View Article Online
View Journal

Food & Function

Linking the chemistry and physics of food with health and nutrition

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: I. Ferrarese, M. G. Lupo, I. Rossi, G. Marodin, H. Chen, S. Sut, N. Ferri and S. Dall'Acqua, *Food Funct.*, 2025, DOI: 10.1039/D5FO01511A.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/food-function

View Article Online
DOI: 10.1039/D5FO01511A

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

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

[a] Department of Pharmaceutical and Pharmacological Sciences, University of Padova, 35131 Padova, Italy;

[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

* Corresponding author

View Article Online
DOI: 10.1039/D5FO01511A

Abstract

Peel (MPLE), pulp extract (MPPE) and the essential oil (MPO) of *Citrus x tangelo* were studied for their chemical composition and for their ability to act on the expression of low-density lipoprotein (LDL) receptor and proprotein convertase subtilisin/kexin 9 (PCSK9), two key players of cholesterol metabolism, in human hepatocarcinoma cell line Huh7. MPPE shows ability to induce LDL receptor and to reduce PCSK9 respectively. Chemical characterization of the extracts was obtained by LC-DAD-MS for the MPLE and MPPE while by GC-MS for MPO. The main compounds in MPLE and MPPE were isolated and 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 suppress 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.

View Article Online
DOI: 10.1039/D5FO01511A

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide, imposing substantial health and economic burden. The first line of intervention is to recoomed an healthy diet low in saturated fat with a focus on wholegrain 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, accounting for >4 million global deaths. Nevertheless, the large majority of 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, recently the European Food Safety Authority (EFSA) has reduced the maximal dosage of monacolin K, the active compound of 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 the mild hypercholesterolemia for primary prevention in the general population. Here, we investigated the potential lipid lowering effect of 'Mapo', as starting point for the development of a new nutraceutical product.

'Mapo' tangelo (*Citrus* x *tangelo*) is a citrus Italian cultivar developed in 1950 by crossing 'Avana' mandarin (*Citrus deliciosa* Ten.) and 'Duncan' grapefruit (*Citrus paradisi* Macf.) [3-5]. Tangelo hybrid exists in a large number of cultivars [6] and they are tangerine-grapefruit or pummelo hybrids (*C. reticulata* x *C. paradisi* or *C. reticulata* x *C. grandis*) [7]. Citrus fruits peels represent in general the 40-50% of the total fruit mass but this part is not edible so is considered a waste. Peels are a source of health enhancing compounds as phenolic compounds, terpenoids. Most common are hydroxycinnamic derivatives as caffeic, p-coumaric, ferulic and sinapic acid, the flavanones as naringin and hesperidin and the polymethoxylated flavones as nobiletin and tangeretin [8]. The peels are rich in many volatile terpenoids as well as in non-terpenoid derivatives as polymethoxyflavones that are detected in relevant amount compared to edible fruit part [8-10]. Among flavonoids, flavanones are the most represented in Citrus tangelo and the glycosyl derivatives as naringenin, narirutin, eriocitrin, neoeriocitrin, poncirin, hesperidin, neohesperidin, and didymin are in general prevalent than the aglycones that are commonly the hesperetin, eriodictyol and naringenin [11]. Several *C*-glycosyl flavones and *O*-triglycosyl flavanone were detected in tangelo juice[9]. Among

View Article Online
DOI: 10.1039/D5FO01511A

polymethoxyflavones, nobiletin, tangeretin and sinensetin are the most abundant in citrus peels [10]. Tangelos are tangerine-grapefruit or pummelo hybrids [7] and, differently from grapefruit, these varieties contain traces or not 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 polymethoxyflavones pharmacological activity and mechanism of action 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 as 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 an hypolipidemic effect throw 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 tangelos varieties Honeyball, K-Early, Minneola and Seminole, flavanones are the predominant compounds, being in concentration of 30 mg/100 g juice [7]. Other authors reported that hesperidin (21.1 mg/L), narirutin (6.3 mg/L) and vicenin-2 (3.9 mg/L) 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, as three studies reported exclusively a characterization of the volatile constituents [3, 4, 15].

In the present study we investigated the effect 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 of 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 isolated compounds and the most abundant constituents of the essential oil namely D-limonene, and γ-terpinene were all tested for their ability to modulate the expression of the LDL receptor and PCSK9.

View Article Online
DOI: 10.1039/D5FO01511A

2. Material and methods

2.1. Fruit Samples

The fruits of *Citrus* x *tangelo* ('Mapo' tangelo) were supplied in 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)

Microwave-assisted extraction (MAE)

A Microwave-Assisted Extraction (MAE) system was used in order 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 accomplished using microwave in two times. 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 with 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).

Gas Chromatography-Mass Spectrometric (GC–MS) analysis

For the analysis a Varian 3900 gascromatograph equipped with autosampler and coupled to a Varian Saturn 2100T Ion-trap (MS/MS) was used. As stationary phase an Agilent HP-INNOVAX column (30m x 0.250 mm x 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. Total run time: 50 min. The flow rate carrier gas (helium) was 1.0 mL min^{-1}. A spitless injection was used. 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. Essential oil sample was diluted in diethyl ether in a concentration of 10 microliter of MPO diluted in 25 mL of solvent, as internal standard nonanol was used. Compounds were identified by matching the following criteria, at first comparison of the experimental MS spectra with data from mass spectra 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 reference solution of standard compounds was finally used to confirm identity of the compound.

For quantification purposes nonanol was used as an internal standard and mixture of *n*-nonanol and D-limonene, germacrene D and sclareol were obtained. Semi-quantification of the compounds was obtained using D-limonene for the monoterpene, Germacrene D for the sesquiterpenoids and

View Article Online
DOI: 10.1039/D5FO01511A

Sclareol for the diterpenoid. Quantification of compounds 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 20 % of 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) were obtained. Then, mapo pulps (200 g) were hand-squeezed and the obtained juice was frozen and freeze-dried by a Lyovapor L200 (BUCHI Ibérica S.L.U., 08960 Sant Just Desvern, Barcelona, Spain). 40 g of mapo pulp extract (MPPE) were 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 loaded on a Sephadex column (4 X 70 cm). Elution of the Sephadex column with methanol 0.5 ml/min yielded 144 fractions which were grouped in 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_20, 10:5:1 as solvent mixture. Purified fractions were evaporated to dryness under vacuum in a rotary evaporator at 55 °C. The obtained fractions weigh respectively: 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 finally 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 stationary phase, a Zorbax SB C-18 column (particle size 15µm, 21.2×150 mm) (Agilent Technologies, Santa Clara, CA, USA), was used. A mixture of 0.1 % formic acid in water (A), methanol (B) was used as mobile phase, and gradient was as follows: 0 min, 80 % A; 15 min, 20 % B.

Structure elucidation was achieved by 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, 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 50 % methanol (25 mL) using ultrasound bath and analysed using an LC-DAD-MS^n system. The system consisted of an Agilent 1260

View Article Online
DOI: 10.1039/D5FO01511A

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, Zorbax SB C18 column (250 × 4.6 mm, 5 µm) (Agilent Technologies, Santa Clara, CA, USA), was used. As mobile phase, a mixture of 0.1 % formic acid in water (A), acetonitrile (B) and methanol (C) was used, and 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. 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. Identification of compounds was obtained based on comparison with the literature and reference compounds, when available. For compounds quantification, rutin, naringenin, catechin, sinensetin and ferulic acid were used. Standard solutions were prepared in the concentration ranges 1–100 µg/ mL and calibration curves were built and used for quantification purposes. Quantitative data are mean of three repeated analyses.

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

MPLE, MPPE, MPO extract were dissolved in DMSO to a stock concentration of 20 mg/ml and used at 100 µg/ml 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 50 mM stock concentration and used at 5 µM as previously reported [18]. If not used, all the stocks 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 a stock solution of 100 mg/ml and 80 mM. Simvastatin was obtained by 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 manufacturers. The solution was then

View Article Online
DOI: 10.1039/D5FO01511A

sterilized by filtration. Huh7 cell line were 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 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.

2.7.3. Sulphorodamine B cell viability assay

Cell viability of the extract and the isolated compounds was assessed by the means of sulphorodamine (SRB) assay according to the protocol established by Skehan et al. [19]. Briefly, $8 \cdot 10^3$ cells/ well were seeded in a 96 well-tray in 100 µL/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 of the appropriate % of DMSO to be comparable with compounds and mapo extracts treatments. After 72 h of incubation, SRB assay was performed and absorbances measured at 570 nm with Victor Nivo multiplate reader by PerkinElmer.

2.7.4. Western blotting for intracellular PCSK9 and LDL receptor detection

Nontoxic compounds and extract concentrations (100 µM or 25 µM and 100 µg/ml or 50 µg/ml, respectively) were used for the western blotting analysis. $5 \cdot 10^5$ cells/well were seeded in a 60 mm cell culture dishes in 3 mL/dish of complete medium. The following day the old media was replaced by fresh media 10 % FBS containing treatments after a wash with sterile PBS. Simvastatin 5 µM was used as positive control as PCSK9 and LDL receptor inducer. Untreated controls and Simvastatin controls were supplied of the appropriate % of DMSO to be comparable with compounds and mapo extracts 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 Abcam recipe). Protein quantification in samples was carried out with bicinchoninic acid assay (SERVA) and samples for the electrophoretic run equalized to the same concentration by dilution with opportune amount of lysis buffer and the addition of a home-made Laemli loading buffer (prepared according

View Article Online
DOI: 10.1039/D5FO01511A

to Abcam recipe). Protein denaturation was then enhanced by 5 min at 95 °C. A total amount of ≥20 µg of protein/samples were loaded into the SDS-PAGE wells and left to separate under denaturing conditions (Bio- Rad apparatus). Proteins were then semi-dry transferred into a nitrocellulose membrane (Bio-Rad apparatus), upon which membranes were blocked with a 5 % non-fat skim milk solution in TBS-Tween 20 1X (hereafter named blocking solution) for 1 h at room temperature. To follow, an overnight incubation with primary antibodies for PCSK9, or LDL receptor, or GAPDH as loading control. The day after, membranes were washed three times (15 min each) with TBS-Tween 20 1X (TBST20 1X) and then incubated with HRP-conjugated secondary antibodies for 90 min at room temperature, followed by three further washes with TBST20 1X 15 min each, and ECL challenging at 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. 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') 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 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.

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 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 Kovats index and comparison of MS data as well as confirmation by standard injection. As expected, D-limonene (60.1 %) and ϒ-terpinene (4.2 %) were the most abundant monoterpenoids while δ-cadinene (3.7 %) and β-elemene (2.2 %) resulted the most represented sesquiterpenoids. The diterpene cembrene (3.9 %), sclareol (2.8 %) and α-muurolol (2.4 %) were on the other hand the most present in our sample of MPO. The quali-quantitative data are in good agreement with previous published analytical measurements on mapo essential oil [3].

Table 1. Table represents characterisation of volatile constituents of Mapo peel essential oil (MPO). Calculated retention indices are reported for each compound, (*) indicate confirmation of the identification by standard injection.

peak number	retention time (minutes)	compound	%	RI
1	5.4	β-pinene*	0.26	1111.1
2	5.7	sabinene	0.12	1122.2
3	7.2	α-terpinene*	1.08	1177.8
4	8.2	D-limonene*	60.12	1212.1
5	9.6	ϒ-terpinene	4.20	1254.5
6	9.8	β-ocimene	0.07	1260.6
7	10.3	m-cymene*	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*	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*	1.18	1558.8
16	21.1	β-elemene*	2.16	1588.2
17	21.5	β-gurjunene	0.04	1600.0
18	21.7	terpinen-4-ol*	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*	0.36	1660.6
22	23.8	β-farnesene	0.20	1669.7
23	24.2	ϒ-muurolene	0.24	1681.8
24	24.3	cedrene	0.08	1684.8

View Article Online
DOI: 10.1039/D5FO01511A

25	24.8	germacrene D*	0.61	1700.0
26	24.9	ϒ-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*	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*	2.77	not calculated

View Article Online
DOI: 10.1039/D5FO01511A

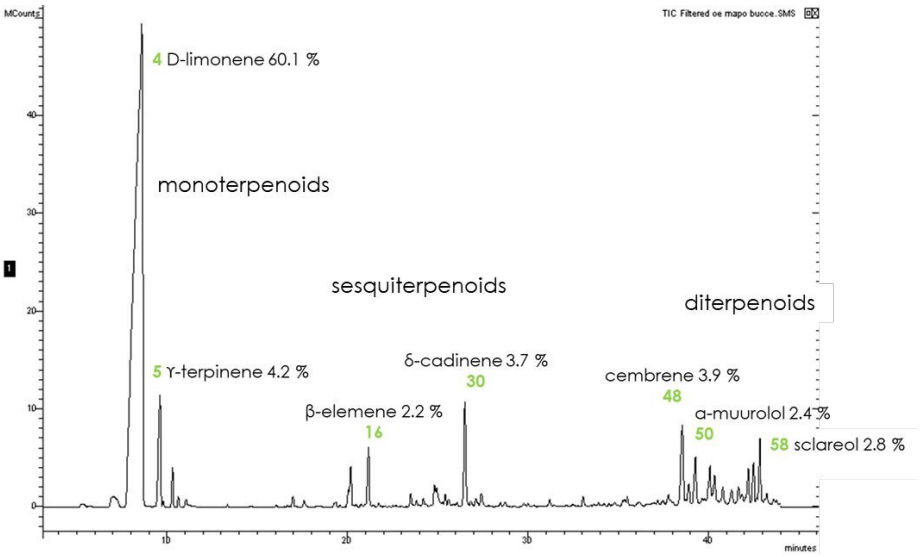
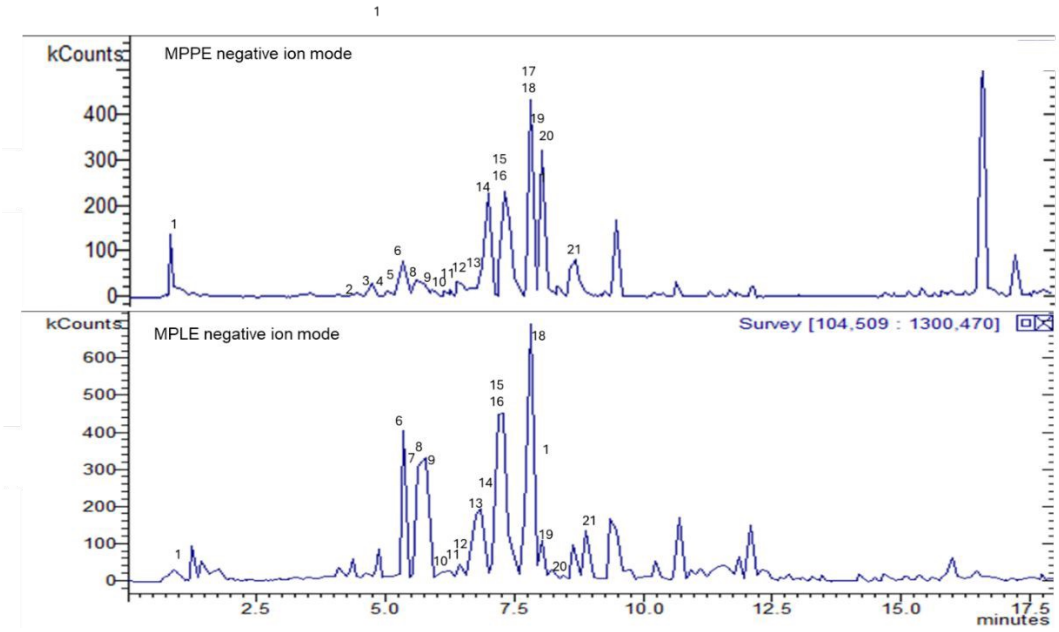


Figure 1. GC-MS chromatogram of mapo peel essential oil (MPO).

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 shown



View Article Online
DOI: 10.1039/D5FO01511A

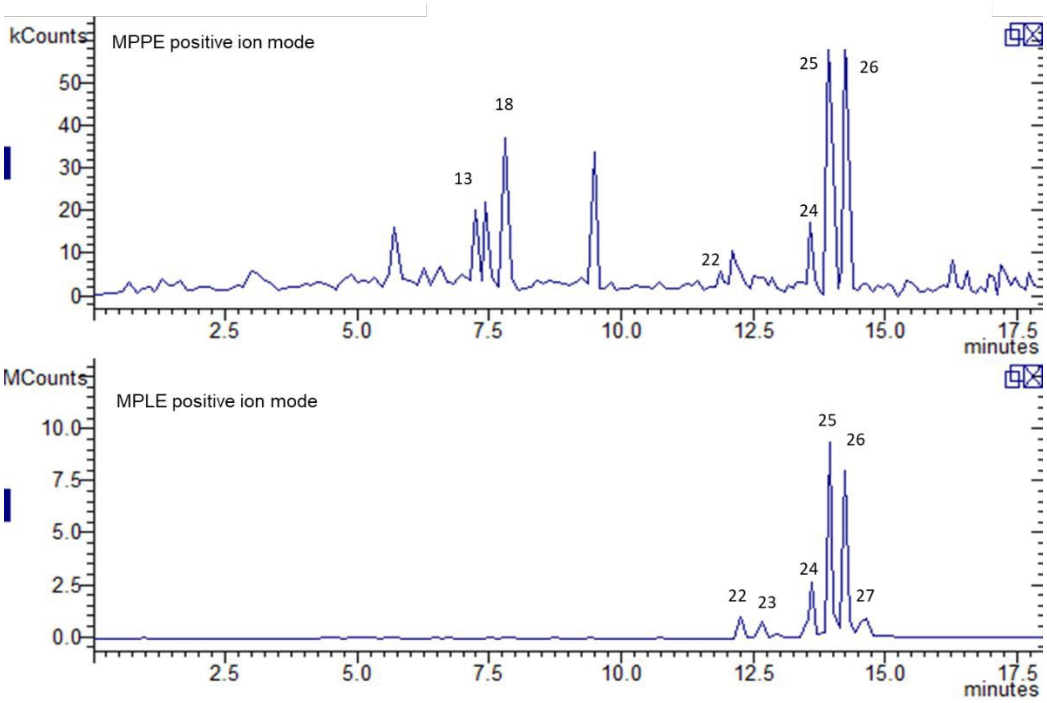


Figure 2. Exemplificative chromatograms of MPLE and MPPE in negative and positive ion mode, peaks are indicated with the number related to the identified compounds reported in the table 2.

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 3-hydroxy-3-methyl-glutaryl (HMG) moiety. More lipophilic compounds as polymethoxyflavones, were detected only in peels. Naringenin-7-*O*-glucoside-*O*-HMG and 3-methoxynobiletin were detected for the first time in tangelo species. Considering the two extracts data of the analysed samples revealed several *O*-glycoside flavonoids that have been detected mainly in pulps as taxifolin and eriodyctiol and luteolin glycosides, while diosmetin di-C-glucoside is detected mostly in peels. Data about the phytochemical composition agree with the previously published paper [11].

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

View Article Online
DOI: 10.1039/D5FO01511A

Table 2. HPLC-DAD-MSn characterization of BE constituents

				MPLE	MPPE
	compound	$[M-H]^-/[M+H]^+$	Fragments	mg/g	mg/g
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 *	$[M-H]^-$ 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 *	$[M+H]^+$ 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

*Firstly, identified in tangelos

3.3. Isolation and structure elucidation of phytoconstituents from MPLE

Six compounds were isolated from MPLE, and their structure was elucidated using NMR technique. The obtained structures are reported in Figure 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].

View Article Online
DOI: 10.1039/D5FO01511A

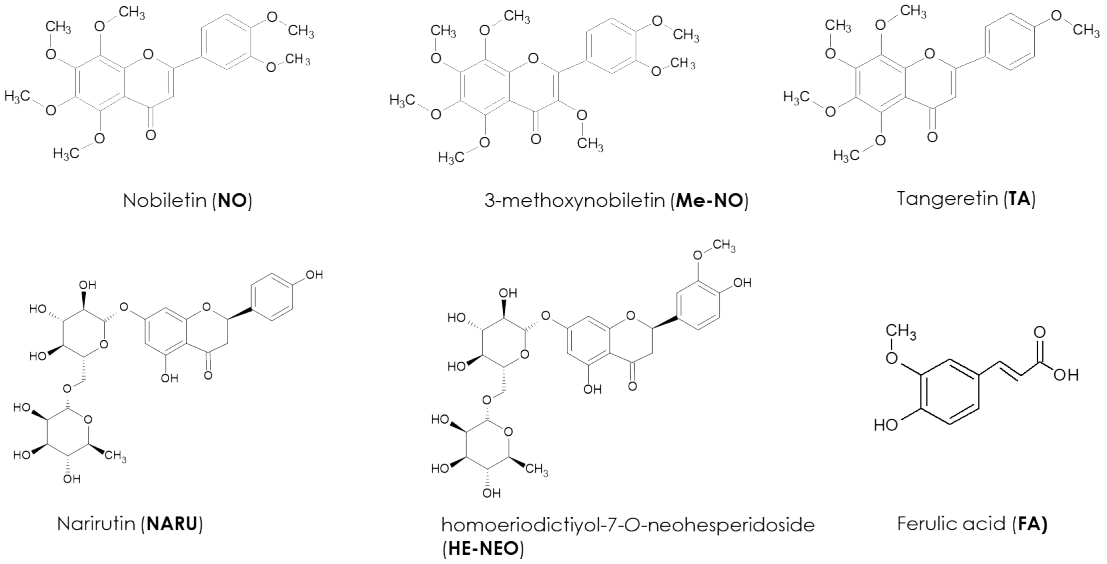


Figure 3. Isolated compounds MPLE.

3.4. Effect of MPO, MPLE, MPPE and isolated compounds on LDL receptor and PCSK9 expression in HuH7 cell

An *in vitro* model of 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 starting point MPO, MPLE, MPPE were tested on both targets after 72 h of cell exposure by western blot analysis from total protein extracts. As positive control, simvastatin was used being an inhibitor of the β-Hydroxy β-methylglutaryl-coenzyme A (HMG-CoA), and inducer of both LDL receptor and PCSK9 *in vitro* [21].

View Article Online
DOI: 10.1039/D5FO01511A

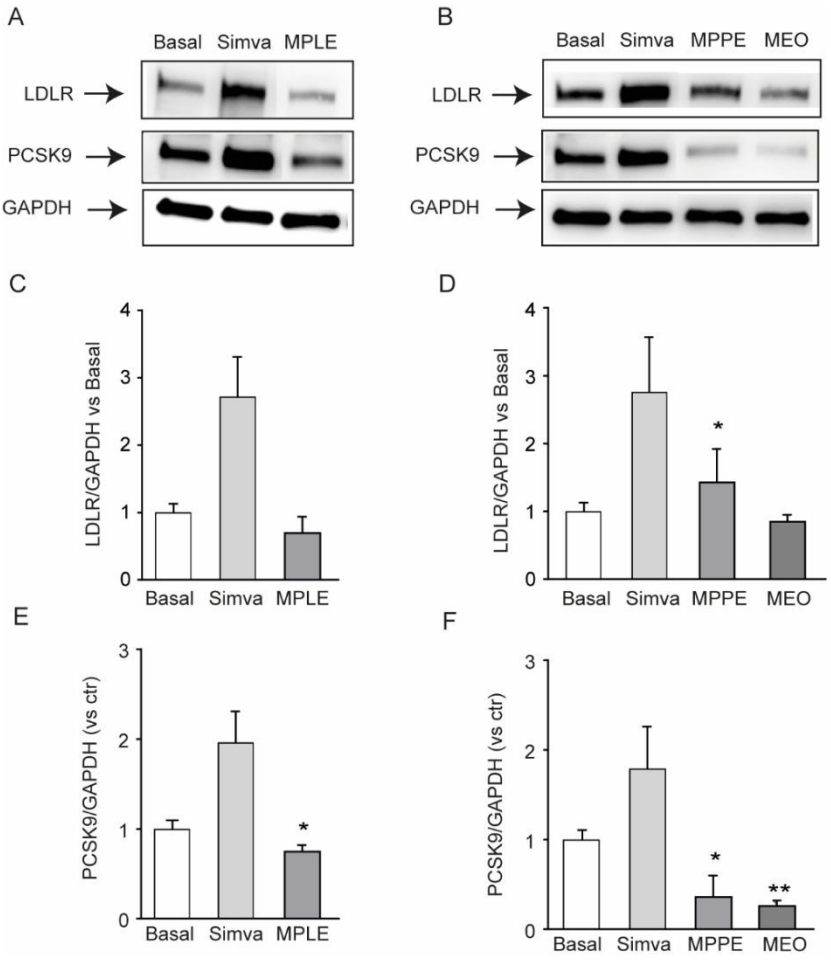


Figure 4. Effect of MPLE, MPPE and MEO on LDL receptor and PCSK9 expression in HuH7 human cell line. Cells were incubated with simvastatin 5 µM or 100 µg/ml of extract or 25 µM of the PMFs or 100 µM of the flavanones isolated from mapo peel extract for 72 h. A and B. Representative western blotting analysis for the expression of LDL receptor and PCSK9 upon treatments. GAPDH was used as loading control; C and D. Densitometric analysis of LDL receptor/GAPDH ratio; E and F. Densitometric analysis of PCSK9/GAPDH ratio. Data are presented as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***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; GAPDH: glyceraldehyde phosphate dehydrogenase.

As shown in Figure 4 the incubation of 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 reduce the PCKS9 expression (-25±7% and -74±6% vs basal for MPLE and MEO, respectively) (Figure 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 a potential hypocholesterolemic activity. For this reason, we extended the evaluation of the bioactivity on the two protein targets to the most abundant constituents.

View Article Online
DOI: 10.1039/D5FO01511A

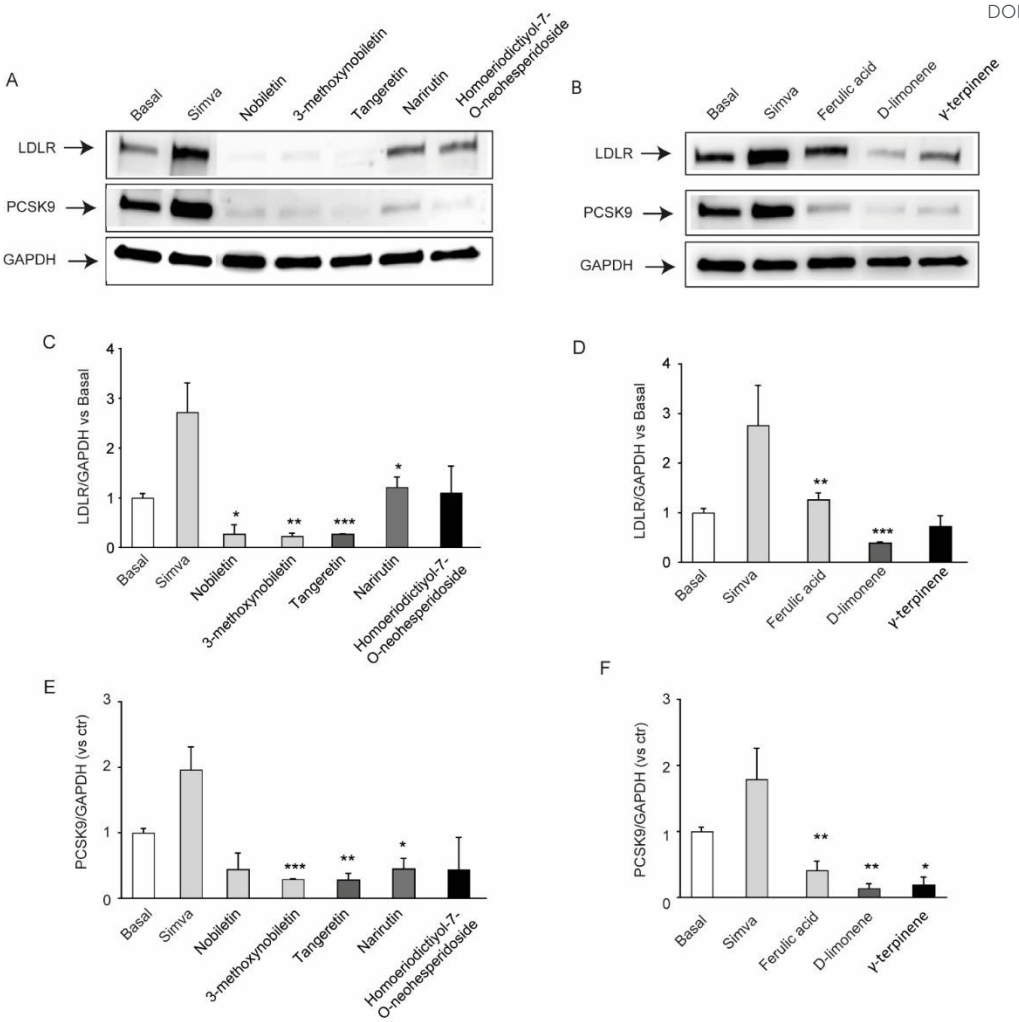


Figure 5. Effect of isolated compounds on LDL receptor and PCSK9 expression in HuH7 human cell line. Cells were incubated with simvastatin 5 µM or 100 µg/ml of extracts or 50 µg/ml of the monoterpenes or 100 µM of ferulic acid for 72 h. A and B. Representative western blotting analysis for the expression of LDL receptor and PCSK9 upon treatments. GAPDH was used as loading control; C and D. Densitometric analysis of LDL receptor/GAPDH ratio; E and F. Densitometric analysis of PCSK9/GAPDH ratio. Data are presented as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***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; GAPDH: glyceraldehyde phosphate dehydrogenase.

Nobiletin and tangeretin are particularly abundant in the peel extract while the terpenoid D-limonene, and γ-terpinene that are the most abundant constituent 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) (Figure 5). γ-terpinene only reduced the PCSK9 levels (-81±12%). Thus, some of these compounds may be considered as possible models for the search of new small molecules able to inhibit PCSK9.

View Article Online
DOI: 10.1039/D5FO01511A

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) being valuable as possible model compound for the search of hypocholesterolemic agents due to the contemporaneous activity on the two targets (Figure 5). This observation was previously done by our research group during the evaluation of 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.

The ferulic, the most abundant compounds 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 (Figure 4).

3.5. Effect of MPO, MPLE, MPPE and selected compounds on LDLR mRNA expression in HuH7 cell

To further investigating 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 of Huh7 cell line. As predicted, simvastatin induced LDL receptor by 2-fold (Figure 6). Very similarly, we observed that MPPE significantly increased the mRNA expression of LDL receptor. Me-NO and TA showed a similar effect although the induction of LDL receptor mRNA did not reach the statistical significance (Figure 6).

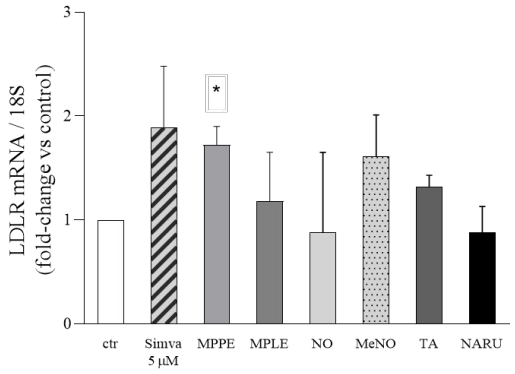


Figure 6. Effect of mapo extracts and isolated compounds on LDLR mRNA expression in Huh7 cells. LDLR mRNA expression was evaluated by RT-qPCR analysis and normalized with 18S. Data are presented as mean ± SD of three independent experiments. *p < 0.05vs control by Student's T-test. Ctr: control; Simva: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

View Article Online
DOI: 10.1039/D5FO01511A

4. Discussion

'Mapo' is an Italian cultivar of *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]. Furthermore, we here 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 are ferulic acid and homoeriodichtyol-7-*O*-neoesperidoside. The extracts not only present difference in the chemical composition but also in their bioactivities. The *in vitro* effects of MPO, MPLE on the LDL receptor and PCSK9 expression appear limited, while MPPE present 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 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 as most abundant compounds of MPO, D-limonene and ϒ-terpinene and the 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 reduces that of PCSK9, such mutual effect on protein expression may suggest that narirutin acts by the same mechanism as berberine [23]. Likewise, homoeriodictiyol-7-*O*-neohesperidoside showed a similar but not significant trend.

Ferulic acid is present in high amount in MPPE and is also detected in MPLE although in less 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 HNF1-α transcription factor regulating PCSK9 expression [24]. Since a previous report showed that ferulic acid acts decreasing plasma lipids (total cholesterol and triglycerides) and liver cholesterol in a *in vivo* rat model [25], our results further support the potential use of this compound as cholesterol-lowering agent. These results indicate that the MPPE extract can be considered as a new potential nutraceutical for controlling cholesterol levels. Differently from MPPE, MPLE extract downregulated both LDL receptor (although not significantly) and PCSK9.

View Article Online
DOI: 10.1039/D5FO01511A

5. Conclusions

The results on MAPO extract reveal that MPPE present some advantageous properties on two key targets of cholesterol homeostasis namely inducing the LDL receptor and reducing PCSK9 *in vitro* at 100 ug/mL. These data indicate that MPPE extracts can be useful as new hypocholesterolemic nutraceutical to be utilized in conjunction to 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 allow to demonstrate a significant effect for the ferulic acid that at 100 µM was able to induce 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 and further mode of action and to assess potential efficacy *in vivo*.

6. 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, *Metabolism: clinical and experimental*, 2024, **161**, 156028.
3. G. Dugo, A. Cotroneo, A. Verzera, G. Dugo and G. Licandro, “Mapo” tangelo essential oil, *Flavour and Fragrance Journal*, 1990, **5**, 205-210.
4. G. Ruberto, D. Biondi, P. Rapisarda, A. Renda and A. Starrantino, Essential Oil of Cami, a New Citrus Hybrid, *Journal of Agricultural and Food Chemistry*, 1997, **45**.
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., *JOURNAL OF FOOD SCIENCE*, 2005, **70**.
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, *Journal of Food Composition and Analysis*, 2006, **19**.
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, *Journal of Functional 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, *Scientia Horticulturae*, 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, *Journal of the Science of Food and Agriculture*, 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 and Fragrance Journal*, 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, *Journal of Essential Oil Research*, 2012, **24**, 159-169.
16. N. Ferri, M. Ruscica, S. Fazio and A. Corsini, Low-Density Lipoprotein Cholesterol-Lowering Drugs: A Narrative Review, *Journal of clinical medicine*, 2024, **13**.

View Article Online
DOI: 10.1039/D5FO01511A

17. V. I. Babushok, P. J. Linstrom and I. G. Zenkevich, Retention Indices for Frequently Reported Compounds of Plant Essential Oils., *Journal of Physical and Chemical Reference Data*, 2011, **40**.
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, *Nutrition, metabolism, and cardiovascular diseases : NMCD*, 2019, DOI: 10.1016/j.numecd.2019.06.001.
19. N. Squillace, V. Cogliandro, E. Rossi, G. Bellelli, M. Pozzi, F. Luppi, M. Lettino, M. G. Strepparava, C. Ferrarese, E. Pollastri, E. Ricci, P. Bonfanti and S. L.-C. Team, A multidisciplinary approach to screen the post-COVID-19 conditions, *BMC Infect Dis*, 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**.
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, *Journal of Functional Foods*, 2023, **108**.
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. Journal of Food and Drug Analysis, *Journal of Food and Drug Analysis*, 2009, **17**, 123-132.
26. M. L. Fernandez and K. L. West, Mechanisms by which dietary fatty acids modulate plasma lipids, *The Journal of nutrition*, 2005, **135**, 2075-2078.
27. J. M. Dietschy, Dietary fatty acids and the regulation of plasma low density lipoprotein cholesterol concentrations, *The Journal of nutrition*, 1998, **128**, 444S-448S.

dsf
DIPARTIMENTO DI SCIENZE DEL FARMACO
DEPARTMENT OF PHARMACEUTICAL AND PHARMACOLOGICAL SCIENCES

UNIVERSITÀ DEGLI STUDI DI PADOVA

View Article Online
DOI: 10.1039/D5FO01511A

Dear Editor

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