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Bergamot (*Citrus bergamia*) peel extract as new hypocholesterolemic agent modulating PCSK9 expression

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ABSTRACT

Citrus bergamia extracts have been studied for the management of hypercholesterolemia disorders. Up to now limited information is available concerning the activity of its main phytoconstituents towards the main targets of the cholesterol homeostasis. In the present study, the effects of bergamot peel extract and isolated constituents, namely glycosidic and non-glycosidic flavonoids, one coumarin and one limonoid on the low-density lipoprotein receptor (LDLR) and proprotein convertase subtilisin/kexin type 9 (PCSK9) were evaluated by using cultured HuH7 cell line. Furthermore, for the first time the effects of bergamot peel extract were studied to describe a potential hypolipidemic action. Significant differences were observed due to glycosylation and different substitution on flavanone moiety (*O*-methylation). Considering the thirteen isolated compounds, both naringenin-7-*O*-rutinoside (NA-rut) and apigenin-6,8-*C*-glicoside induced the expression of the LDLR while no effect was observed on PCSK9. However, hesperetin (HE) and its derivatives, hesperetin-7-*O*-glucoside and hesperetin-7-*O*-neohesperidoside (HE-glu, HE-neo) and eriodictyol (ER) showed a statin-like effect since a significant increase of both LDLR and PCSK9 expression were detected. Furthermore, bergamot peel extract (BE) firstly demonstrated a significant reduction of PCSK9 expression, indicating a potential adjuvant action to statins. BE, HE-neo, and NA-rut reduced intracellular sterols and the expression of PCSK9 transcription factor HNF1-α. BE also significantly improved the LDL uptake u Huh7 cells. Based on the present data, bergamot peel constituents can play a role in the management of hypercholesterolemia, on one hand they may produce an adjuvant action in combination with statins, while on the other hand they may have a statin-like effect. Additionally, BE may be a good candidate as an adjuvant to statins action.

1. Introduction

Bergamot (*Citrus bergamia* Risso et Poiteau) of *Rutaceae, Citrus* genus, is endemic in Calabria region, south Italy. It is known for its multiple uses, such as in cosmetic and perfume industry (Maruca, Laghetti, Mafrica, Turiano, & Hammer, 2017), in the food industry (Lagana, Giuffre, De Bruno, & Poiana, 2022) and in the nutraceutical sector (Di Folco et al., 2018). In general, bergamot is known for its health-promoting properties due to its polyphenol content namely phenolic acids, flavanones, flavones, polymethoxylated flavones and limonoids (Mandalari et al., 2006; Russo et al., 2016). Bergamot juice has proven to be highly effective in ameliorate the inflammatory (Risitano et al., 2014) and lipidic profiles (Miceli et al., 2007). On the other hand, bergamot peels extracts shown antioxidant/anti-inflammatory properties (Mandalari et al., 2006; Russo et al., 2016), similarly to many other plant extracts (Mokgalaboni & Phoswa, 2023). Orally-administered bergamot juice has been shown to lower total cholesterol (TC), low-density lipoprotein cholesterol (LDLc), triglycerides (TG) and to increase high density lipoprotein cholesterol (HDLc) levels both in animal models (Miceli et al., 2007), and in dyslipidemic patients (up to 40 % of reduction in TC, LDLc and TG) (Lamiquiz-Moneo et al., 2019; V. Mollace et al., 2019), accompanied by a significant decrease in blood glucose (up

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to 23 %) (V. Mollace et al., 2011; V. Mollace et al., 2019). Furthermore, treatment with bergamot juice exerts cholesterol reduction with protective effects against hypercholesterolemic-induced renal injury in a rat model (Trovato et al., 2010).

On the other hand, bergamot peel, consisting of an outer layer (flavedo), and an internal layer (albedo) (Rosa et al., 2022), represents around 60 % of the total weight of the fruit and is a by-product of essential oil production (Mandalari et al., 2006). Bergamot peels contain significant quantities of pectins and fiber, limonoids and flavonoids (Mandalari et al., 2006; Russo et al., 2016). The most representative flavonoid derivatives are flavanone rutinosides and neohesperosides derived from naringenin, eriodictyol, and hesperetin (Mandalari et al., 2006) and several studies demonstrated hypolipidemic and antiatherogenic activities of these constituents. Naringin exhibit an antiatherogenic effect through the inhibition of intercellular adhesion molecule-1 in hypercholesterolemic rabbits (Choe, Kim, Jeong, Bok, & Park, 2001) and, naringenin has shown hypolipidemic activity and antiadiposity effects in rats through PPAR-α regulation mechanism (Cho, Kim, Andrade, Burgess, & Kim, 2011). Therefore, neoeriocitrin has exhibited activity as inhibitor of LDL oxidation (Gliozzi et al., 2013; Yu et al., 2005). Moreover, it has been demonstrated that a mixture of naringin and hesperidin significantly lowers the HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase and the ACAT (acyl CoA:cholesterol *O*-acyltransferase) activities in experimental high-cholesterol diet fed male rats (Bok et al., 1999).

Beside these aspects, *in vitro* and *in vivo* studies have been conducted on the cholesterol-lowering activity of citrus flavonoid derivatives and extracts on LDL receptor (LDLR) expression and its main negative modulator, proprotein convertase subtilisin/kexin type 9 (PCSK9). The administration of a bergamot polyphenolic fraction in rats fed with a hyperlipidemic diet was associated with a significant reduction of PCSK9 serum levels in comparison to the PCSK9 serum levels of hyperlipidemic rats treated with a Red Yeast Rice extract (Mollace et al., 2022). Furthermore, a flavanones (3-hydroxy-3-methyl-glutaryl flavanones) enriched fraction from bergamot fruit increases the LDLR expression (mRNA and protein) in a hypocholesterolemic rat model (Di Donna et al., 2014). On the other hand, naringin and naringenin increased LDLR expression in HepG2 cell lines *via* enhancing sterol-regulatory binding protein 2 (SREBP2) pathway (Bawazeer et al., 2017; J. Liang et al., 2016). Naringin produced the same effect in an obese mice model and the same study showed the naringin ability in reducing PCSK9 plasma levels (Sui, Xiao, Lu, & Sun, 2018). Additionally, the incubation of hesperetin, predominant flavonoid in citrus fruits, in human hepatoma HepG2 cells increases LDLR mRNA levels *via* regulation of LDLR gene transcription by SREBP1a and SREBP2 (Barreca et al., 2017; Bawazeer et al., 2016).

To date, several studies have considered the correlation between the activity of bergamot flavonoid derivatives and extracts and cholesterol through investigating LDL-receptor induction or PCSK9 regulation mechanisms (Di Donna et al., 2014; Mollace et al., 2022). However, there are no works examining, simultaneously, bergamot peel isolated constituents under the same experimental conditions and investigating both regulatory mechanisms of LDLR and PCSK9. Furthermore, no papers investigating the cholesterol-lowering properties of bergamot peel extracts are present. This may be attractive since bergamot peels are a waste product of essential oil industry and they may be good candidates for the production of new hypocholesterolemic agents.

In the present study, we evaluated the effects of bergamot peel extract and 13 compounds isolated from bergamot peel extract, on the expression of LDLR and PCSK9 in cultured hepatoma cell line Huh7 as predictive model for a potential hypocholesterolemic action.

2. Materials and methods

2.1. Cell culture

The studies were carried out on HuH7 cells, a human hepatoma cell line. The cells were cultured in 100 mm Petri dishes with Minimum Essential Medium (MEM) supplemented with 10 % fetal bovine serum (FBS), 1 % L-glutamine 200 mM, 1 % penicillin (10.000U/ml)/streptomycin (10 mg/ml) solution, 1% non-essential amino acids, 1 % sodium pyruvate and maintained at 37% °C, under 95 % air and 5 % CO_2 conditions. Once reached the 80 % of confluence, cells were passed. All reagents were purchased by EuroClone S.p.A., plastics from Corning.

2.2. Bergamot peel extract and compound isolation

Bergamot fruits were obtained in local market and 3 Kg of fruits were used to collect peels. Peels were grinded in methanol/water mixture (1:1 vol/vol) and resulting material was sonicated for 20 min. Liquid was filtered and dried under vacuum. 2 g of bergamot peel extract (BE) were dispersed in 40 mL of water and a liquid-liquid partition was performed with 40 mL of ethyl acetate. The separation was repeated three times. Ethyl acetate fractions were collected and filtered in $MgSO_4$ and dried under vacuum. The ethyl acetate extract (180 mg) was used for isolation of compound. A preparative HPLC was performed using a Zorbax C18 column and water 0.1% formic acid (A) and acetonitrile (B) was used as mobile phase, 3.5 mL/min. Solvent gradient starts with A:B 90:10, in 40 min A:B 15:85, and after 5 min A:B 10:90. 38 fractions were collected and were pulled together on the base of their behavior by TLC. From fraction 15, TLC was performed using chloroform-methanol 4:1 and 3-(2-glucosyloxy-4-methoxyphenyl) propanoic acid was isolated (Chan, Hwang, Kuo, Hung, & Wu, 2017). From fraction 19 and 20 TLC was performed using chloroform-methanol 4:1 and naringenin-7-*O*-glucoside (NA-glu) was isolated. Minor spots in TLC were pulled together and preparative HPLC was performed in the same preparative column mentioned before using as solvent 30:70 water 0.1 % formic acid and acetonitrile, isocratic. Hesperetin-7-*O*-neoesperidoside (HE-Neo) was isolated. Fractions 30 and 31 was purified by TLC using as solvent chloroform-methanol 98:2, and the fractions were pulled together on the base of TLC behavior. From fraction 30 and 31, naringenin (NA), eriodictyol (ER) and hesperetin (HE) were isolated. Fractions 37 and 38 were pooled together and bergapten (BER) was isolated by TLC using diethyl ether-toluene-ethyl acetate 40:50:10 as solvent.

2.3. HPLC-DAD-MS^n analysis of bergamot peel extract

Bergamot peel extract (50 mg) was solubilised in methanol (25 mL) using ultrasound bath and analysed using an HPLC-DAD-MS^n system. The HPLC-MS^n system consisted of an Agilent 1260 quaternary pump coupled to 1260 Agilent diode array detector (DAD) pump (Agilent Technologies, Santa Clara, CA, USA) and a Varian MS 500 mass spectrometer (Varian, Santa Clara, CA, USA) equipped with electrospray (ESI) ion. As stationary phase, 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, esperidin, nomilin, bergapten were used. Standard solutions were prepared in the concentration

ranges 1–100 μg/ mL and calibration curves were built.

2.4. Bergamot peel extract and isolated compounds solubilization and treatment

Bergamot peel extract was dissolved in DMSO to a stock concentration of 80 mg/ml and used at 100 μg/ml in culture medium; the isolated compounds were dissolved in DMSO to get a stock of 80 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 40 μM as previously reported (Ferri et al., 2017). If not used, all the stocks were stored at −20 °C.

2.5. 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. (Skehan et al., 1990). 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 0.4 % FBS containing treatments after a wash with sterile PBS. Controls were supplied of the appropriate % of DMSO to be comparable with compounds and BE treatments. After 24 h of incubation, SRB assay was performed and absorbances measured at 570 nm with Victor Nivo multiplate reader by PerkinElmer.

2.6. Western blotting for intracellular PCSK9, LDLR, SREBP-2 and HNF-1α detection

Nontoxic compounds and extract concentrations (100 μM and 100 μg/ml, respectively) were used for the western blotting analysis. $3{\cdot}10^5$ cells/well were seeded in a 6 well-tray in 2 mL/well of complete medium. The following day the old media was replaced by fresh media 0.4 % FBS containing treatments after a wash with sterile PBS. Simvastatin 40 μM was used as positive control as PCSK9 and LDLR inducer. Untreated controls and Simvastatin controls were supplied of the appropriate % of DMSO to be comparable with compounds and bergamot extract treatments. After incubation, the cell monolayer was washed twice with cold PBS, then lysed on ice for 30 min with an 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 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 denaturating 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 LDLR, or SREBP2, or HNF-1α, 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-LDLR (Millipore, Darmstat, Germany; mouse monoclonal antibody, clone 2H7.1; dilution 1:1000); anti-PCSK9 (Abcam, cod. ab181142; dilution 1:1000), anti-SREBP2 (GeneTex, cod. GTX82865; dilution 1:1000), anti-HNF-1α (GeneTex, cod. GTX113850; 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. Fluorescent LDL uptake cell-based assay

Huh7 cells were seeded in 6-well tray ($3{\cdot}10^5$ cells/well in a complete medium) and after 24 h treated in MEM/ 0.4 % FBS media. 24 h after treatment, cells were washed with PBS and incubated with 10 μg/mL of LDL-DyLight™ 550 in 0.4 % FCS media. After 3 h of incubation at 37 °C, cells were washed with PBS, detached with trypsin and resuspended in MEM/ 10 % FBS. After centrifugation (4 min at 3500 rpm), the pellet is resuspended in PBS and each sample were transferred to a cytofluorometer tube. The fluorescence was measured by using a flow cytometer (BD FACSAria™ IIIu, DB Life Sciences, San Jose, CA, 95131, USA) at excitation and emission wavelength of 484 nm.

2.8. Cholesterol determination

Huh7 cells were incubated under the same experimental conditions described for western blot analysis. At the end of the period of incubation, cell monolayers were washed with PBS (phosphate buffer saline) and incubated for 2 h at RT with 0.1 M NaOH. An aliquot of these samples was used for protein determination (BCA assay) and a second aliquot for cholesterol analysis. The total cholesterol content of cells treated with the different compounds was measured using liquid chromatography coupled with mass spectrometry with atmospheric pressure chemical ionization ion source (LC-APCI-MS). The system for analysis was an Agilent 1260 Liquid chromatograph, coupled with a Varian mass spectrometer MS 500 with ion trap analyzer. For the chromatographyc separation an Agilent XDB C-18 3.0 × 150 mm was used (5μ). Elution was performed using a mixture of acetonitrile 87 % Methanol 10% and water 0,1 % formic acid 3 %, in isocratic mode for 15 min. Spectra were acquired in the range *m/z* 350–550. Cholesterol was detected as $[M-H_2O + H]^+$ at *m/z* 369.5. A cholesterol calibration curve was created in the range 50.0–0.5 μg/mL. Samples were prepared as follows: a liquid/liquid partition was performed adding chloroform to lysates. Samples were dried and then were diluted with equal volume of chloroform and finally used for chromatography.

2.9. 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. A probability value of $p < 0.05$ was considered statistically significant. The experiments were performed in triplicate.

3. Results

3.1. Chemical characterization of bergamot peel extract.

A total of 19 constituents were identified by HPLC-DAD-MS^n analysis (Table 1). Of these, 17 compounds corresponding to flavonoid derivatives, four flavones and fourteen flavanones, were detected. On the other hand, non-flavonoid derivatives, a limonoid (nomilin), and a furocumarin (bergapten), were idenified. Considering flavonoids, the most abundant are hesperetin-7-*O*-neohesperidoside (88.70 mg/g), naringenin-7-*O*-rutinoside (44.78 mg/g) and eriodictyol-7-*O*-neohesperidoside (33.51 mg/g). 3-hydroxy-3-methyl-glutaryl flavanones are also present in considerable amount, particularly eriodictyol-7-*O*-neohesperidoside-6″-*O*-HMG is 21.12 mg/g and melitidin 19.30 mg/g. Of non-flavonoids, nomilin is present in modest amount, 0.88 mg/g, and bergapten is present in low amount 0.40 mg/g.

Table 1
HPLC-DAD-MS^n characterization and quantification of BE constituents.

	flavonoids					
retention time	***flavones***	***[M−H]$^-$***	***[M + H]$^+$***	***fragmentation***	***mg/g***	***ident.*** [b]
7.7	luteolin-6,8-di-*C*-glucoside	609		519 489 399 369	2.28 ± 0.05	(Baron et al., 2021)
8.2	**apigenin-6–8-di-*C*-glucoside** [a] (AP-di-glu)	593		503 473 383 353	5.61 ± 0.04	(Baron et al., 2021)
8.5	diosmetin-6–8-di-*C*-glucoside	623		503 413 383	12.19 ± 0.06	(Baron et al., 2021)
9.0	luteolin-7-*O*-neohesperidoside	593		285	3.06 ± 0.06	(Baron et al., 2021)
	flavanones					
8.7	**eriodictyol-7-*O*-glucoside** (ER-glu)	477		287	5.38 ± 0.05	(Baron et al., 2021)
9.4	eriodictyol-7-*O*-rutinoside	595		477 461 459 417 357 287	2.4 ± 0.02	(Baron et al., 2021)
9.8	eriodictyol-7-*O*-neohesperidoside	595		477 461 459 357 271 235	33.51 ± 0.03	(Baron et al., 2021)
10.0	eriodictyol-7-O-neohesperidoside-6″-*O*-HMG	739		677 637 595	21.12 ± 0.03	(He et al., 2018)
10.5	**naringenin-7-*O*-rutinoside** (NA-rut)	579		459 433 417 271	44.78 ± 0.06	(Baron et al., 2021)
10.6	**naringenin-7-*O*-glucoside** (NA-glu)	433		271	11.78 ± 0.05	(Baron et al., 2021)
11.0	**hesperetin-7-*O*-neohesperidoside** (HE-neo)		611	575 449 303	88.70 ± 0.10	(Baron et al., 2021)
11.1	**hesperetin-7-*O*-glucoside** (HE-glu)	463		301	1.88 ± 0.02	(Baron et al., 2021)
11.2	**melitidin** (MEL)	723		621 579 459 271	19.30 ± 0.04	(Baron et al., 2021)
11.6	**brutieridin** (BRU)	753		689 651 609 301	12.28 ± 0.05	(Baron et al., 2021)
12.1	hesperetin-7-*O*-glucoside-6′-O-HMG	607		543 503 463 301	0.78 ± 0.02	(Baron et al., 2021)
13.1	**eriodictyol** (ER)	287			2.79 ± 0.04	(Baron et al., 2021)
14.4	**naringenin** (NA)	271		177 151	4.02 ± 0.03	(Baron et al., 2021)
14.9	**hesperetin** (HE)	301			1.95 ± 0.03	(Baron et al., 2021)
	non-flavonoids					
	limonoids					
19.6	**nomilin** (NO)		515	411 369 187 161	0.88 ± 0.01	std
	furocoumarins					
18.1	**bergapten** (BERG)		217	202 174 146 118 90	0.40 ± 0.01	std

[a] constituents in bold were isolated
[b] *ident.*, identification method: [reference] or std (standard).

3.2. Isolation and structure elucidation of flavonoid and non-flavonoid constituents from bergamot peel.

Extensive chromatographic procedures allowed the isolation of three naringenin, two eriodictyol, three hesperetin derivatives and the melitidin and brutieridin, two hydroxymethylglutaryl esters. Furthermore, one di-C-glycoside of apigenin, the furocumarin bergapten and the limonoid nomilin were isolated in sufficient amount (>10 mg) and good purity (>98 % based on HPLC assay) to perform the biological assays. Structures of isolated compounds are reported in Fig. 1.

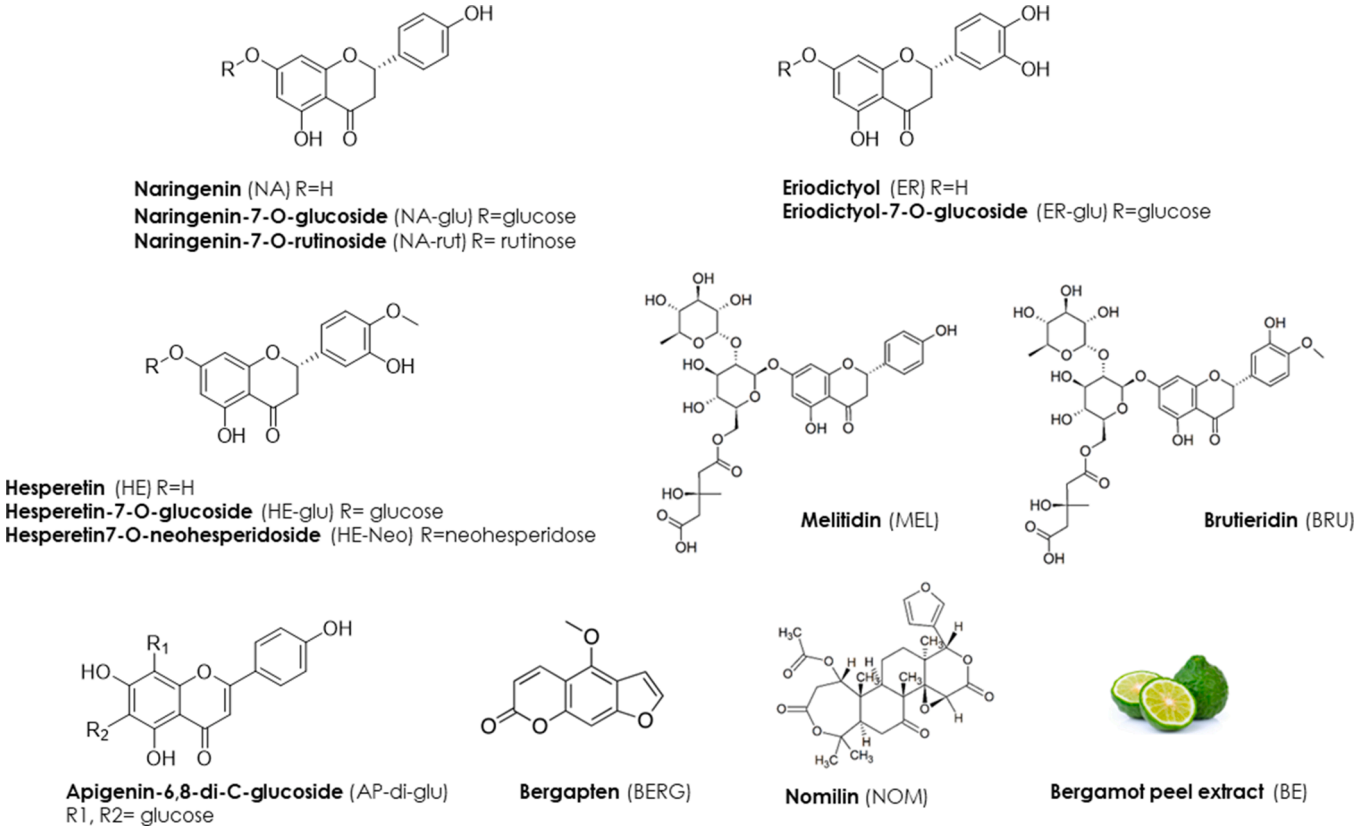

Fig. 1. Chemical structures of isolated compounds from BE. The flavanones class includes NA, NA-glu, NA-rut, ER, ER-glu, HE, HE-glu, HE-Neo, MEL, BRU; the flavones class include AP-di-glu; the non-flavonoids class include BERG, NOM.

3.3. Bergamot peel extract increases the LDLR/PCSK9 ratio.

The not toxic concentration (determined by SRB analysis, Figure S1) of 100 μg/ml of bergamot extract has been tested on HuH7 human hepatoma cell line and its effect was compared with simvastatin 40 μM, a known positive modulator of both LDLR and PCSK9. After 24 h incubation, the expression of LDLR was marginally, and not significantly, reduced compared to control ($p = 0.1$), conversely to what observed with simvastatin (+4.9-fold, $p = 0.001$) (Fig. 2A and 2B). On the other hand, bergamot peel extract produced a significant decrease in PCSK9 protein levels compared to control (−74 %, $p = 6.15E-06$), showing an opposite behavior versus simvastatin (+6-fold vs ctr, $p = 0.0008$) (Fig. 2A and 2C). Thus, these results demonstrate a net favorable LDLR/PCSK9 ratio in bergamot extract treated cells compared to control cells (Fig. 2A).

3.4. Flavanones and flavones isolated from bergamot extract differentially affect LDLR and PCSK9 expression in HuH7 cell.

The aglycone and glycosylate flavanones isolated from bergamot peel (Fig. 1) were tested on HuH7 cells for 24 h at the not toxic concentration (Figure S1), and their effect on LDLR and PCSK9 protein expression was compared with the activity of simvastatin 40 μM on the same targets (LDLR: +5-fold, $p < 0.001$ vs ctr; PCSK9: +11-fold, $p < 0.05$ vs ctr) (Fig. 3A-C). Naringenin-7-*O*-glucoside (NA-glu), produced a 0.5-fold decrease in LDLR expression compared to control ($p < 0.01$) (Fig. 3A and B), and similar effect was observed with its aglycone counterpart, naringenin (NA), (−0.58-fold, $p < 0.01$ vs ctr) (Fig. 3A and B), while they seem to produce an opposite effect on PCSK9 expression, with NA that produced a positive induction (+3-fold, $p < 0.001$ vs ctr) while NA-glu determined a slight inhibition, compared to control (−25%, $p < 0.01$) (Fig. 3A and C).

Eriodictyol (ER) strongly enhanced LDLR (+4-fold, $p < 0.01$) and PCSK9 expression (+7-fold, $p < 0.05$), compared to control (Fig. 3A and B), thus showing a statin-like behavior. On the other hand, its glucoside, eriodictyol-7-*O*-glucoside (ER-glu), did not produced any significant change on LDLR (Fig. 3A and B), nor on PCSK9 protein levels (Fig. 3A and C), if compared to control. However, worth-of-note was the opposite mode of action of ER and ER-glu on PCSK9 (Fig. 3A and C). The incubation with naringenin-7-*O*-rutinoside (NARI), also known as narirutin, produced a 4.8-fold increase in LDLR expression (Fig. 3A and B; $p < 0.01$ vs ctr) and a 2-fold increase in PCSK9 protein levels compared to control (Fig. 3A and C), showing a different behavior compared to its aglycone, naringenin, and naringenin-7-*O*-glucoside (NA-glu) (Fig. 3). A similar activity was shown by apigenin-6,8-di-C-glucoside (AP-di-glu), showing a significant + 4.6-fold change in LDLR expression if compared to control (Fig. 3A and B; $p < 0.01$) and a 2.2-fold increase in PCSK9 intracellular levels.

Hesperetin (HE) and its glycosylated derivatives hesperetin-7-*O*-glucoside (or hesperidin) (HE-glu) and hesperetin-7-*O*-neohesperidoside (Neohesperidin) (HE-Neo) were isolated and successfully tested at the non-toxic concentration of 100 μM.

HE is the 4′-methoxy derivatives of ER, and significantly induced both LDLR (+8.3-fold, $p < 0.01$ vs ctr) and PCSK9 (+7.89-fold change vs ctr, $p < 0.01$) protein levels (Fig. 4A-C), similarly to ER (Fig. 3A-C). The addition of a neohesperidose sugar to HE in 7-*O* position (HE-Neo) stimulated more the LDLR protein expression than HE (+9.7-fold, $p < 0.01$ vs ctr), but with lower efficacy, compared to HE, on PCSK9 levels (+5.11-fold change vs ctr, $p < 0.01$). In addition, the 7-*O* glycosylation of HE (HE-glu) produced a statin-like behavior, by increasing both the LDLR (+4.27-fold vs ctr) and PCSK9 (+4.41-fold vs ctr, $p < 0.01$) proteins compared to control, even if to a minor extent compared with HE, HE-Neo, and simvastatin (LDLR: +8.12-fold vs ctr, $p < 0.001$; PCSK9: +9.12-fold vs ctr, $p < 0.001$) (Fig. 4A-C).

Melitidin (MEL) and brutieridin (BRU) are flavanones diglycosides of NA and HE carry the 3-hydroxy-3-methylglutaric acid (HMG) moiety (Fig. 1). Indeed, both showed a statin-like behavior. MEL produced a +3.7-fold increase in LDLR and a +4.32-fold change in PCSK9 ($p < 0.05$) expression levels vs control (Fig. 4A-C) and BRU stimulated the LDLR expression by +3.1-fold if compared with control (Fig. 4A and 4B), while no effect was observed on PCSK9 levels (Fig. 4A and 4C).

3.5. Non-flavonoid compounds isolated from bergamot extract reduce PCSK9 expression in HuH7 cell.

Two non-flavonoid compounds have been isolated from bergamot extract, namely bergapten (BERG), belonging to furanocoumarins family, and nomilin (NOM), a limonoid (i.e., a highly oxygenated triterpene). Compared to simvastatin 40 μM, that strongly stimulated LDLR (+4.9-fold, $p < 0.01$ vs ctr) and PCSK9 (+7.34-fold, $p < 0.01$ vs ctr) as expected, BERG and NOM produced a significant decrease in PCSK9 intracellular levels (-30 %, $p < 0.01$; −53 %, $p < 0.01$, respectively) but no effects on LDLR (Fig. 5A-C), thus balancing the LDLR/PCSK9 ratio in favor of LDLR.

Our *in vitro* approach aimed to identify possible bergamot constituents with hypocholesterolemic effect. The predictive lipid-lowering effect is certainly related to the levels of expression of the LDLR, by which all the different classes of drugs exert their action for controlling LDL

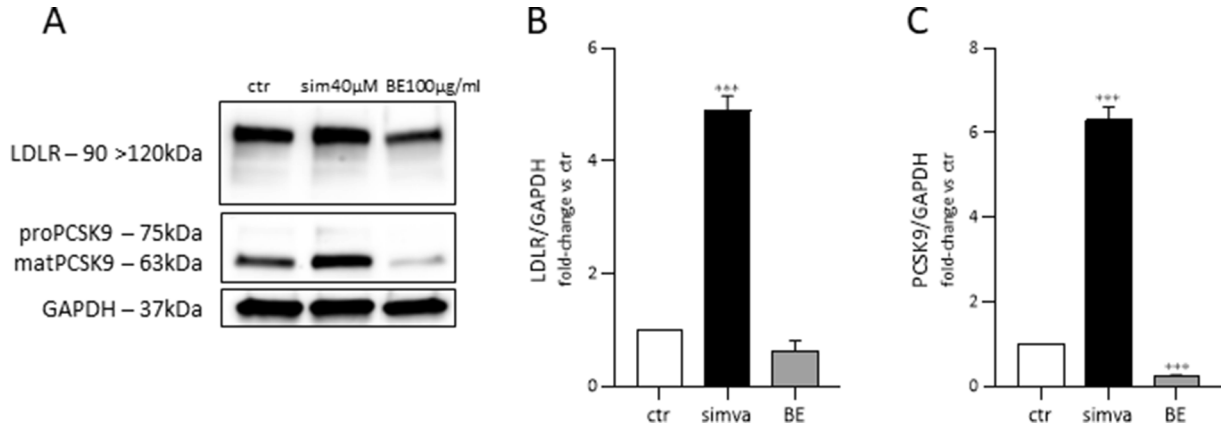

Fig. 2. BE effect on LDLR and PCSK9 expression in HuH7 human cell line. Cells were incubated with simvastatin 40 μM or bergamot peel extract 100 μg/ml for 24 h. A. Representative western blotting analysis for the expression of LDLR and PCSK9 upon treatments. GAPDH was used as loading control; B. Densitometric analysis of LDLR/GAPDH ratio; C. Densitometric analysis of PCSK9/GAPDH ratio. Data are presented as mean ± SD of three independent experiments. *** $p < 0.001$ vs control by Student's T-test. Ctr: control; simva: simvastatin; BE: bergamot peel extract; LDLR: low-density lipoprotein receptor; proPCSK9: not mature protein convertase subtilisin/kexin type 9; matPCSK9: mature PCSK9; GAPDH: glyceraldheyde phosphate dehydrogenase.

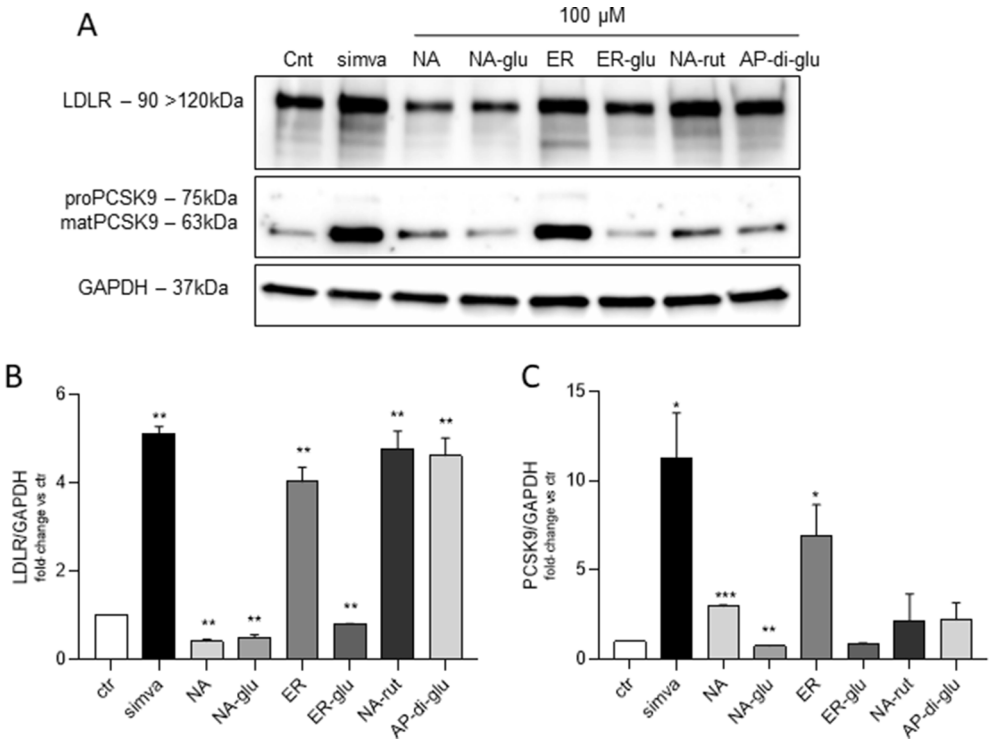

Fig. 3. BE flavanones and flavones activity on LDLR and PCSK9 expression in HuH7 human cell line – Part I. Cells were incubated with simvastatin 40 μM or 100 μM of the flavanones or flavones isolated from bergamot extract for 24 h. ER and ER-glu were used at 12.5 μM and 25 μM, respectively. A. Representative western blotting analysis for the expression of LDLR and PCSK9 upon treatments. GAPDH was used as loading control; B. Densitometric analysis of LDLR/GAPDH ratio; C. 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; NA: naringenin; NA-glu: naringenin-7-*O*-glucoside; ER: eriodictyol; ER-glu: eriodictyol-7-*O*-glucoside; NA-rut: Naringenin-7-*O*-rutinoside; AP-di-glu: apigenin-6,8-C-glicoside; LDLR: low-density lipoprotein receptor; proPCSK9: not mature protein convertase subtilisin/kexin type 9; matPCSK9: mature PCSK9; GAPDH: glyceraldehyde phosphate dehydrogenase.

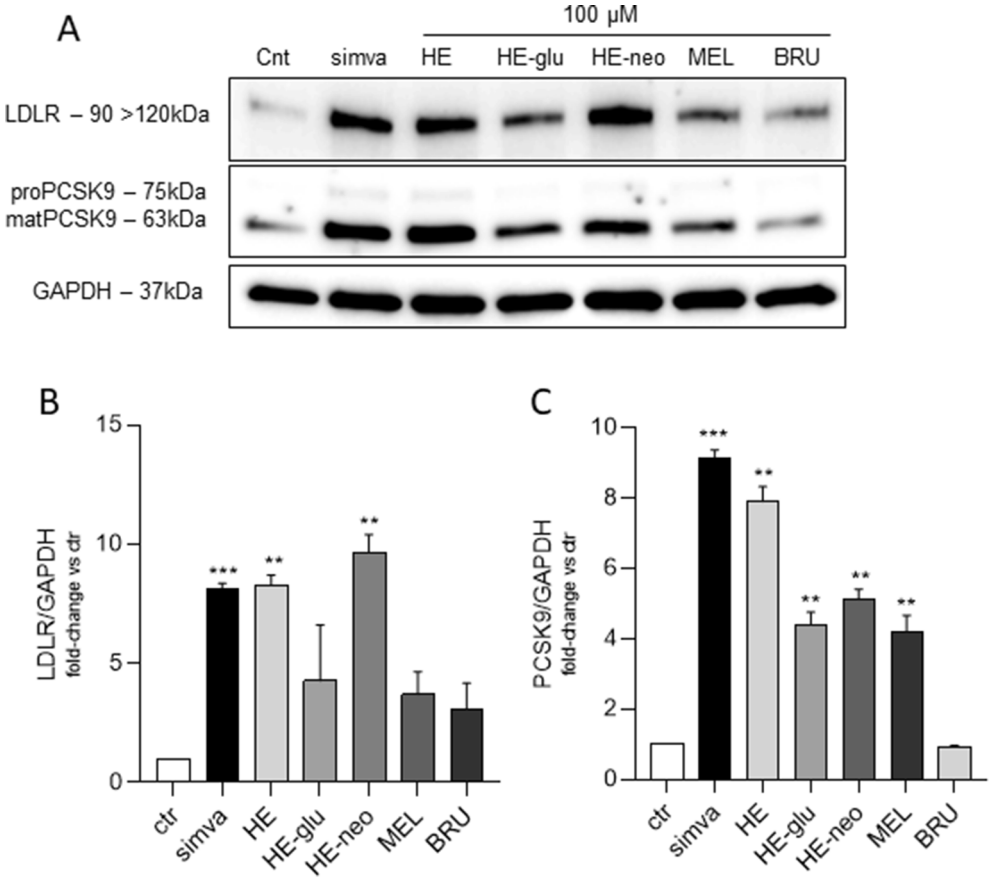

Fig. 4. BE flavanones activity on LDLR and PCSK9 expression in HuH7 human cell line – Part II. Cells were incubated with simvastatin 40 μM or 100 μM of the flavanones isolated from bergamot extract for 24 h. A. Representative western blotting analysis for the expression of LDLR and PCSK9 upon treatments. GAPDH was used as loading control; B. Densitometric analysis of LDLR/GAPDH ratio; C. Densitometric analysis of PCSK9/GAPDH ratio. Data are presented as mean ± SD of three independent experiments. **$p < 0.01$, ***$p < 0.001$ vs control by Student's T-test. Ctr: control; simva: simvastatin; HE: hesperetin; HE-glu: hesperetin-7-*O*-glucoside; HE-Neo: hesperetin-7-*O*-neohesperidoside; MEL: melitidin; BRU: brutieridin; LDLR: low-density lipoprotein receptor; proPCSK9: not mature protein convertase subtilisin/kexin type 9; matPCSK9: mature PCSK9; GAPDH: glyceraldehyde phosphate dehydrogenase.

cholesterol levels, including statins. On the other hand, and differently from statins, an inhibitory effect on PCSK9 levels would also be predictive of an hypocholesterolemic effect and, at the same time, this effect could be beneficial as synergist to statin action. For this reason, the effects on the two pathways were expressed as LDLR/PCSK9 ratio (Table 2) and compared to the reference drug simvastatin.

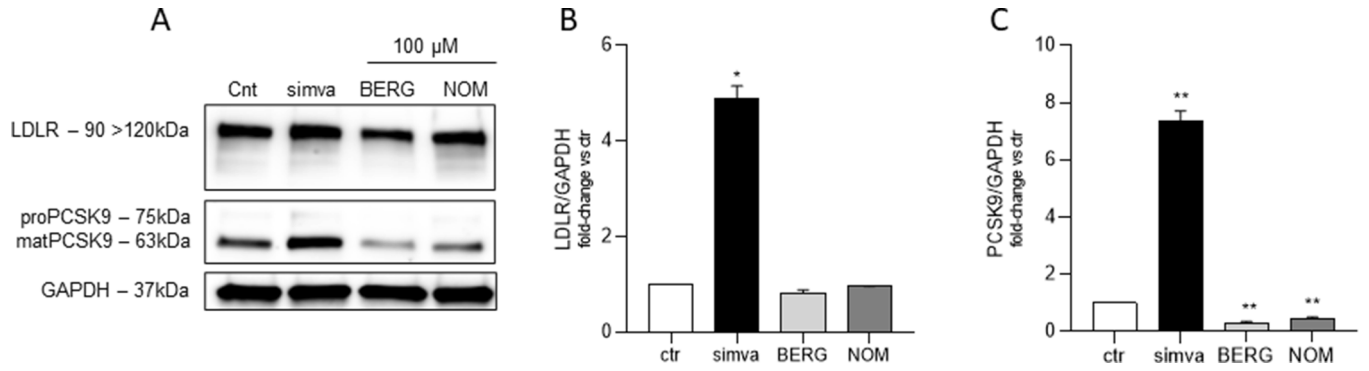

Fig. 5. BE non-flavonoids activity on LDLR and PCSK9 expression in HuH7 human cell line – Part III. Cells were incubated with simvastatin 40 μM or 100 μM of the non-flavanoids isolated from bergamot extract for 24 h. A. Representative western blotting analysis for the expression of LDLR and PCSK9 upon treatments. GAPDH was used as loading control; B. Densitometric analysis of LDLR/GAPDH ratio; C. Densitometric analysis of PCSK9/GAPDH ratio. Data are presented as mean ± SD of three independent experiments. **p < 0.01, ***p < 0.001 vs control by Student's T-test. Ctr: control; simva: simvastatin; BERG: bergapten; NOM: nomilin; proPCSK9: not mature proprotein convertase subtilisin/kexin type 9; matPCSK9: mature PCSK9; GAPDH: glyceraldheyde phosphate dehydrogenase.

Table 2
LDLR/PCSK9 expression levels ratio after incubation with indicated compounds. Cont: control; Simva: simvastatin; BE: Bergamot peel extract; NA: naringenin; NA-glu: naringenin-7-*O*-glucoside; NA-rut: naringenin-7-*O*-rutinoside; ER: eriodictyol; ER-glu: eriodictyol-7-*O*-glucoside; AP-di-glu: apigenin-6,8-C-glicoside; HE: hesperetin; HE-glu: hesperetin-7-*O*-glucoside; HE-Neo: hesperetin-7-*O*-neohesperidoside; MEL: melitidin; BRU: brutieridin; BERG: bergapten; NOM: nomilin. *p < 0.01; ***p < 0.001 vs control.

Sample	LDLR/PCSK9 ratio	*P* vs Cont
Cont	1.00 ± 0.00	
Simva	0.67 ± 0.21	*
BE	2.44 ± 0.68	ns
NA	0.14 ± 0.01	***
NA-glu	0.69 ± 0.09	*
NA-rut	3.11 ± 2.42	ns
ER	0.59 ± 0.10	*
ER-glu	0.98 ± 0.08	ns
AP-di-glu	2.35 ± 1.20	ns
HE	1.05 ± 0.01	*
HE-glu	0.95 ± 0.46	ns
HE-neo	1.89 ± 0.04	***
MEL	0.89 ± 0.32	ns
BRU	3.40 ± 1.38	ns
BERG	2.77 ± 0.27	ns
NOM	2.04 ± 0.19	*

The comparison of the flavanone revealed that in general the glycosylation improves the ratio LDLR/PCSK9 especially for naringenin and eriodictyol, while for hesperetin this is observed only with the neohesperidose. On the other hand, slight changes in the flavanone structure appear to be critical on this selectivity index and hesperetin and derivatives present selectivity values around 1. Table 2 allow to establish the different activity of the derivatives compared to simvastatin, such as brutieridin, and naringenin-7-*O*-rutinoside which present values >3.

3.6. *Effect of bergamot peel extract, Na-rut and He-neo on intracellular cholesterol levels and SREBP2, HNF-1α expression.*

To begin to investigate the mechanism of action of BE and the most effective isolated compounds, we determined the intracellular cholesterol levels of Huh7 cells after 72 h incubation with BE, Na-rut and He-neo. As expected, simvastatin strongly reduced by more than 80% the intracellular cholesterol levels. Similar effect was also observed after the incubation with BE, Na-rut and He-neo (Fig. 6A). The reduction of intracellular sterols in response to simvastatin and He-neo was associated to increased expression of the activated form of SREBP2 (Fig. 6B). In particular, SREBP2 levels were increased by 1.6-fold and 1.8-fold after simvastatin and He-neo of Huh7 cells. A minor effect was observed with Na-rut and no effect with BE. However, BE and its active

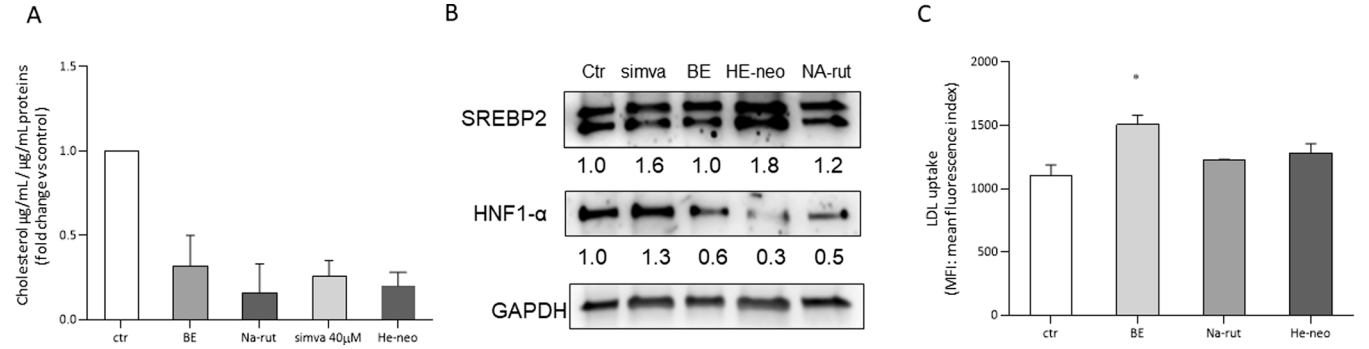

Fig. 6. Effect of BE, Na-rut and He-neo on intracellular cholesterol, SREBP2 and HNF1-α expression and LDL uptake in Huh7 cell line. Cells were incubated in MEM/0.4 % FCS with simvastatin 40 μM or in the presence or absence of BE (100 μg/ml) or isolated compounds (100 μM). A) After 24 h, total lipids were extracted, and cholesterol levels determined by mass spectrometry analysis. These values were normalized by cellular protein concentrations. Each bar represents the mean ± SD of three independent determinations. B) Representative western blotting analysis for the expression of SREBP2 and HNF1-α upon 24 h treatments. GAPDH was used as loading control; C) After 24 h incubation with BE, Na-rut and He-neo, cells were incubated with 10 μg/mL of LDL-DyLight and fluorescence intensity (MFI: mean fluorescence index) determined by flow cytometry analysis after 3 h incubation. Data are given as the mean ± SD of three determinations. Differences between treatments were assessed by Student's *t* test. *p < 0.05 vs control. Ctr: control; simva: simvastatin; BE: bergamot peel extract; Na-rut: Naringenin-7-O-rutinoside; HE-Neo: hesperetin-7-O-neohesperidoside.

constituents behaved differently from simvastatin by strongly suppressing the HNF1-α expression (Fig. 6B), a pivotal transcriptional regulator of PCSK9 expression (Shende et al., 2015). Interestingly, only BE was capable to significantly increase the LDL-DyLight™ 550 uptake by Huh7 cells, suggesting a potential lipid lowering effect (Fig. 6C).

4. Discussion

Bergamot peels are a rich source of bioactive constituents, especially flavonoid derivatives, (flavanones and flavones) limonoids and furocoumarins which are highly concentrated (Mandalari et al., 2006; Russo et al., 2016). Although hypolipidemic activity of bergamot flavonoid derivatives (Cho et al., 2011; Choe et al., 2001) and extracts (Lamiquiz-Moneo et al., 2020; Miceli et al., 2007; V. Mollace et al., 2019) has been reported, the molecular mechanism involved needs to be further clarified. Given this, the investigation of the effects of bergamot peel extract and isolated compounds from bergamot peel on the two key players of cholesterol homeostasis, LDLR and PCSK9, may be a valid *in vitro* assay to predict a potential hypocholesterolemic activity.

Differently from previous papers, here we tested at once the effects of 13 isolated constituents from bergamot peels and bergamot peel extract, on *in vitro* HuH7 cell line at the same experimental conditions (24 h of incubation, MEM with 0.4 % FBS). Furthermore, no studies have reported experiments testing simultaneously the effects of bergamot constituents or extracts on both LDLR and PCSK9. Regarding this, up-regulation of LDLR levels has been reported in the literature to achieve hypolipidemic properties (Luo, Yang, & Song, 2020). Moreover, differently from statins and several classes of drugs, cholesterol-lowering effects are also given by a decrease in PCSK9 levels (Adorni, Zimetti, Lupo, Ruscica, & Ferri, 2020; Luo et al., 2020; Seidah, Abifadel, Prost, Boileau, & Prat, 2017). For this reason, in this work LDLR/PCSK9 ratio was used to express the effects of bergamot compounds and peel extract on the two key mediators of cholesterol metabolism. Furthermore, this value may be useful to compare the activity of the reference therapeutic drug, simvastatin, with new potential hypocholesterolemic agents and, more generally, to evaluate a potential synergistic action of constituents and bergamot extract with statins activity. In this regard, the literature has demonstrated the enhanced effects of combining nutraceuticals with simvastatin in patients with ischemic heart disease (Campolongo et al., 2016). In addition, the potential hypolipidemic effects of bergamot peel extracts have not been investigated so far, although pulp extracts have been extensively studied (Lamiquiz-Moneo et al., 2019; Miceli et al., 2007; V. Mollace et al., 2019).

In this study we isolated ten flavanones (seven glycosylates and three aglycones) and one glycosylates flavone. Among these, five demonstrated a significant induction of the LDLR, i.e., naringenin-7-*O*-rutinoside, eriodictyol, apigenin-6,8-*C*-glicoside, hesperetin and hesperetin-7-*O*-neohesperidoside. However, eriodictyol, hesperetin and hesperetin-7-*O*-neohesperidoside induced PCSK9 expression, an effect similar to simvastatin.

More in detail, we observed that hesperetin-7-*O*-neohesperidoside and, less potently, naringenin-7-*O*-rutinoside induced the expression of SREBP2, the main transcription factor involved in the regulation of the LDLR and significantly reduced the intracellular sterol levels. These effects were similar to simvastatin, thus suggesting a possible impairment of cholesterol synthesis. However, both compounds strongly affected the HNF1-α, which instead is mainly involved in PCSK9 expression. The final balance between these two transcription factors may have determined the differential effect on LDLR and PCSK9 by hesperetin-7-*O*-neohesperidoside and naringenin-7-*O*-rutinoside. On the contrary, BE did not alter the SREBP2 pathway but strongly reduced HNF1-α expression thus providing a possible explanation for its inhibitory action on PCSK9.

Considering only the compounds that more selectively indued the LDLR, their glycosylation seems too be required for their activity as compared to their aglycone counterparts. In this respect, several studies demonstrated the higher stability of glycosylated flavonoids, compared to aglycones. Quercetin 3-*O*-rhamnosylglucoside (rutin) showed higher stability on aqueous solution (100 °C in aqueous model system) (Buchner, Krumbein, Rohn, & Kroh, 2006), under oxidative conditions (phosphate buffer, at pH 8.0, bubbling air into the solution) and under oxidative degradation (addition of Fe^{2+} and Cu^{2+}) (Makris & Rossiter, 2000) compared to quercetin. On the other hand, glycosylation showed to improve stability of flavonoids when incubated in Dulbecco's modified Eagle's medium (DMEM) at 37 °C (Al-Sharea et al., 2016). Furthermore, it was demonstrated that conjugation of sugars can increase the solubility of drugs and it can ameliorate *in vitro* absorption (Fernandez et al., 2000).

Regardless the role of the glycosylation, the most promising compounds we identified with potential adjuvant action with statins, such as those that do not alter the PCSK9 expression, are naringenin-7-*O*-rutinoside, and apigenin-6,8-*C*-glucoside.

On the contrary, hesperetin, hesperetin-7-*O*-glucoside, hesperetin-7-*O*-neohesperidoside induced significantly both LDLR and PCSK9 protein expression, thereby exerting statin-like effects. For this reason, LDLR/PCSK9 ratio are lower than other constituents and extract and similar to simvastatin. These flavanones have a hydroxyl substitution that seems to change significantly the effect on our biological targets, i.e. LDLR and PCSK9. Previous studies indicate that *O*-methylation metabolically stabilizes flavonoids, besides increasing their *in vitro* bioavailability and tissue distribution (Banach et al., 2018; Xiao & Hogger, 2015). Moreover, the hepatic metabolic stability and intestinal absorption are more favorable in methylated polyphenols than unmethylated polyphenols (Wen & Walle, 2006). Even if these constituents showed lower LDLR/PCSK9 ratio than the others, they may be useful as statin-like agents.

Brutieridin, a diglycoside of hesperetin, carrying the 3-hydroxy-3-methylglutaric acid (HMG) moiety, showed non-significant effect on LDLR and PCSK9 levels. Our results appear to be in contrast with previous studies where the *in vivo* treatments with 3-hydroxy-3-methyl-glutaryl flavanones enriched fraction (62 % of brutieridin) led to an increase of LDLR, HMGR (3-hydroxy-3-methylglutaryl-coenzyme A reductase) and FASN (fatty acid synthase) mRNAs and proteins expression respect to hypercholesterolemic group (Di Donna et al., 2014). The most reasonable explanation of the lack of effect in our *in vitro* system could be related to a low cellular bioavailability due to its high hydrophilicity, similarly to statins (Corsini, Raiteri, Soma, Fumagalli, & Paoletti, 1991).

Finally, eriodictyol, a non-*O*-methylated flavanone aglycone, showed a potent statin-like effect with significant induction of both LDLR and PCSK9. Besides flavanones and flavones, we isolated two non-flavonoids compounds, namely bergapten and nomilin. Bergapten, firstly isolated from bergamot essential oil, has been widely associated with many biological effects, including the modulation of the LDLR in HepG2 cells (Pattanayak, Bose, Sunita, Siddique, & Lapenna, 2018). Pattanayak et al observed a decrease in LDLR by an LXR-mediated increase of IDOL (inducible degrader of LDLR) levels, a known modulator of LDLR, thus envisioning a role for bergapten in the treatment of cholesterol overload in hepatocellular carcinoma. These observed differences may probably be due to the cell line utilised in these studies as well as the *in vitro* experimental conditions. In addition, here we demonstrated, for the first time, that bergapten significantly decrease PCSK9 levels, thus bringing the LDLR/PCSK9 ratio (2.77) in favor of LDLR, suggesting a possible additive effect with statins. However, given the known phototoxicity of bergapten, this compound is not a prime choice for a potential use as cholesterol-lowering agent (Y. Liang et al., 2021).

Nomilin is a typical limonoid of *Citrus* species which demonstrated hypoglycemic effect and improved glucose tolerance in high-fat diet-fed mice (Ono, Inoue, Hashidume, Shimizu, & Sato, 2011), along with a trend in decrease of triglycerides. In this work, we added insight into nomilin hypolipidemic activity, showing a very effective PCSK9 inhibitory action, while no evident effects on LDLR levels. Nomilin LDLR/PCSK9 ratio was 2.04, making it valuable synergist to the action of

statins. This evidence paved the way to further *in vivo* investigations on the hypocholesterolemic effects of nomilin.

Besides this evidence, bergamot peels were not yet investigated for potential hypocholesterolemic effects. Our results showed that bergamot peel extract significantly decrease of PCSK9 expression and, more importantly, increased the LDL uptake by Huh7 cells, thus supporting its use for the management of hypercholesterolemia, with potential adjuvant action to statins.

5. Conclusions

In conclusion, based on our data we can place *Citrus bergamia* peel extract and its constituents as good candidates for the development of new cholesterol-lowering agents for the prevention of mild hypercholesterolemia.

In particular, the most promising compounds acting as potential adjuvant-action with statins are naringenin-7-*O*-rutinoside and apigenin-6,8-*C*-glucoside, while hesperetin and its derivatives namely, hesperetin-7-*O*-glucoside, hesperetin-7-*O*-neohesperidoside, and eriodictyol demonstrated a significant statin-like effect placing them as potential natural agents for the management of cholesterol homeostasis. Finally, here bergamot peel extract was firstly investigated for its potential hypolipidemic effects and showed a potential adjuvant action to statins.

We acknowledge that our *in vitro* analysis cannot predict the effect in *in vivo* study. Thus, our conclusion that bergamot peel extract may improve the hypolipidemic effect of statins must be confirmed in clinical setting.

CRediT authorship contribution statement

Irene Ferrarese: Investigation, Writing – original draft, Writing – review & editing. **Maria Giovanna Lupo:** Conceptualization, Software, Writing – review & editing, Writing – original draft. **Ilaria Rossi:** Investigation. **Stefania Sut:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Francesca Loschi:** Investigation. **Pietro Allegrini:** Conceptualization, Supervision. **Antonella Riva:** Conceptualization, Supervision. **Nicola Ferri:** Conceptualization, Funding acquisition, Writing – review & editing, Supervision. **Stefano Dall'Acqua:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105724.

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