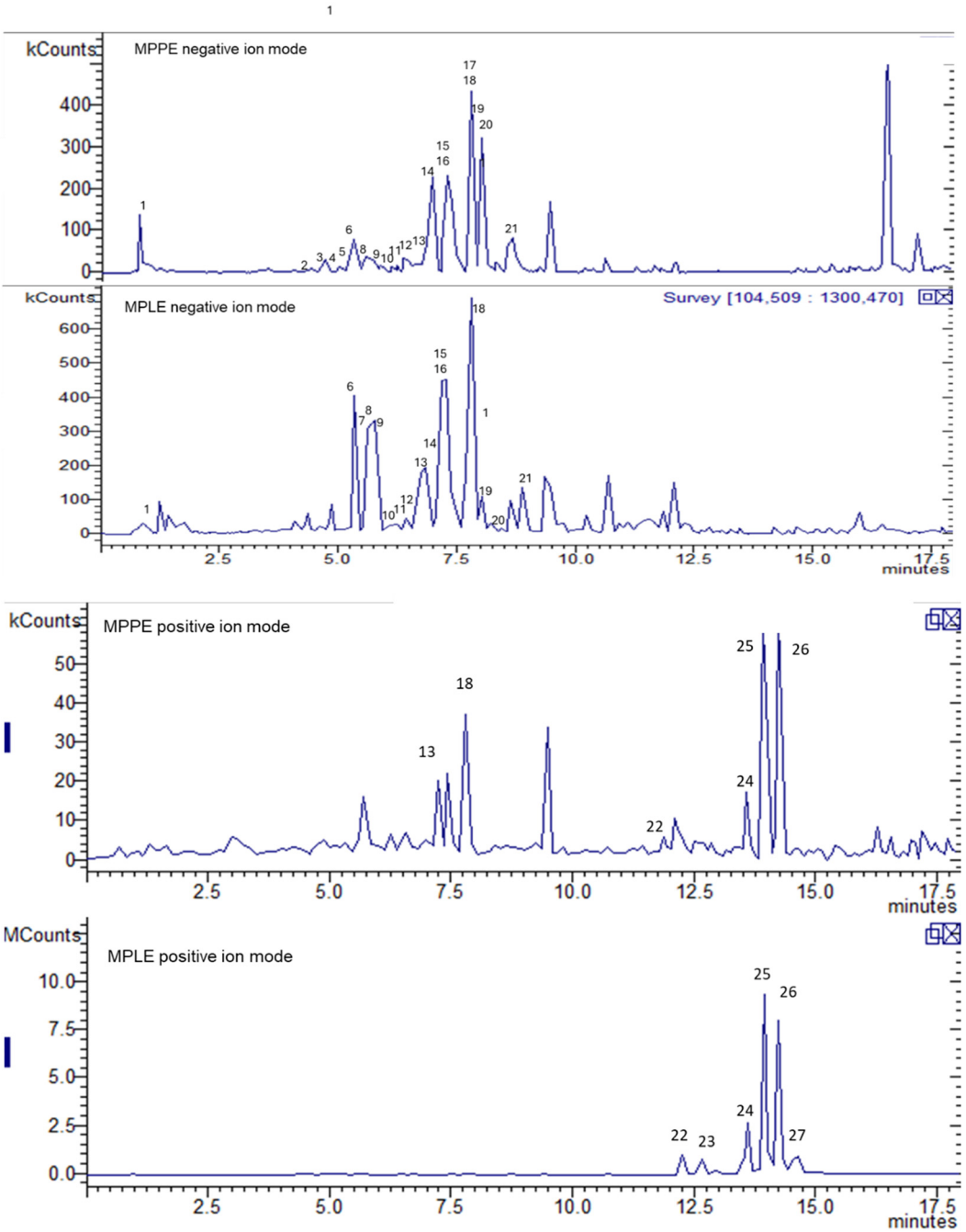

Fig. 2 Exemplificative chromatograms of MPLE and MPPE in negative and positive ion modes. Peaks are indicated with numbers corresponding to the identified compounds reported in Table 2.

3.3. Isolation and structure elucidation of phytoconstituents from MPLE

Six compounds were isolated from MPLE, and their structures were elucidated using the NMR technique. The obtained structures are reported in Fig. 3. The isolated constituents are three polymethoxyflavones (nobiletin, 3-methoxynobiletin, tangeretin),[11] two glycosylated flavonoids (narirutin, homoeriodictiyol-7-*O*-neohesperidoside), and a hydroxycinnamic acid derivative (ferulic acid).[20]

3.4. The effects of MPO, MPLE, MPPE, and isolated compounds on LDL receptor and PCSK9 expression in HuH7 cells

An *in vitro* model of the human hepatoma cell line Huh7 was used to evaluate the potential hypocholesterolemic activities of Mapo extracts and isolated compounds, evaluating the induction of the LDL receptor, or the inhibition of PCSK9; both these conditions could influence the cholesterol homeostasis. As a starting point, MPO, MPLE, and MPPE were tested on both targets after 72 h of cell exposure by western blot analysis

Table 2 HPLC-DAD-MSn characterization of BE constituents

	Compound	$[M-H]^-/[M+H]^+$	Fragments	MPLE (mg g^{-1})	MPPE (mg g^{-1})
1	Citric acid	$[M-H]^-$ 191	111 129 173 87	1.73	0.65
2	Taxifolin-*O*-hexoside-deoxyhexoside	$[M-H]^-$ 611	303 285 241 175	0.10	0.00
3	Quercetin-*O*-hexoside-deoxyhexoside-*O*-hexoside	$[M-H]^-$ 711	609 301 270	0.06	0.00
4	Eriodictyol-*O*-hexoside-deoxyhexoside	$[M-H]^-$ 595	567 287 259 215 173 125	1.51	0.06
5	Luteolin O-rutinoside *O*-hexoside	$[M-H]^-$ 755	593 285 255 242 229	0.01	0.00
6	Apigenin 6.8 di-*C*-glucoside	$[M-H]^-$ 593	503 473 383 353	10.37	2.51
7	Diosmetin 6.8-di-*C*-glucoside	$[M-H]^-$ 623	503 443 383 312	13.45	0.10
8	Syringic acid derivative	$[M-H]^-$ 403	343 241 197	1.28	0.03
9	Rutin	$[M-H]^-$ 609	301 241	2.46	3.34
10	Luteolin *O*-hexoside-deoxyhexoside	$[M-H]^-$ 593	285 217 199 175	4.89	0.22
11	Naringenin-*O*-hexoside-*O*-3-hydroxy-3-methylglutary-*O*-hexoside	$[M-H]^-$ 739	577 475 433 271	7.25	1.59
12	Limonin glucoside	$[M-H]^-$ 649	65 461 443	0.73	0.45
13	Narirutin/isonaringin/naringenin 7-*O*-rutinoside	$[M-H]^-$ 579/$[M+H]^+$ 581	313 271 151	25.59	17.82
14	Deacetyl nomilin acid glucoside	$[M-H]^-$ 669	609 401 333 257	1.17	0.67
15	Isorhoifolin/apigenin-7-*O*-rutinoside	$[M-H]^-$ 577	269	8.19	2.58
16	3-*O*-Methylquercetin-*O*-hexoside-deoxyhexoside	$[M-H]^-$ 623	315 300 271 255 243	3.73	0.89
17	Homoeriodichtiyol-7-*O*-neoesperidoside	$[M-H]^-$ 609	301 240	28.75	39.44
18	Ferulic acid	$[M+H]^+$ 195		24.07	63.68
19	3 hydroxy 3 methylglutaryl syringetin hexoside	$[M-H]^-$ 651	507 345 302	6.52	8.21
20	Naringenin-7-*O*-glucoside-*O*-HMG[a]	$[M-H]^-$ 577	515 475 433 271	1.50	0.26
21	Kaemferol-*O*-rutinoside	$[M-H]^-$ 593	(628 Cl adduct) 285 270 164	8.95	3.27
22	Sinensetin	$[M+H]^+$ 373	359 343 329 315	26.22	0.44
23	Nobiletin isomer 1	$[M+H]^+$ 403	387 373 357 355 343 327	3.10	0.00
24	Nobiletin	$[M+H]^+$ 403	387 373 357 355 343 327	9.92	0.03
25	3-Methoxynobiletin[a]	$[M+H]^+$ 433	419 403 385 373	27.32	0.48
26	Tangeretin	$[M+H]^+$ 373	359 343 312	25.75	0.48
27	Nobiletin isomer 2	$[M+H]^+$ 403	387 373 369 355 341 329	4.48	0.00
			Total amount	264.10	147.22

[a] First identified in tangelos.

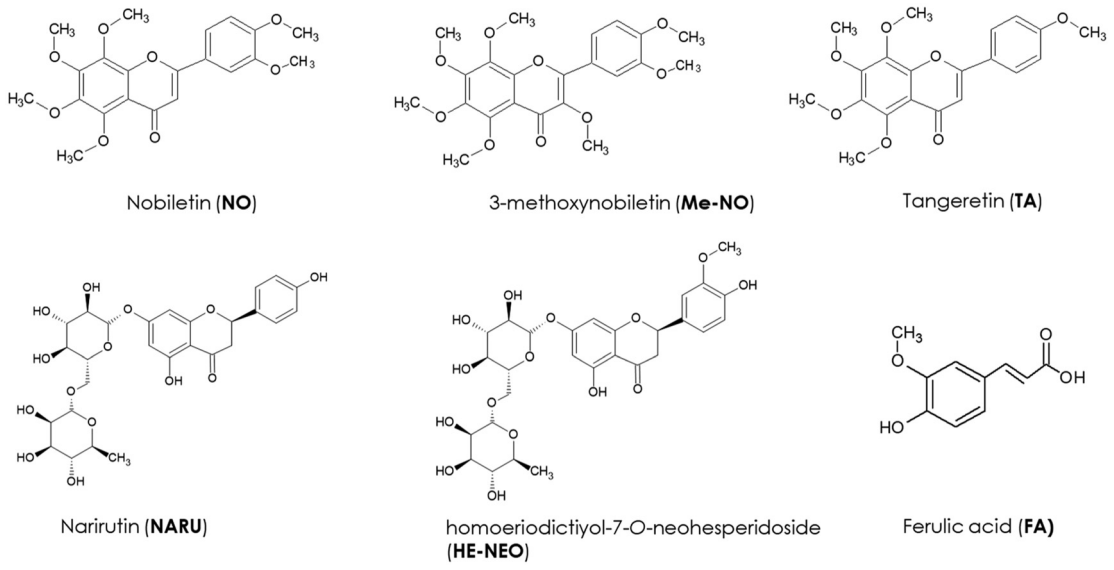

Fig. 3 Compounds isolated from MPLE.

from total protein extracts. Simvastatin was used as a positive control, being an inhibitor of the β-hydroxy β-methylglutaryl-coenzyme A (HMG-CoA), and inducer of both the LDL receptor and PCSK9 *in vitro*.[21]

As shown in Fig. 4, the incubation of the Huh7 cell line for 72 h with the extracts revealed that MPLE and MEO did not show any significant effect on LDL receptor expression, while they both reduced the PCKS9 expression (−25% ± 7% and

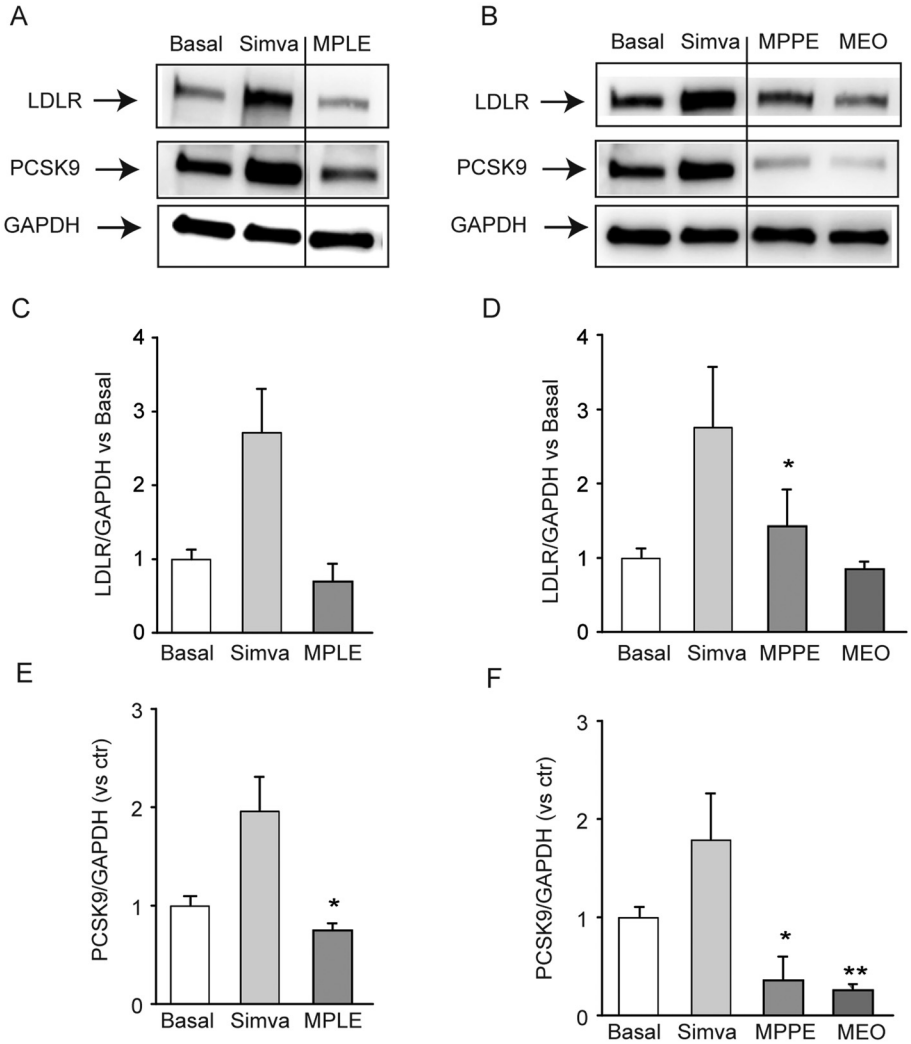

Fig. 4 Effects of MPLE, MPPE and MEO on LDL receptor and PCSK9 expression in the HuH7 human cell line. Cells were incubated with 5 μM simvastatin, 100 μg ml^{-1} extract, 25 μM PMFs or 100 μM flavanones isolated from Mapo peel extract for 72 h. (A and B). Representative western blotting analysis for the expression of the LDL receptor and PCSK9 upon treatment. GAPDH was used as a loading control. (C and D). Densitometric analysis of the LDL receptor/GAPDH ratio. (E and F). Densitometric analysis of the PCSK9/GAPDH ratio. Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ *vs.* control by Student's *T*-test. Ctr: control; Simva: simvastatin; MPLE: Mapo peel extract; MPPE: Mapo pulp extract; MEO: Mapo essential oil; LDLR: low-density lipoprotein receptor; proPCSK9: not mature protein convertase subtilisin/kexin type 9; matPCSK9: mature PCSK9; and GAPDH: glyceraldehyde phosphate dehydrogenase.

−74% ± 6% *vs.* basal for MPLE and MEO, respectively) (Fig. 4). On the other hand, MPPE significantly induced the LDL receptor (+1.43 ± 0.49-fold *vs.* basal), and suppressed PCSK9 levels (−64% ± 24% *vs.* basal), thus suggesting potential hypocholesterolemic activity. For this reason, we extended the evaluation of the bioactivity on the two protein targets to the most abundant constituents.

Nobiletin and tangeretin are particularly abundant in the peel extract, while the terpenoids D-limonene and γ-terpinene are the most abundant constituents of the essential oil (Table 2). We observed that nobiletin, tangeretin and limonene at the tested concentrations significantly reduced the expression of the LDL receptor (−73% ± 19%, −73% ± 1%, and −61% ± 2% *vs.* basal, for nobiletin, tangeretin and limonene, respectively) but also reduced the expression of PCSK9 (−56% ± 25%, −72% ± 10%, −86% ± 7%, for nobiletin, tangeretin and limonene, respectively) (Fig. 5). γ-Terpinene only reduced the PCSK9 levels (−81% ± 12%). Thus, some of these compounds may be considered as possible models in the search for new small molecules that can inhibit PCSK9.

On the other hand, narirutin showed a significant, although moderate, induction of the LDL receptor expression (+1.21 ± 0.21-fold *vs.* basal) and a significant reduction of PCSK9 expression (−55% ± 16% *vs.* basal), making it valuable as a possible model compound in the search for hypocholesterolemic agents due to the contemporaneous activity on the two targets (Fig. 5). This observation was previously made by

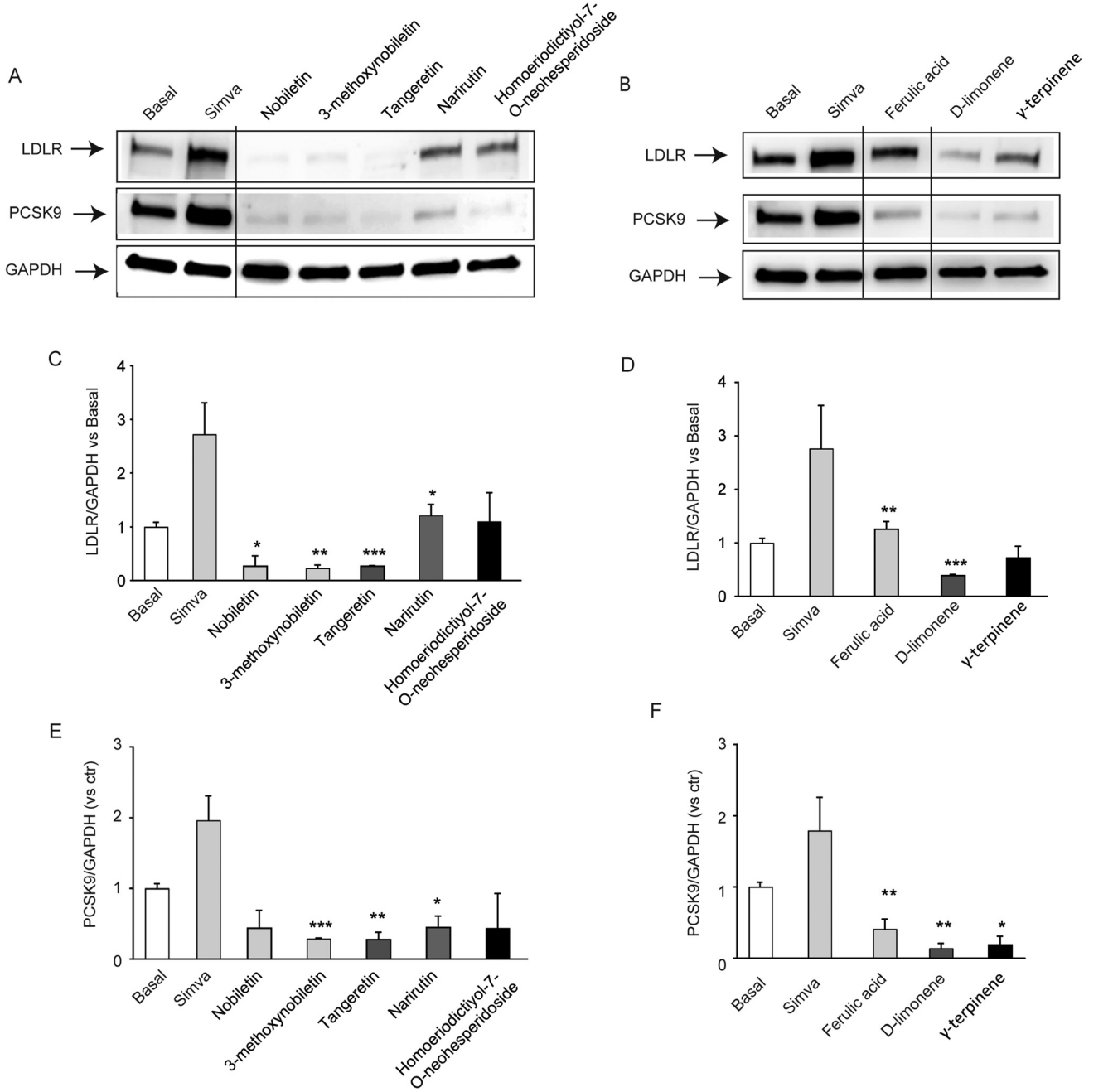

Fig. 5 Effects of isolated compounds on LDL receptor and PCSK9 expression in the HuH7 human cell line. Cells were incubated with 5 μM simvastatin, 100 μg ml^{-1} extracts, 50 μg ml^{-1} monoterpenes or 100 μM ferulic acid for 72 h. (A and B). Representative western blot analysis for the expression of the LDL receptor and PCSK9 upon treatment; GAPDH was used as a loading control. (C and D). Densitometric analysis of the LDL receptor/GAPDH ratio. (E and F). Densitometric analysis of the PCSK9/GAPDH ratio. Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ *vs.* control by Student's *T*-test. Simva: simvastatin; LDLR: low-density lipoprotein receptor; proPCSK9: not mature protein convertase subtilisin/kexin type 9; matPCSK9: mature PCSK9; and GAPDH: glyceraldehyde phosphate dehydrogenase.

our research group during the evaluation of the hypocholesterolemic activity of bergamot extract.[22] We also observed for the homoeriodichtiyol-7-*O*-neoesperidoside the tendency to upregulate the LDL receptor and downregulate PCSK9, but the observed effects were not statistically significant compared to control cells.

Ferulic acid, the most abundant compound in the MPPE, significantly induced the LDL receptor (+1.26 ± 0.14-fold *vs.* basal) and suppressed PCSK9 levels (−59% ± 14% *vs.* basal), thus suggesting a potential hypocholesterolemic activity and explaining, at least in part, the observed effect for the MPPE (Fig. 4).

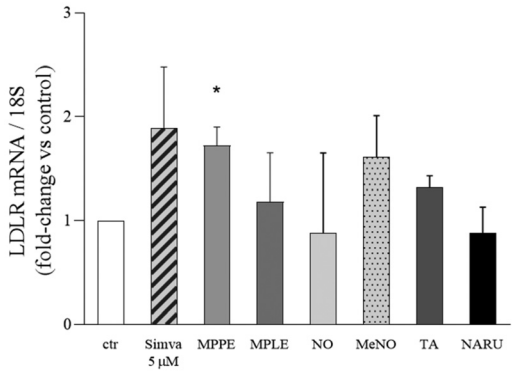

Fig. 6 Effects of Mapo extracts and isolated compounds on LDLR mRNA expression in Huh7 cells. LDLR mRNA expression was evaluated by RT-qPCR analysis and normalized to 18S. Data are presented as mean ± SD of three independent experiments. *$p < 0.05$ *vs.* control by Student's *T*-test. Ctr: control; Simva: simvastatin; MPPE: Mapo pulp extract; MPLE: Mapo peel extract; NO: nobiletin; Me-NO: 3-methoxynobiletin; TA: tangeretin; and NARU: narirutin.

3.5. The effects of MPO, MPLE, MPPE, and selected compounds on LDLR mRNA expression in HuH7 cells

To further investigate the molecular mechanisms underlying the effect of Mapo extracts and isolated compounds on genes related to cholesterol metabolism, we measured the mRNA levels of LDL receptor by real-time qPCR after 24 h exposure to the Huh7 cell line. As predicted, simvastatin induced LDL receptor by 2-fold (Fig. 6). Very similarly, we observed that MPPE significantly increased the mRNA expression of the LDL receptor. Me-NO and TA showed a similar effect, although the induction of LDL receptor mRNA did not reach statistical significance (Fig. 6).

4. Discussion

'Mapo' is an Italian cultivar of the *Citrus tangelo* hybrid. In our work, we analysed the composition of different extracts. The MPO was characterized by GC-MS, showing the large presence of D-limonene and γ-terpinene, and revealing results similar to those previously published by other groups.[3,4,15] Herein, we identified and quantified the phytoconstituents showing that in MPLE, the most abundant compounds are ferulic acid, homoeriodichtiyol-7-*O*-neoesperidoside, naringenin 7-*O*-rutinoside, 3-methoxynobiletin, sinensetin, and tangeretin. For MPPE, the most abundant are ferulic acid and homoeriodichtyol-7-*O*-neoesperidoside. The extracts not only present differences in the chemical composition but also their bioactivities. The *in vitro* effects of MPO and MPLE on the LDL receptor and PCSK9 expression appear limited, while MPPE presents valuable effects with an induction of LDL receptor and inhibition of PCSK9. In addition, we found that MPPE induced the expression of the mRNA of the LDL receptor, thus indicating a regulatory mechanism at transcriptional levels. However, the exact mechanism of action still needs to be determined.

To establish the role of the main constituents, we considered the most abundant compounds of MPO; D-limonene, γ-terpinene, and the other compounds significantly decreased the expression of PCSK9, an effect that may improve the effect of statins or red yeast rice extracts on the LDL receptor.[23] Considering the MPPE and MPLE constituents, naringenin 7-*O*-rutinoside and homoeriodictiyol-7-*O*-neohesperidoside are present in both extracts. Naringenin 7-*O*-rutinoside significantly increased the expression of LDL receptor and reduced that of PCSK9; such a mutual effect on protein expression may suggest that narirutin acts by the same mechanism as berberine.[23] Homoeriodictiyol-7-*O*-neohesperidoside showed a similar but not significant trend.

Ferulic acid is present in high amounts in MPPE and was also detected in MPLE, but in a smaller quantity. We demonstrated, for the first time, that ferulic acid enhanced LDL receptor protein expression and downregulated PCSK9, showing potential cholesterol-lowering properties with a mechanism of action that may involve the inhibition of the HNF1-α transcription factor regulating PCSK9 expression.[24] Since a previous report showed that ferulic acid acts by decreasing plasma lipids (total cholesterol and triglycerides) and liver cholesterol in an *in vivo* rat model,[25] our results further support the potential use of this compound as a cholesterol-lowering agent. These results indicate that the MPPE extract can be considered as a new potential nutraceutical for controlling cholesterol levels. Different from MPPE, the MPLE extract downregulated both the LDL receptor (although not significantly) and PCSK9.

5. Conclusions

The results from the MAPO extract revealed that MPPE presents some advantageous properties on two key targets of cholesterol homeostasis, namely, inducing the LDL receptor and reducing PCSK9 *in vitro* at 100 μg mL^{-1}. These data indicate that MPPE extracts can be useful as new hypocholesterolemic nutraceuticals to be utilized in conjunction with a healthy diet containing unsaturated fatty acids that increase hepatic LDL receptor activity, protein, and mRNA abundance, which will increase the clearance of LDL from the circulation.[26,27] The chemical analysis and isolation of main constituents and the assay of isolated compounds showed a significant effect for the ferulic acid, which at 100 μM was able to induce the LDL receptor and reduce PCSK9, thus at least in part explaining the effect observed for the MPPE. Furthermore, other constituents of peels and the main terpene of the essential oil showed some significant effects as PCSK9 inhibitors, thus indicating that other investigations are needed to fully understand the effects of the extracts. Mapo tangelo can be considered as a valuable source of bioactive compounds with potential usefulness in hypercholesterolemia. More studies are needed to establish doses, safety, further modes of action, and to assess potential efficacy *in vivo*.

Conflicts of interest

There are no conflict of interest do declare.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its ESI.

References

1 F. Mach, C. Baigent, A. L. Catapano, K. C. Koskinas, M. Casula, L. Badimon, M. J. Chapman, G. G. De Backer, V. Delgado, B. A. Ference, I. M. Graham, A. Halliday, U. Landmesser, B. Mihaylova, T. R. Pedersen, G. Riccardi, D. J. Richter, M. S. Sabatine, M. R. Taskinen, L. Tokgozoglu, O. Wiklund and E. S. C. S. D. Group, 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk, *Eur. Heart J.*, 2020, **41**, 111–188.

2 X. F. Zeng, K. A. Varady, X. D. Wang, G. Targher, C. D. Byrne, R. Tayyem, G. Latella, I. Bergheim, R. Valenzuela, J. George, C. Newberry, J. S. Zheng, E. S. George, C. W. Spearman, M. D. Kontogianni, D. Ristic-Medic, W. A. F. Peres, G. Y. Depboylu, W. Yang, X. Chen, F. Rosqvist, C. S. Mantzoros, L. Valenti, H. Yki-Jarvinen, A. Mosca, S. Sookoian, A. Misra, Y. Yilmaz, W. Kim, Y. Fouad, G. Sebastiani, V. W. Wong, F. Aberg, Y. J. Wong, P. Zhang, F. J. Bermudez-Silva, Y. Ni, M. Lupsor-Platon, W. K. Chan, N. Mendez-Sanchez, R. J. de Knegt, S. Alam, S. Treeprasertsuk, L. Wang, M. Du, T. Zhang, M. L. Yu, H. Zhang, X. Qi, X. Liu, K. Pinyopornpanish, Y. C. Fan, K. Niu, J. C. Jimenez-Chillaron and M. H. Zheng, The role of dietary modification in the prevention and management of metabolic dysfunction-associated fatty liver disease: An international multidisciplinary expert consensus, *Metab., Clin. Exp.*, 2024, **161**, 156028.

3 G. Dugo, A. Cotroneo, A. Verzera, G. Dugo and G. Licandro, "Mapo" tangelo essential oil, *Flavour Fragrance J.*, 1990, **5**, 205–210.

4 G. Ruberto, D. Biondi, P. Rapisarda, A. Renda and A. Starrantino, Essential Oil of Cami, a New Citrus Hybrid, *J. Agric. Food Chem.*, 1997, **45**, 3206–3210.

5 M. S. Ladaniya, Commercial fresh citrus cultivars and producing countries, in *Citrus Fruit: Biology, Technology and Evaluation*, Elsevier, 2008, DOI: **10.1016/b978-012374130-1.50004-8**.

6 W. Widmer, One Tangerine/Grapefruit Hybrid (Tangelo) Contains Trace Amounts of Furanocoumarins at a Level Too Low To Be Associated with Grapefruit/Drug Interactions, *J. Food Sci.*, 2005, **70**, c419–c422.

7 J. J. Peterson, J. T. Dwyer, G. R. Beecher, S. A. Bhagwat, S. E. Gebhardt, D. B. Haytowitz and J. M. Holden, Flavanones in oranges, tangerines (mandarins), tangors, and tangelos: a compilation and review of the data from the analytical literature, *J. Food Compos. Anal.*, 2006, **19**, S66–S73.

8 B. Singh, J. P. Singh, A. Kaur and N. Singh, Phenolic composition, antioxidant potential and health benefits of citrus peel, *Food Res. Int.*, 2020, **132**, 109114.

9 Z. Gao, W. Gao, S. L. Zeng, P. Li and E. H. Liu, Chemical structures, bioactivities and molecular mechanisms of citrus polymethoxyflavones, *J. Funct. Foods*, 2018, **40**, 498–509.

10 Z. Zhao, S. He, Y. Hu, Y. Yang, B. Jiao, Q. Fang and Z. Zhou, Fruit flavonoid variation between and within four cultivated Citrus species evaluated by UPLC-PDA system, *Sci. Hortic.*, 2017, **224**, 93–101.

11 D. Barreca, C. Bisignano, G. Ginestra, G. Bisignano, E. Bellocco, U. Leuzzi and G. Gattuso, Polymethoxylated, C- and O-glycosyl flavonoids in tangelo (Citrus reticulataxCitrus paradisi) juice and their influence on antioxidant properties, *Food Chem.*, 2013, **141**, 1481–1488.

12 Y. Deng, Y. Tu, S. Lao, M. Wu, H. Yin, L. Wang and W. Liao, The role and mechanism of citrus flavonoids in cardiovascular diseases prevention and treatment, *Crit. Rev. Food Sci. Nutr.*, 2022, **62**, 7591–7614.

13 G. G. Pan, P. A. Kilmartin, B. G. Smith and L. D. Melton, Detection of orange juice adulteration by tangelo juice using multivariate analysis of polymethoxylated flavones and carotenoids, *J. Sci. Food Agric.*, 2002, **82**, 421–427.

14 M. S. Njeroge, H. Koaze, M. Mwaniky, N. T. Minh Tu and M. Sawamura, Essential oils of Kenyan Citrus fruits_ volatile components of two varieties of mandarins (Citrus reticulata) and a tangelo (C. paradisi × C. tangerina), *Flavour Fragrance J.*, 2005, **20**, 74–79.

15 S. Fabroni, G. Ruberto and P. Rapisarda, Essential oil profiles of new Citrus hybrids, a tool for genetic citrus improvement, *J. Essent. Oil Res.*, 2012, **24**, 159–169.

16 N. Ferri, M. Ruscica, S. Fazio and A. Corsini, , Low-Density Lipoprotein Cholesterol-Lowering Drugs: A Narrative Review, *J. Clin. Med.*, 2024, **13**, 943.

17 V. I. Babushok, P. J. Linstrom and I. G. Zenkevich, Retention Indices for Frequently Reported Compounds of Plant Essential Oils, *J. Phys. Chem. Ref. Data*, 2011, **40**, 043101-1–043101-45.

18 M. G. Lupo, C. Macchi, S. Marchiano, R. Cristofani, M. F. Greco, S. Dall'Acqua, H. Chen, C. R. Sirtori, A. Corsini, M. Ruscica and N. Ferri, Differential effects of red yeast rice, Berberis aristata and Morus alba extracts on PCSK9 and LDL uptake, *Nutr., Metab. Cardiovasc. Dis.*, 2019, **29**, 1245–1253.

19 P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney and M. S. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, *J. Natl. Cancer. Inst.*, 2023, **23**, 54.

20 B. Nayak, F. Dahmoune, K. Moussi, H. Remini, S. Dairi, O. Aoun and M. Khodir, Comparison of microwave, ultrasound and accelerated-assisted solvent extraction for recovery of polyphenols from Citrus sinensis peels, *Food Chem.*, 2015, **187**, 507–516.

21 S. Sut, I. Ferrarese, M. G. Lupo, N. De Zordi, E. Tripicchio, N. Ferri and S. Dall' Acqua, The Modulation of PCSK9 and LDLR by Supercritical CO(2) Extracts of Mentha longifolia and Isolated Piperitone Oxide, an In Vitro Study, *Molecules*, 2021, **26**, 3886.

22 I. Ferrarese, M. G. Lupo, I. Rossi, S. Sut, F. Loschi, P. Allegrini, A. Riva, N. Ferri and S. Dall'Acqua, Bergamot (Citrus bergamia) peel extract as new hypocholesterolemic agent modulating PCSK9 expression, *J. Funct. Foods*, 2023, **108**, 105724.

23 M. G. Lupo, C. Macchi, S. Marchiano, R. Cristofani, M. F. Greco, S. Dall'Acqua, H. Chen, C. R. Sirtori, A. Corsini, M. Ruscica and N. Ferri, Differential effects of red yeast rice, Berberis aristata and Morus alba extracts on PCSK9 and LDL uptake, *Nutr., Metab. Cardiovasc. Dis.*, 2019, **29**, 1245–1253.

24 H. Li, B. Dong, S. W. Park, H. S. Lee, W. Chen and J. Liu, Hepatocyte nuclear factor 1alpha plays a critical role in PCSK9 gene transcription and regulation by the natural hypocholesterolemic compound berberine, *J. Biol. Chem.*, 2009, **284**, 28885–28895.

25 Y. H. Yeh, Y. T. Lee, H. S. Hsieh and D. F. Hwang, Dietary caffeic acid, ferulic acid and coumaric acid supplements on cholesterol metabolism and antioxidant activity in rats, *J. Food Drug Anal.*, 2009, **17**, 123–132.

26 M. L. Fernandez and K. L. West, Mechanisms by which dietary fatty acids modulate plasma lipids, *J. Nutr.*, 2005, **135**, 2075–2078.

27 J. M. Dietschy, Dietary fatty acids and the regulation of plasma low density lipoprotein cholesterol concentrations, *J. Nutr.*, 1998, **128**, 444S–448S.