JOURNAL OF FUNCTIONAL FOODS XXX (2014) XXX–XXX

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Hypocholesterolaemic activity of 3-hydroxy-3-methyl-glutaryl flavanones enriched fraction from bergamot fruit (*Citrus bergamia*): "*In vivo*" studies

Leonardo Di Donna[a,1], *Domenico Iacopetta*[b,1], *Anna R. Cappello*[b], *Giselda Gallucci*[a], *Emanuela Martello*[b], *Marco Fiorillo*[b], *Vincenza Dolce*[b,*,2], *Giovanni Sindona*[a,*,2]

[a]*Dept. of Chemistry, University of Calabria, 87036 Arcavacata di Rende, (CS), Italy*
[b]*Dept. of Pharmacy, Health and Nutritional Sciences, University of Calabria, 87036 Arcavacata di Rende, (CS), Italy*

ARTICLE INFO

Article history:
Received 30 July 2013
Received in revised form
20 December 2013
Accepted 23 December 2013
Available online xxxx

Keywords:
Bergamot fruit
Brutieridin
High-density lipoprotein
Low-density lipoprotein
Melitidin
Statin like compounds

ABSTRACT

Statins are the pharmacological inhibitors of cholesterol biosynthesis, acting on key enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). They are the most effective class of drugs used to treat cardiovascular disease, the main cause of mortality in westernized countries. Our major goal was to investigate the hypocholesterolaemic effects of the 3-hydroxy-3-methyl-glutaryl flavanones enriched fraction (HMGF), extracted from bergamot fruit, in comparison with one of the most used statins, i.e. simvastatin, in a rat model. HMGF and simvastatin reduced the total cholesterol (TC), triacylglycerols (TG), very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) levels, whereas an increase of the high-density lipoproteins (HDL) content was observed exclusively in the HMGF treated rats. Additionally, an appreciable increase of HMGR, LDL receptor (LDLR) and fatty acid synthase (FASN) was evidenced, at mRNA and protein levels. HMGF did not exhibit genotoxic effects and was cytotoxic only at elevated concentrations. The daily supplementation of HMGF in the diet could be very effective for the treatment of hypercholesterolaemia.

1. Introduction

Cholesterol is a molecule of primary importance in animals, including humans, but is not required in the diet because hepatocytes can synthesize it starting from acetyl-CoA. The HMGR is the rate-limiting enzyme in endogenous cholesterol biosynthesis and catalyzes the conversion of HMG-CoA into mevalonate (Goldstein & Brown, 1990). Inhibition of HMGR has proven to be one of the most effective approaches for lowering plasma LDL and reducing cardiovascular event rates (Ross et al., 1999). As part of a compensatory mechanism due to cholesterol depletion in the liver, inhibition of HMGR leads to an increased synthesis of itself and low-density lipoprotein receptors (LDLR); this latter process allows a subse-

* *Corresponding authors.* Address: Dept. Pharmacy, Health and Nutritional Sciences, University of Calabria, Arcavacata di Rende, (CS) 87036, Italy. Tel.: +39 (0) 984 493119; fax: +39 (0) 984 493107 (V. Dolce). Dept. Chemistry and Chemistry Technologies, University of Calabria, Arcavacata di Rende, (CS) 87036, Italy. Tel.: +39 (0) 984 492083; fax: +39 (0) 984 492044 (G. Sindona).
E-mail addresses: vincenza.dolce@unical.it (V. Dolce), giovanni.sindona@unical.it (G. Sindona).

[1] These authors contributed equally to this work.

[2] Joint senior authors.

Abbreviations: HMGF, 3-hydroxy-3-methyl-glutaryl flavanones enriched fraction; FASN, fatty acid synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; H, hypercholesterolaemic; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LDLR, low-density lipoprotein receptors; S, simvastatin; TC, total cholesterol; TG, triglycerides; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

1756-4646/$ - see front matter
http://dx.doi.org/10.1016/j.jff.2013.12.029

quent clearance of LDL from systemic circulation (Brown & Goldstein, 1986), lowering the risk of atherosclerosis and coronary heart diseases (Balbisi, 2006; Miller, 1996).

The HMGR inhibitors (statins) are the most effective, practical and largely prescribed class of drugs for reducing LDL concentrations, because they are pretty safe, well tolerated and highly efficient, nevertheless cardiovascular disease remains the main cause of mortality in westernized countries. The pursuit of novel therapies targeting the residual risk is focused on preventing the increase of LDL concentration and favouring the increase of HDL (deGoma & Rader, 2011). Some natural compounds found in the human diet have been shown to possess therapeutic and pharmacologic properties, in particular, the daily consumption of citrus fruit juice has been shown to positively influence plasma lipid levels and reduce the risk of coronary heart disease (Gorinstein et al., 2004; Srinivasan & Pani, 2013). Hypolipidaemic effects can be correlated to several components of citrus juice, such as flavonoids (naringin and hesperidin), pectins, and ascorbic acid, which have a high antioxidant potential and interfere with cholesterol metabolism (Chen, Ma, Liang, Peng, & Zuo, 2011; Chinapongtitiwat, Jongaroontaprangsee, Chiewchan, & Devahastin, 2013; Gorinstein et al., 2005; Monforte et al., 1995).

Bergamot, the common name of the fruit *Citrus bergamia* Risso, belongs to the family Rutaceae, subfamily Esperidea, and is widespread in the Mediterranean area for centuries. The botanical and geographical origins are still uncertain; probably bergamot is native of Calabria, deriving by mutations of other citrus species, or even arrived *in loco* from Berga (hence the name of bergamot) otherwise from Antilles, Greece and Canary Islands. Under an ethno-botanical point of view, the history of calabrian bergamot cultivation is of interest because several cultures rule in the past (Greeks, Byzantines, Arabs) and the awareness of the "subsistence", due to the poorly developed road network and the isolation of villages (Passalacqua, De Fine, & Guarrera, 2006). These events, together with the optimal habitat, promoted the intensification of bergamot cultivation that became a flagship product of calabrian agriculture, although the essential oil characteristics were not known and appreciated at the beginning (Rapisarda & Germanò, 2013). Afterwards, the terpene-rich essential oil, extracted from the peel of the pear-shaped fruit, and its volatile fraction have been largely employed in the cosmetic and perfumery products. In the early 18th century, Jean Paul Féminis created the "Aqua mirabilis", a distillate exhibiting digestive and antiseptic properties which was subsequently used as cologne; after that, many famous perfumers (Guerlain, Roudnitska) widely exploited bergamot essential oil for their creations (Bijaoui, 2013).

Bergamot and its derivatives have also been used in calabrian folk medicine as fever palliative, antiseptic, anthelminthic, wound healing, anti-inflammatory and hypocholesterolaemic agent (Mollace et al., 2011; Trombetta et al., 2010; www.rc.camcom.gov.it). To date, bergamot essences are used in the food industry for the preparation of teas, jams, sherbet and other commodities, but also for the domestic preparation of liquors, ice creams and pastries by the calabrian confectionery industry (www.rc.camcom.gov.it). Bergamot juice has been considered, for a long time, a secondary and waste by-product of the essential oil extraction and, because of its organoleptic properties and its bitter taste, did not reach the popularity of other citrus juices but was used to fortify fruit juice instead of synthetic additives (Di Donna et al., 2009). Later on, the discovery of the abundance and variety of bioactive compounds in the juice (e.g. naringin, neoeriocitrin, and neohesperidin), led to the development of several nutraceuticals (capsules, pills or soluble granular powders) in the global market (Lo Curto, 2013). Furthermore, other flavonoids, such as rhoifolin, neodiosmin, and some chryosoeriol derivatives, have been found in smaller amounts (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2007; Dugo et al., 2005; Gattuso et al., 2006) and different tissues of the fruit produce the flavonoids diosmin and poncirin (Nogata et al., 2006).

We undertook a detailed analysis of dry extract from bergamot peel fruit. Some statin-like components such as brutieridin and melitidin (Di Donna et al., 2009, 2013), were found and isolated from the dry extract. The main aim was to investigate the effects of the extract (i.e. HMGF) on the total lipid profile of hypercholesterolaemic rats in comparison with simvastatin, examining the hepatic and serum lipid contents and the expression of the HMGR, LDLR and FASN. Our study demonstrates the ability of HMGF to parallel the simvastatin effects on the lipid profile and, more importantly, to increase the HDL levels. Lastly, HMGF is cytotoxic only at very high doses and no genotoxic effects have been evidenced.

2. Materials and methods

2.1. *Preparation of 3-hydroxy-3-methyl-glutaryl flavanones enriched fraction*

A dry extract of bergamot fruit (*C. bergamia* Risso) provided from Gioiasucchi s.r.l. (Gioia Tauro, Italy) was submitted to flash chromatography using the VersaFlash™ system from Supelco (St. Louis, MO, USA); 35 g of C_{18} 200–400 mesh (Aldrich, St. Louis, MO, USA) were used as stationary phase into a 40 × 37.5 mm column (Supelco, St. Louis, MO, USA). The column was activated eluting in sequence 200 mL of EtOH, 200 mL of H_2O/EtOH (50:50, v/v), 200 mL of H_2O/EtOH (75:25, v/v), 200 mL of H_2O/EtOH (87.5:12.5, v/v), and finally 500 mL of H_2O, maintaining the flow rate at 25 mL/min. The column was loaded pouring 2 g of dry extract dissolved in 10 mL of H_2O with a syringe; 100 mL of water were then eluted at 5 mL/min and the flow rate was gradually increased to 50 mL/min. The elution of the components of the dry extract was conducted using 4 L of H_2O collecting fraction of 0.5 L each, then 1 L of H_2O/EtOH (90:10, v/v) and 500 mL of H_2O/EtOH (70:30, v/v) were used, collecting fraction of 50 mL each. The first two fractions of the elution step with H_2O/EtOH (70:30, v/v) were combined and evaporated to dryness under vacuum. One hundred twenty-five milligrams of a mixture containing 62% of brutieridin, 14% of melitidin and 15% of another HMG-flavanone conjugated (i.e., HMG-neoeriocitrin, unpublished data) (Fig. 1) were obtained in a single run.

All fraction were monitored using a FractionLynx semi-preparative system from Waters (Milford, MA, USA) in analytical mode equipped with an UV detector and a Luna C_{18} column 5 mm particle size, 25 cm × 4.6 mm (Supelco, St. Louis, MO, USA). The run time was 105 min, while the flow

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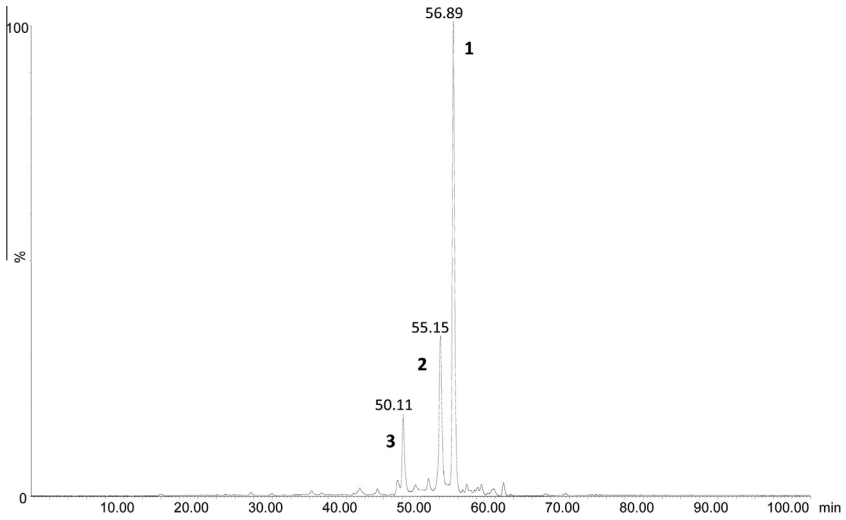

Fig. 1 – 280 nm HPLC/UV Chromatogram of the HMGF. The revealed molecules were: brutieridin (1, r.t. 56.89), melitidin (2, r.t. 55.15) and neoeriocitrin HMG conjugated (3, r.t. 50.11).

rate was set at 1 mL/min and the following eluents and gradient conditions were used: 0.1% HCOOH in H_2O (solvent A) and CH_3OH (solvent B); 10 min isocratic 80% A; 2 min linear gradient from 80 to 74% A; 65 min linear gradient from 74 to 31% A; 18 min linear gradient from 31 to 80%; 10 min isocratic 80% A. Twenty milliliters of each fraction were injected into the loop, while the absorption wavelength of the UV detector was set at 280 nm (Di Donna et al., 2009).

2.2. *Animals and diets*

Male Wistar strain rats, weighing about 150 g each, were purchased from Charles River (Lecco, Italy). All of the animal experiment protocols followed the institutional guidelines of the Italian Ministry of Health for Animal Care (D.M. 116/1992). Animals were housed 1 rat per cage, in an air conditioned room with a 12 h light–dark cycle and with *ad libitum* access to food and water. Before the experiment, all the animals were allowed to stabilize by being fed with regular rodent chow, then randomly divided into four groups of twelve animals each: group N (control) received the regular diet for 3 weeks; group H (hypercholesterolaemic) received the hypercholesterolaemic diet (regular diet +2% cholesterol +0.2% cholic acid) for 3 weeks; group H + S received the hypercholesterolaemic diet (regular diet +2% cholesterol +0.2% cholic acid) for 3 weeks; from the 2nd to the 3rd week each rat was administered by gavage with simvastatin (20 mg/kg bw/day) and group H + HMGF received the hypercholesterolaemic diet (regular diet +2% cholesterol +0.2% cholic acid) for 3 weeks; from the 2nd to the 3rd week each rat was administered by gavage with the HMGF (60 mg/kg bw/day).

Regular and hypercholesterolaemic diets were supplied by Charles River (Lecco, Italy), simvastatin was purchased from Sigma Aldrich (Milan, Italy).

During the experiment, rats were weighed daily and the 24 h food consumption was recorded. At the end of the study, the animals were killed by decapitation, under chloral hydrate anesthesia, and blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-treated tubes, centrifuged at 2500*g* for 15 min and the serum was separated and stored at −20 °C until analyzed (Rai, Sharma, & Tiwari, 2009); liver was excised and immediately frozen in liquid nitrogen, then stored at −80 °C until used.

2.3. *Biochemical estimations*

The livers were cut into small pieces, washed several times in a cold buffer (0.25 M sucrose, 3 mM EDTA, 20 mM Tris–HCl, pH 7.0), next homogenized in the same buffer and treated as described in Muci et al. (1992) for TC extraction. Briefly, an aliquot of 10 mg protein sample was saponified with alcoholic KOH for 90 min at 85–90 °C. Unsaponifiable constituents were extracted three times (5 mL each) with light petroleum (b.p. 40–60 °C). The pooled extracts were then evaporated to dryness under N_2 and the residue, dissolved in 2-propanol (Muci et al., 1992). Total lipids were extracted from liver homogenate (10 mg protein) with chloroform/methanol (1:1, v/v) according to Bligh and Dyer (1959). The hepatic TC and TG levels were measured respectively by HPLC and direct enzymatic assays from (Chematil Srl, Salerno, Italy).

The TG, TC, HDL, LDL and VLDL levels were evaluated respectively by direct enzymatic assays using Trigliceridi Kit and Colesterolo kit (Chematil srl, Salerno, Italy), HDL Kit (Intermedical srl, Naples, Italy), LDL Kit (Biogemina srl, Catania, Italy) and VLDL ELISA Kit (antibodies-online GmbH, Aachen, Germany), according to manufacturer's instruction with some modifications.

2.4. *RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction*

RNAs were extracted from liver as described by Zara et al. (2007) and aliquots of 1 μg were reverse-transcribed as reported in Iacopetta et al. (2011). Quantitative real-time polymerase chain reaction (RT-PCR) was performed with the obtained complementary DNAs (cDNAs) using the Applied Biosystems StepOne™. Real-Time PCR System (Applied Biosystems, Monza, Italy). Primers based on the cDNA sequences

of the genes of interest were designed using Primer Express (Applied Biosystems, Monza, Italy). In each sample of 20 μL real-time PCR reaction 10 ng of cDNA, 10 μL of the Power SYBR® PCR Master Mix (Applied Biosystems, Monza, Italy) and 0.3 μL (150 nM) of the specific primers for each genes analyzed were used. Each experiment was repeated at least 3 times. The comparative threshold cycle method was used in relative gene quantification as previously described (Lunetti et al., 2013) using 18S gene as the endogenous control.

2.5. *Microsomal fraction extraction and Western blot analysis*

The liver homogenate, obtained as previously described, was centrifuged at 800*g* for 8 min, the pellet was discarded and supernatant centrifuged for 10 min at 12,000*g*. The pellet containing the mitochondrial fraction was discarded and the supernatant was first centrifuged at 20,000*g* for 20 min and then at 105,000*g* for 60 min to obtain the cytosolic fraction and pelleted microsomes. The latter were re-suspended in the homogenizing medium and centrifuged again under the same conditions. Contamination of microsomal preparation by other subcellular fractions, ranging from 5 to 9%, was determined by the assay of marker enzymes as described (Caputi Jambrenghi et al., 2007) and protein concentration was determined as in (Madeo et al., 2009). Western blot analysis was conducted as described previously (Cappello et al., 2012; Iacopetta et al., 2010). Antibodies anti-HMGR (H-300), fatty acid synthase (FASN, H-300), LDL receptor (C-20), anti-calnexin (C-20), anti-β-tubulin (D-10), anti-GAPDH (FL-335) were supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and used following the manufacturer's instructions. Anti-calnexin, anti-β-tubulin or anti-GAPDH antibodies were used to confirm the equal loading of proteins.

2.6. *HPLC analysis of total cholesterol in liver samples*

The analyses of samples were performed following a method from literature (Duncan, Culbreth, & Burtis, 1979).

2.7. *Cytotoxicity assays*

The potential toxicity of the HMGF was investigated on HepG2 and 3T3-L1 cell lines, which were cultured in DMEM (Dulbecco's modified Eagle's medium) with phenol red, supplemented with 10% FBS and 100 μg/mL penicillin/streptomycin. Cell proliferation using a Countess™ automated cell counter (Invitrogen) and MTT viability assays were performed, as reported elsewhere (Knutson et al., 2012; Santolla et al., 2012), in order to determine the median cytotoxic concentration (CC_{50}). CC_{50} is defined as the concentration of HMGF which reduces the viability/number of the cells such

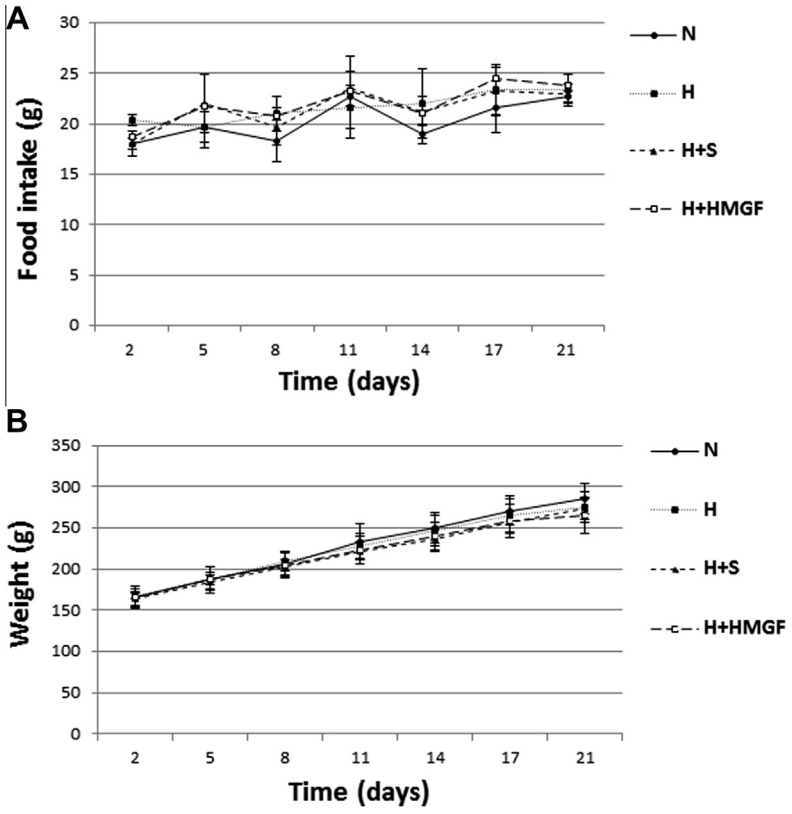

Fig. 2 – Summary of daily food intake and body weight changes over the study period for each group of rats. Hypercholesterolaemic rats group (filled squares, H) was fed and then treated with simvastatin (triangles, H + S) or HMGF (open squares, H + HMGF), as described in par 2.2. Regular diet fed rats group is indicated as N (dots). Values represent the mean ± SD of the daily chow intake (panel A) and of the daily weight (panel B) for each rat respect to the group.

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that only 50% of the cells survive, in comparison with the untreated group (only vehicle). Different concentrations, i.e. six to eight serial dilutions, of HMGF were tested in the experiments and the obtained data were used to construct a sigmoidal dose–response curve with variable slope. A non-linear regression curve fitting the sigmoidal model was performed employing GraphPad PRISM5 (GraphPad Software, La Jolla, CA, USA) to calculate CC_{50}. All cytotoxicity assays were performed in duplicate.

2.8. Genotoxicity experiments

Genotoxicity experiments were performed using the SOS-Chromotest™ kit (EBPI, Mississauga, Ontario, Canada) following the manufacturer's instructions, in presence or absence of rat liver S9 activation enzymes (EBPI, Mississauga, Ontario, Canada), used as exogenous metabolic activation system. The induction factor (IF) was calculated as the ratio of β-gal activity/Alkaline Peroxidase activity (determined in presence of increasing HMGF concentrations) and the β-gal activity/AP activity (in the absence of the HMGF). The enzyme activities were detected colorimetrically (Quillardet & Hofnung, 1993). In the SOS Chromotest assay, the compounds are classified as non-genotoxic, if the SOS IF remains <1.5, marginally genotoxic in the IF ranges between 1.5 and 2, and as genotoxic if IF exceeds 2.0 (Kevekordes et al., 1999). The experiments were performed in triplicate.

2.9. Statistical analysis

All data are presented as means ± SD (n = 12 rats/group). Data were analyzed by one-way ANOVA, followed by Dunnett's test using the GraphPAD Prism5 software (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Food intake and body weight gain

The data on food intake and body weight are shown in Fig. 2. In Fig. 2, panel A, it is summarized the daily food intake respect to the whole studies period for each group of rats. As positive treatment control in rats, we used one of the most prescribed statins in man, simvastatin. No significant differences in food intake were observed for each group (i.e. simvastatin or HMGF treated rats versus untreated). Fig. 2, panel B, shows the overview of changes in body weight over the study period for the different rat groups. For each rat the body weight normally increased during the studies period, without

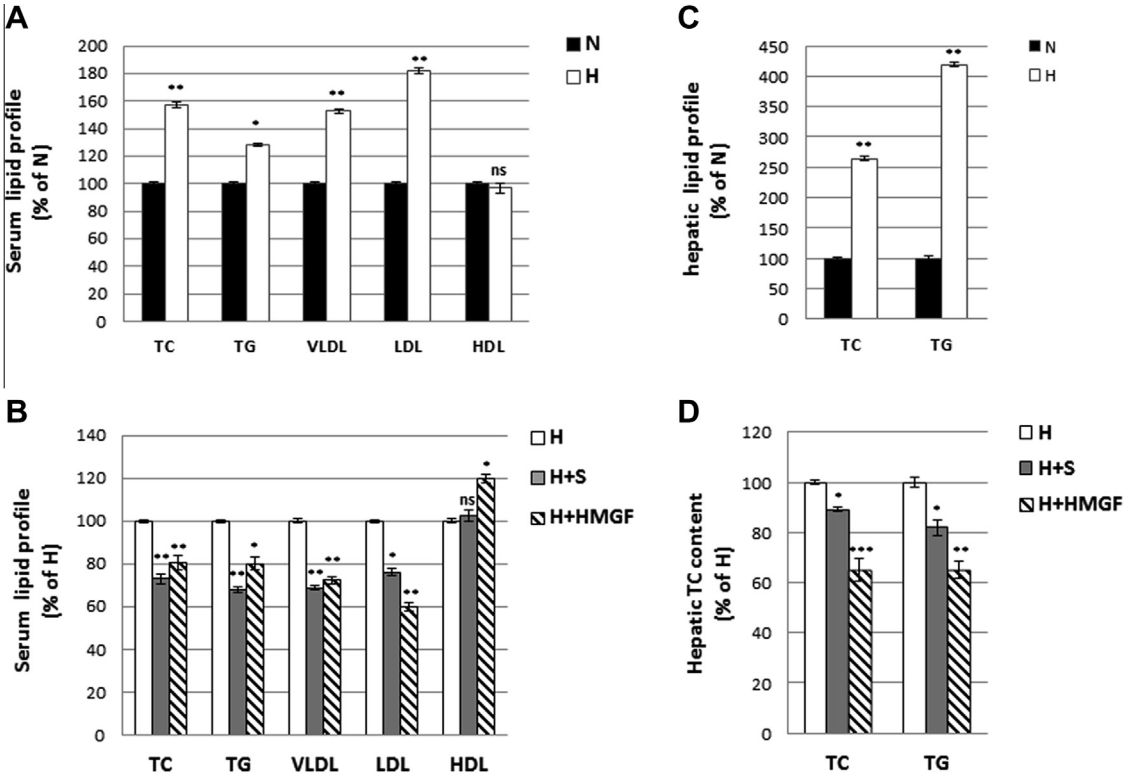

Fig. 3 – Serum and hepatic lipid profile variations between normal, hypercholesterolaemic and treated rats Normal (black bars, N), hypercholesterolaemic (white bars, H), simvastatin treated (grey bars, H + S) and HMGF treated (striped bars, H + HMGF) rats groups were fed as described in par 2.2. The total cholesterol (TC), triglycerides (TG), very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) levels were measured in N and H serum (panel A, N as control) and in H + S and H + HMGF serum (panel B, H as control). TC and TG were also measured in N and H liver (panel C, N as control) and H + S and H + HMGF liver (panel D, H as control). The obtained results were plotted as percentage and columns are mean ± SD of three independent experiments performed in duplicate. $^{*}P = 0.01$ *vs.* control; $^{**}P < 0.01$ *vs.* control; $^{***}P < 0.005$ *vs.* control; ns, nonsignificant.

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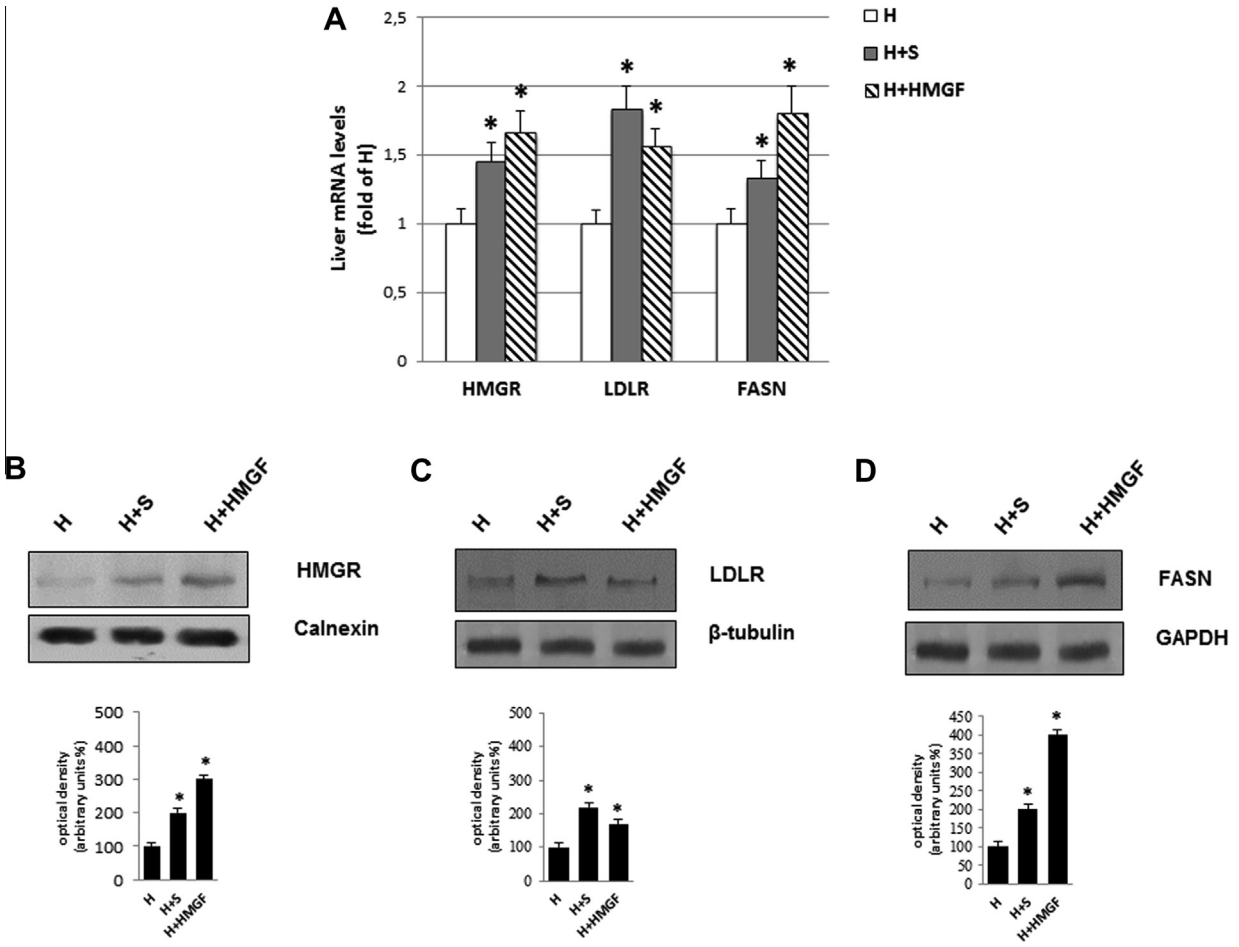

Fig. 4 – Effect of simvastatin and HMGF on HMGR, LDLR and FASN mRNAs and proteins expression in experimental rats. Panel A: livers were isolated from hypercholesterolaemic untreated rats (white bars, H), hypercholesterolaemic treated with simvastatin (grey bars, H + S) or with HMGF (striped bars, H + HMGF). The HMGR, LDLR and FASN mRNAs levels were analyzed by RT-PCR and normalized to that of 18S. The values are plotted as fold of H group and are representative of three independent experiments. *P < 0.01 *vs.* H. Panels B–D: protein levels of HMGR (97 kDa, panel B), LDLR (160 kDa, panel C) and FASN (270 kDa, panel D) are shown. A total of 100 μg of microsomes or cellular liver extracts were used for Western blot analysis; Calnexin (90 kDa), β-tubulin (55 kDa) or GAPDH (37 kDa) were used as a control for equal loading and transfer. Densitometric analyses of the blots are also shown. The immunoblots are representative of three separate experiments. *P < 0.01 *vs.* H.

significant differences in weight within the same group or between the different groups. Moreover, the livers excised from each treated group rats were not significantly different in weight from those of the induced hypercholesterolaemic rats (data not shown). These observations indicate that the diet and treatments used in these studies were well tolerated in rats, given that no physical alteration, body weight loss or food intake reduction occurred over the period of the study. A group of regular diet fed rats was taken under evaluation as control to verify the induced hypercholesterolaemia.

3.2. *Effect of simvastatin and HMGF on serum and hepatic lipid content*

To examine the concentrations of TC, TG, VLDL, LDL and HDL in rat serum, we used a colorimetric assay, whereas the hepatic TC and TG were measured by HPLC and colorimetric assay respectively. Firstly, we compared the serum and hepatic lipid profiles of the regular diet fed rats (group N) and the hypercholesterolaemic ones (group H, Fig. 3, panels A and C), in order to establish whether the hypercholesterolaemic rat model has been achieved. The results showed a clear increase of the lipid profile in serum (Fig. 3, panel A) and liver (Fig. 3, panel C) of H group rats. In particular TC, TG, VLDL and LDL serum levels were increased of 57, 28, 53 and 82% respectively, compared to the N group, which values were 79.85 ± 5.6 mg/dl for TC, 104.7 ± 9.3 mg/dl for TG, 19.02 ± 1.5 mg/dl for VLDL and 35.03 ± 3.1 mg/dl for LDL, whereas no significant variation has been found for HDL levels (for N group the HDL value was 43.52 ± 3.3 mg/dl). A marked raise of the hepatic TC and TG levels has been observed in H group (164 and 319% respectively) compared to N group, which values were 1.98 ± 0.9 mg/g liver for TC

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Table 1 – Genotoxic effect of the HMGF in the absence of the exogenous metabolizing system S9 in the SOS Chromotest.

Sample	μg/mL	*E. coli* PQ37		
		β-galactosidase (units)	Alkaline phosphatase (units)	IF
4-NQO	20	4.37 ± 0.02	12.99 ± 0.09	7.350615858
HMGF	0	0.60 ± 0.03	13.11 ± 0.12	1
HMGF	10	0.62 ± 0.01	13.08 ± 0.11	1.035703364
HMGF	30	0.65 ± 0.05	13.05 ± 0.08	1.050797182
HMGF	60	0.67 ± 0.03	12.99 ± 0.09	1.03553029
HMGF	90	0.68 ± 0.02	13.01 ± 0.07	1.01336515

Data are expressed as the mean ± SD of three independent experiments. 4-NQO was used as a positive control. IF = inhibition factor.

and 4.87 ± 0.39 mg/g liver for TG. These results confirmed that H group can be used as a hypercholesterolaemic model in the following experiments.

After that, we investigated the effects elicited by the simvastatin and HMGF treatments on serum and hepatic lipid levels of hypercholesterolaemic rats and we observed a significant decrease of the serum TC (of about 30 and 20%, respectively) compared to H group (Fig. 3, panel B). We also observed a reduction in liver TC content of 11% in H + S group and a more significant reduction of 35% in H + HMGF group (Fig. 3, panel D). It is well known that an elevated concentration of LDL in blood is associated with a high risk of coronary heart disease and atherosclerosis plaque lesions (Berliner & Heinecke, 1996; Ross, 1993), thus we also evaluated serum levels of LDL and VLDL (Marzetta, Foster, & Brunzell, 1990). A significant reduction of VLDL and LDL was measured in H + S (33 and 24% lower, respectively) and H + HMGF (28 and 40% lower, respectively) groups (Fig. 3, panel B). Conversely, any significant difference in the HDL cholesterol was found in the H + S group, whereas, surprisingly, a significant increase was observed in the H + HMGF group (20% higher) respect to H group (Fig. 3, panel B).

Since HMGR inhibitors have been shown to contribute in decreasing plasmatic TG levels in experimental animals (Krause & Newton, 1995), we investigated this feature in our model. We measured serum and hepatic TG content observing that, in both cases, there was a decrease in H + S and H + HMGF groups respect to H group of about 32 and 20%, respectively, for serum TG (Fig. 3, panel B), and of about 18 and 35%, respectively, for hepatic TG (Fig. 3, panel D).

3.3. *Effect of simvastatin and HMGF administration on hepatic HMGR, LDLR and FASN mRNAs and proteins levels*

In order to further investigate the HMGF effects in H groups, we evaluated the transcriptional levels of two main proteins involved in cholesterol metabolism, i.e. HMGR and LDLR, and the expression of the main enzyme of fatty acid biosynthesis, i.e. FASN (Dolce, Cappello, Lappano, & Maggiolini, 2011). As shown in Fig. 4, panel A, simvastatin and HMGF treatments produced a similar modulation on the gene expression of the above mentioned proteins. Particularly, simvastatin administration, respect to H group, brought to an increased transcription of HMGR, LDLR and FASN genes in an extent of 1.45, 1.83 and 1.33-fold respectively. As well HMGF treatment increased the expression of HMGR, LDLR and FASN genes of 1.66, 1.56 and 1.80-fold respectively. In all cases the mRNAs increase is appreciable respect to the untreated H group. These data were additionally verified by Western blot analyses conducted on the microsomal or cytoplasmic fractions or on liver homogenate extract, considering the different sub-localization of proteins analyzed. As shown in Fig. 4, panels B–D, according to RT-PCR results, both simvastatin and HMGF treatments led to a clear increase of HMGR, LDLR and FASN proteins respect to H group.

3.4. *Bergamot and HMGF safety*

Bergamot fruit is generally considered safe and no evidences exist about its toxicity and the juice has been granted for human nutrition (www.salute.gov.it). Furthermore, animal and human studies highlighted the protective effects of bergamot juice or extracts (Mollace et al., 2011; Trovato et al., 2010) and did not report any toxic effects. Conversely, since no literature data have been published about the HMGF toxicity, we carried out experiments to test the safety of our fraction. The potential cytotoxicity was tested on HepG2 and 3T3-L1 cell cultures as reported in Section 2 (par 2.7), and the results showed a low cytotoxic activity, as evidenced by CC_{50} values of 54.04 ± 2.3 and 51.09 ± 1.8 mg/mL, respectively, for HepG2 and 3T3-L1 cells. Additionally, in order to establish if HMGF could exert some genotoxic and/or mutagenic effects by itself or even their metabolic derivatives, we performed the SOS Chromotest assay, in presence or absence of an exogenous metabolic activation system S9 from rat liver. The outcomes, shown in Tables 1 and 2, demonstrated that HMGF, used at doses up to 90 μg/mL, did not produce any DNA damage, also after being metabolized by hepatic enzymes.

4. Discussion

The cholesterol homeostasis is subtly regulated at several levels as intestinal absorption, hepatic uptake of LDL, *de novo* synthesis and excretion. When its blood concentration raise over certain levels, the incidence of atherosclerosis and cardiovascular/cerebrovascular diseases becomes higher (Steinberg, 1987), hence it followed the need to develop many statin classes, as advanced pharmacological treatment, during the twentieth century (Witztum, 1996). Besides, it became pretty clear that a more correct daily diet could prevent the hypercholesterolaemia, lowering the risk of the associated-diseases onset, as already reported from traditional cures

Table 2 – Genotoxic effect of the HMGF in the presence of the exogenous metabolizing system S9 in the SOS Chromotest.

Sample	μg/mL	*E. coli* PQ37 + (S9)		
		β-galactosidase (units)	Alkaline phosphatase (units)	IF
4-NQO	20	4.33 ± 0.03	13.01 ± 0.08	7.142009299
HMGF	0	0.61 ± 0.01	13.09 ± 0.11	1
HMGF	10	0.61 ± 0.03	13.07 ± 0.12	1.001530222
HMGF	30	0.63 ± 0.02	13.08 ± 0.10	1.050797182
HMGF	60	0.65 ± 0.04	12.96 ± 0.09	1.03553029
HMGF	90	0.66 ± 0.002	13.02 ± 0.10	1.01336515

Data are expressed as the mean ± SD of three independent experiments. 4-NQO was used as a positive control. IF = inhibition factor.

employed in folk medicine (Miceli et al., 2007). Concerning this, the right habit of eating foods containing many bioactive compounds, as flavonoids, pectins or ascorbic acid (Gorinstein et al., 2005; Monforte et al., 1995), has been shown to positively influence serum lipid levels and, most importantly, to reduce atherogenic lipoproteins. Recent studies have shown the presence of some statin-like compounds in the *C. bergamia* Risso (Di Donna et al., 2009; Mollace et al., 2011), to which can be ascribed the beneficial effects exerted on human health.

Starting from the latter data, in the present study we demonstrated that the three statin-like flavanones, extracted from bergamot peel and contained in HMGF, exert a similar behaviour respect to commercial simvastatin on a model of hypercholesterolaemic rats. The achievement of this model has been validated by the serum and liver lipid profile comparison of N *vs*. H group, which has been used in the subsequent experiments in order to test HMGF effects. The daily diet supplementation of H group with HMGF, over the period of the study, led to a decrease of serum TC and TG, as in H + S group, but exhibited a higher ability in decreasing LDL levels, accompanied by a significant increase in serum HDL content. The latter peculiarity represents a favourable event, given that HDL are able to picking up cholesterol from peripheral tissues or cells and carries it back to liver (deGoma & Rader, 2011), where they are readily catabolized contributing to prevent atherosclerosis. It has been reported that elevated LDL levels and decreased HDL levels in serum represent independent risk factors for the onset of atherosclerosis associated diseases (Arsenault et al., 2009), whereas the data reporting the VLDL role in atherosclerotic plaque formation are still controversial (Stancu, Toma, & Sima, 2012). In our experiments, HMGF was found able to exert its beneficial actions on the LDL/HDL ratio but also to decrease VLDL serum levels, the latter event represents another very interesting feature. Then, we investigated the TC and TG hepatic levels, observing a drop of both in simvastatin treated rats hepatocytes and, in a higher extent, in HMGF treated group. The TG and TC diminished availability determines the observed fall in VLDL liver secretion, required for cholesteryl ester transport to extra-hepatic districts. The effects on serum and hepatic lipid contents are strictly related to the variation of hepatic key enzymes and proteins involved in TC and TG metabolism. Particularly, we evaluated the expression level of HMGR and LDLR transcripts and proteins, as main indicators of cholesterol metabolism, together with the transcript and protein levels of FASN gene, mainly involved in TG metabolism. We found an up-regulation in HMGR and LDLR

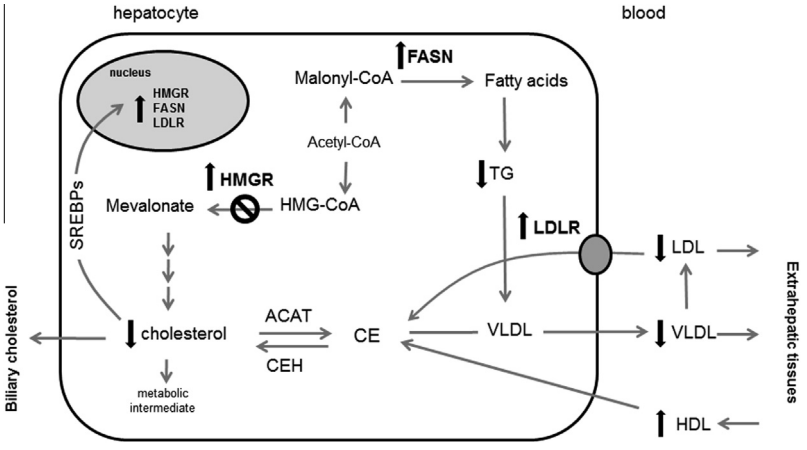

Fig. 5 – Model depicting HMGF effects on lipids metabolism elicited in rat hepatocytes. Black arrows indicate HMGF effects on genes, enzymes and metabolites levels. ⊘ indicates enzymatic inhibition. ACAT, Acyl-CoA:cholesterol acyltransferase; CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; FASN, fatty acid synthase; HDL, high-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; SREBPs, sterol response element binding proteins; TG, triglycerides; VLDL, very low-density lipoprotein.

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genes transcription under simvastatin treatment, according to previously published data (Kong et al., 2008) and also a significant induction of FASN gene transcription which should be included in the knowledge of simvastatin induced effects, not yet reported. Similar results have been obtained in H + HMGF group, but in a higher extent, confirming one more time the HMGF statin-like behaviour. This positive gene regulation consequently affects the proteins expression in liver indeed, in our experimental model, the increase in HMGR, LDLR and FASN gene transcription and protein translation under HMGF treatment is clearly evident and justify the hypolipidemic effects observed in rat serum. The blockade of HMGR activity and the induced expression of hepatic LDLR may be considered amongst the events responsible of TC and LDL decrease. As it is known, the HMGR expression and activity may change in response to the content of local cholesterol in cells and/or tissues (Goldstein & Brown, 1990). In our model, HMGR inhibition lead to a reduction of endogenous cholesterol which, in turn, is responsible of the up-regulation of HMGR and LDLR genes transcription, as well of the higher LDLR exposure within the hepatocytes membrane, through a compensating mechanism based on sterol regulatory element-binding proteins (SREBPs) pathway (Scharnagl et al., 2001). It should be recalled that cholesterol depletion below a certain threshold is also responsible of FASN gene transcription increase, via SREBPs activation (Sato, 2010; Scharnagl et al., 2001), which is one of the effects we observed under both simvastatin or HMGF treatments. These outcomes indicate that HMGF could be a high potential control agent in hypercholesterolaemia caused diseases, confirming one more time the ancient use of bergamot, as source of various nutraceutics, for instance flavonoids. Indeed, in industrialized countries there has been a general trend towards the use of traditional medicines; for their pharmacological properties and, as well, for their low toxicity in animals, flavonoids have been considered as a *panacea* in several diseases treatment (Kurzawa-Zegota, Najafzadeh, Baumgartner, & Anderson, 2012; Nijveldt et al., 2001). Effectively, the three flavanones contained in HMGF did not show toxicity *in vitro*, if not at very elevated concentrations, neither a potential genotoxicity. Our results, summed up in Fig. 5, reinforce the traditional use of bergamot fruit by the calabrian population and may be useful to broaden the survey of the literature data about bergamot and its derivatives.

5. Conclusions

The results presented here support the large number of studies regarding the already known hypolipidaemic effects of diet integration with some citrus species, and bring an important contribution to the complexity of the effects exerted by bergamot fruit, focusing the attention on the presence of statin-like compounds which block the HMGR activity. In rats, the daily supplementation with HMGF in the diet was able to significantly reduce the TC, TG, VLDL and LDL and, more importantly, to increase contemporaneously the HDL serum levels. These data establish, in a model that is highly related to humans, that inhibition exerted by HMGF on HMGR is a promising nutraceutical strategy for the control of hypercholesterolaemia, the main factor responsible of the increased cardiovascular diseases risk.

Acknowledgments

The work presented here was carried out in collaboration between all authors. V.D., G.S. and A.R.C. defined the research plan, discussed analyses, interpretations and edited the manuscript. D.I. and A.R.C. designed methods and experiments, carried out the biological assays, analyzed the data, interpreted the results and wrote the paper. L.D.D. designed the chemistry methods, provided the HMGF fraction and collaborated to manuscript writing. E.M., G.G. and M.F. cooperated in laboratory experiments.

This work is part of the Italian National Project, MIUR-PRIN 2009XZ7XAB_003.

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