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Hypolipemic and hypoglycaemic activity of bergamot polyphenols: From animal models to human studies

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ABSTRACT

Bergamot juice produces hypolipemic activity in rats though the mechanism remains unclear. Here we investigated on the effect of bergamot extract (BPF) in diet-induced hyperlipemia in Wistar rats and in 237 patients suffering from hyperlipemia either associated or not with hyperglycaemia. BPF, given orally for 30 days to both rats and patients, reduces total and LDL cholesterol levels (an effect accompanied by elevation of cHDL), triglyceride levels and by a significant decrease in blood glucose. Moreover, BPF inhibited HMG-CoA reductase activity and enhanced reactive vasodilation thus representing an efficient phytotherapeutic approach in combating hyperlipemic and hyperglycaemic disorders.

1. Introduction

Hyperlipidemia or hypercholesterolemia is an important risk factor for the development of atherosclerosis and coronary artery disease [1,2]. Main pathogenic blood parameters are increased concentrations of cholesterol bound to Low-density Lipoprotein (cLDL), total blood cholesterol (totChol) and triglycerides (TG). Conditions of insulin resistance such as impaired glucose tolerance or "prediabetes" are also characterized by a high risk of cardiovascular diseases (CVD) [3]. Majority of therapeutic protocols rely on drugs that belong to statin family. Statins inhibit the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which catalyzes the rate-limiting step in mevalonate biosynthesis, a key intermediate in cholesterol metabolism. This is associated to a decrease in totChol and a switch from cLDL to High-density Lipoprotein (cHDL) fraction. Despite the significant clinical benefits provided by statins [1], many patients, in particular those with metabolic syndrome, do not achieve their recommended low-density and high-density lipoprotein (LDL, HDL) cholesterol target goals with statins [3]. Moreover, the use of statins is forbidden in more than 40% of patients eligible for this therapeutic approach, mostly for the occurrence of side effects including myalgia, myopathy or liver disease and rhabdomyolysis in more severe cases [4,5].

Abbreviations: LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein; BPF, Bergamot Polyphenol Fraction; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA reductase; CVD, Cardiovascular Diseases; totChol, total blood cholesterol; TG, triglycerides.

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This limits the use of statins and suggests the need of alternative therapeutic approaches.

Experimental and epidemiological evidence suggest that dietary polyphenols, in particular flavonoids, may play a role in ameliorating atherosclerosis and pleiotropic anti-oxidative and anti-inflammatory effects have been proposed as an underlying mechanism [6–8]. Yet these compounds when analysed separately show rather weak cholesterol-lowering effects in animal experiments [9]. In contrast, some natural compositions of plant polyphenols seem to possess a good hypolipemic activity both in animal models and in human studies, suggesting that synergistic effects of individual compounds may play a central role in the beneficial effects [6,8,9]. This observation provides the rationale for the identification of optimally synergizing elements and their plant sources with the best ability to prevent hyperlipidemia and cardiovascular complications.

Bergamot (*Citrus bergamia*) is an endemic plant of the Calabrian region in Southern Italy with a unique profile of flavonoid and flavonoid glycosides present in its juice and albedo, such as neoeriocitrin, neohesperidin, naringin, rutin, neodesmin, rhoifolin and poncirin. Bergamot differs from other *Citrus* fruits not only because of the composition of its flavonoids, but also because of their particularly high content [10,11]. Among them naringin, present also in grapefruit, have already been reported to be active in animal models of atherosclerosis [12], while neoeriocitrin and rutin have been shown to inhibit LDL oxidation [13]. Importantly, bergamot juice (BJ) is rich in neohesperidosides of hesperetin and naringenin, such as melitidine and brutieridine. These flavonoids possess 3-hydroxy-3-methylglutaryl moiety with a structural similarity to the natural substrate of HMG-CoA reductase and are likely to exhibit statin-like proprieties [14]. Experimental evidence obtained in animal models of diet-induced hypercholesterolemia and renal damage [15,16] as well as in the rat model of mechanical stress-induced vascular injury [17] supports the hypolipemic and vasoprotective effects of bergamot constituents. However, the therapeutic potential of bergamot has never been investigated in human studies, even though the traditional use of BJ in the Calabrian region suggested since long time its potential beneficial use in counteracting atherosclerosis.

The present study has been carried out to verify, in a rat model of diet-induced hyperlipemia, the effect of bergamot-derived polyphenolic fraction (BPF) on totChol, cLDL, cHDL, TG and blood glucose. This effect was also investigated by giving orally BPF for 30 consecutive days in 237 patients suffering from isolated or mixed hyperlipemia either associated or not with hyperglycaemia. In patients we have also studied the possible contribution of HMG-CoA reductase inhibition by BPF to its hypolipemic activity and this effect was also compared with changes of reactive vasodilation to verify the potential beneficial effect of BPF in modulating the imbalanced endothelial reactivity.

2. Materials and methods

2.1. Plant material

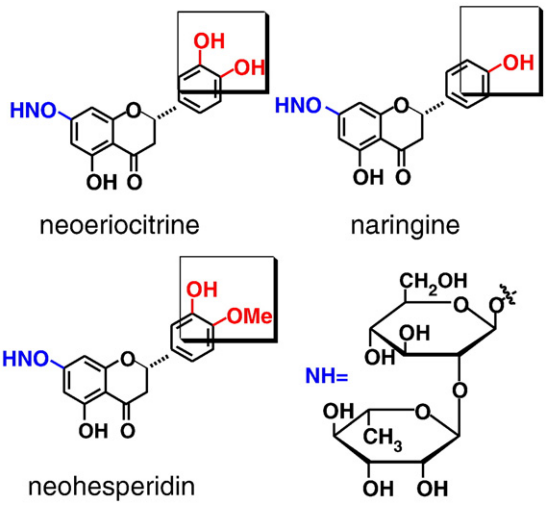
C. bergamia Risso & Poiteau fruits were collected from plantations located between Reggio Calabria and Bianco, in 90 km long costal area in South Italy.

2.2. Preparation of BPF

BJ was obtained from peeled-off fruits by industrial pressing and squeezing. The juice was oil fraction-depleted by stripping, clarified by ultra-filtration and loaded on suitable polystyrene resin columns absorbing polyphenol compounds of MW between 300 and 600 Da (Mitsubishi). Polyphenol fractions were eluted by a mild KOH solution. Next the fitocomplex was neutralized by filtration on cationic resin at acidic pH. Finally it was vacuum dried and minced to the desired particle size to obtain BPF powder. BPF powder was analysed by HPLC for flavonoid and other polyphenol content. In addition, toxicological analyses were performed including heavy metal, pesticide, phthalate and sinephrine content which revealed the absence of known toxic compounds (data not shown). Standard microbiological tests detected no mycotoxins and bacteria. Finally, 500 mg aliquots of the BPF powder supplemented with 50 mg of ascorbic acid as antioxidant were encapsulated with a semi-automatic gelatin encapsulation device by an authorized manufacturer (Plants, Messina, Italy). Tablets containing 500 mg of maltodextrin supplemented with 50 mg ascorbic acid were prepared for placebo studies. All procedures have been performed according to the European Community Guidelines concerning dietary supplements.

2.3. High pressure liquid chromatography (HPLC) analysis

High pressure liquid chromatography (HPLC) analysis was performed on Fast AGILENT 1200 HPLC system, equipped with DAD detector and ZORBAX Eclipse XDB-C18 column, 50 mm. 2 μl of the sample (BPF diluted in 50% ethanol and filtered with 0.2 μm filter) was injected on two solvent gradient of water and acetonitrile. Different gradients were used for the determination of flavonoid content or possible fumocumarin contaminants. The flow-rate was 3 ml/min and the column was maintained at 35 °C. The detector was monitored at 280 nm. Flavonoid and furocumarin pure standards were purchased from Sigma-Aldrich. Brutieridin and melitidin were identified according to Di Donna [14]. . The estimated concentration of 5 main flavonoids was: neoeriocitrin (77,700 ppm), naringin (63,011 ppm), neohesperidin (72,056 ppm), melitidin (15,606 ppm) and brutieridin (33,202 ppm).



2.4. Animal studies

Male Wistar rats (Harlan, Italy), weighing 180–200 g, were used for the experiments. The animals were kept under stable and controlled conditions (temperature, 22 °C; humidity, 60%) with water ad libitum. Animal care was in accordance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with the EC regulations (*Off. J. Eur. Communities* 1986, *L 358*).

The effects of BPF on tChol, cLDL, cHDL, tryglycerides and glucose were evaluated in Wistar rats fed a hypercholesterolemic diet composed of a standard diet (Harlan), supplemented with cholesterol 2% (Sigma-Aldrich, pur. 95%), 0.2% cholic acid (min. 98%, Sigma) and 4.8% palm oil . The rats were divided into four groups of 10 animals each:

- Group I (normolipidemic controls) was kept on a standard diet (Harlan) for 30 days.
- Group II (hyperlipidemic controls) received the hypercholesterolemic diet for 30 days.
- Groups III and IV received the hypercholesterolemic diet for 30 days; from the 1st to the 30th day, each rat was administered by gavage with BPF (10 and 20 mg/kg/rat daily, same route).

During the experiment, animals were weighed weekly, and 24 h food consumption was recorded daily. On day 29, rats were individually housed in metabolic cages. At the end of the study, the animals were fasted overnight; blood samples were collected from the penile vein of the rats and serum was separated and stored at −20 °C until analyzed. The analysis of totChol, cLDL and cHDL was performed as described below.

2.4.1. Fecal neutral sterols and bile acids determination

Neutral sterols and bile acids in the fecal samples of rats fed a hypercholesterolemic diet either untreated or treated with 20 mg/kg of BPF for 30 consecutive days were extracted according to the method described previously [15]. Briefly, feces were freeze-dried for 48 h, minced into fine powder, solubilized in KOH solution and saponified by autoclaving at 120 °C for 1 h; after the addition of NaCl solution, neutral sterols were extracted several times with ethyl ether as described in Ref. [24]. The upper phases were pooled, evaporated with a rotary evaporator, and dried under nitrogen. The concentration of fecal neutral sterols was determined by gas chromatographic (GC) analysis. Before the GC-analysis, all samples were diluted 1:10 v/v in *n*-hexane and R-cholestanol was added as internal standard.

2.5. Human studies

We used a randomized, double-blind, placebo-controlled study design that was approved by the Regional Ethical Committee. All the patients participating in the study signed an informed consensus according to the European Legislation. The randomisation scheme was generated by a computerised procedure. Neither the investigators nor the patients knew the randomisation code, block size or the results of the blood lipid concentrations until after the statistical analysis. Furthermore, the statistical analyses were conducted before breaking the randomisation code.

2.5.1. Intervention and procedures

In the screening visit, conducted 3–21 days before randomisation, all subjects received standardised dietary advice in order to reduce the variability of their baseline lipid values. It was attempted to keep the calorie intake as constant as possible by advising the subjects to reduce their morning and evening meals by approximately the same amount of calories as they received through the study nutrients. There was, however, no detailed diary recording of calorie intake in any of the study phases. Age, gender, and body mass index were matched among all subjects. We recruited 237 patients suffering from hypercholesterolemia from a primary care setting at the Department of Cardiology at University of Rome “Tor Vergata” and at the Vascular Medicine and Atherosclerosis Unit, Cardiology, Villa Salus Medical Center, Marinella di Bruzzano, Italy. Patients enrolled into the study were divided into three groups: group A, 104 subjects with isolated hypercholesterolemia, HC (cLDL levels ≥130 mg/dl); group B, 42 patients with hyperlipidemia (hypercholesterolemia and hypertryglyceridemia, HC/HT) and group C, 59 patients with mixed hyperlipidemia and glycemia over 110 mg/dl, HC/HT/HG. Each group was divided into three subgroups. The first received an oral dose of BPF (500 mg/day; A1, B1 and C1), the second received 1000 mg/day of BPF (A2, B2 and C2) and the third received placebo (APL, BPL, and CPL). The last group “D” or “post-statin” comprised 32 patients who stopped simvastatin therapy due to muscular pain and a significant elevation of serum creatine-phospho-kinase (CPK). Post-statin patients received 1500 mg/day of BPF daily after a washout period of 60 days and were asked to observe a 1600 kcal/day diet.

We excluded patients with overt liver disease, chronic renal failure, hypothyroidism, myopathy, uncontrolled diabetes, severe hypertension, stroke, and acute coronary events within the preceding 30 days, coronary revascularization within the preceding 3 months, or alcohol abuse. None of the patients took hormone replacement therapy or antioxidant or vitamin supplements during the 2 months preceding our study.

All the 237 patients were given placebo or BPF daily (before meal) for consecutive 30 days treatment period. The patients were seen every 7 days during the study and the compliance was monitored. To monitor possible side effects, we measured serum asparate aminotransferase, alanine aminotransferase, creatine kinase, blood urea nitrogen and creatinine and blood cell counts before and after therapy. 18 placebo patients and 24 patients taking BPF took beta adrenergic blockers or calcium channel blockers to control blood pressure. These drugs were withheld for ≥48 h before starting the BPF treatment. No additional medications including aspirin or nonsteroidal anti-inflammatory drugs were allowed during the study period to avoid confounding effects.

2.6. Urinary mevalonic acid detection

Twenty-four-hour urine samples were collected from each patient before and after treatment with BPF or placebo. Total volume was recorded, and aliquots were frozen at −20 °C. Urinary mevalonic acid (MVA) concentrations were determined

by a modified radioenzymatic isotope-dilution method of Popjak [18].

2.7. Endothelial function

Brachial arterial blood pressure was measured with a mercury sphygmomanometer after patients sat rested for 10 min or longer. The mean value of 3 measurements was calculated. Endothelial function was measured from brachial artery flow-mediated vasodilatation with B-mode ultrasound imaging of the brachial artery and by assessing the increase in artery diameter during reactive hyperemia.

2.8. Statistical analysis

In case of homogenous set of data ANOVA was performed to determine the treatment effects, and Dunnett's test was employed as appropriate. In case of heterogeneous data, F test was carried out to determine which pairs of groups are heterogeneous. This was followed by Cochran's or Student's t tests, as appropriate. The analysis was performed by the Statistical analysis add-in component of Microsoft Excel 2007.

3. Results

To assess the nutraceutical proprieties of bergamot flavonoids we concentrated bergamot juice in a form of powder, highly enriched in polyphenols. This was achieved by a standardized procedure of partial purification of bergamot polyphenol fraction (BPF) on a polystyrene resin column. The BPF preparation used in all studies contained 26–28% of 5 main flavonoids. 6–6.15 g of BPF correspond to 1000 ml bergamot juice in terms of flavonoid content. The content of 5 main flavonoids in a standardized BPF, according to HPLC analysis was as follows: neoeriocitrin (7.7%±0.4%), naringin (6.3%±0.33%), neohesperidin (7.2%±0.35%), melitidin (1.56%±0.11%) and brutieridin (3.32%±0.17%).

3.1. Animal studies

In animals fed a hypercholesterolemic diet for 30 consecutive days, an elevation of tChol, cLDL and tryglicerides was found compared to baseline values (n=10; Fig. 1). Administration of BPF (10 and 20 mg/kg/daily; n=10 for each dose) for 30 days in diet-induced hypercholesterolemic rats produced significant reduction in tChol, cLDL and tryglycerides, an effect accompanied by moderate elevation of cHDL (Fig. 1A). No significant difference in weekly mean body weight, in BPF treatment, versus hyperlipidemic controls was found; moreover, no reduction in 24 h food consumption was observed (data not shown).

Moreover, BPF was subjected also to acute (5000 mg/kg) and subchronic animal toxicity studies according to OECD guidelines at 50, 200, 1000 mg/kg per day doses (n=5 for each dose). All performed tests have shown lack of any evident animal toxicity including hematochemical parameters, behavioral tests, histopathological evaluation of liver, kidney and brain tissues (data available as on-line supplementary material, Tables S2 and S3).

3.1.1. Fecal neutral sterols and bile acids

Fecal output of total bile acids and neutral sterols was found to be enhanced significantly, in the BPF-treated group compared to the hyperlipemic group (Fig. 1B), suggesting that bergamot extract enhances the epato-biliary turnover and cholesterol consumption as previously suggested for bergamot juice [15].

3.2. Studies in patients

3.2.1. Effect of BPF on serum lipids and glucose in patients

Treatment with BPF (500 and 1000 mg daily) for 30 consecutive days in patients suffering from isolated HC, (group A), mixed hyperlipidemia (HC/HT, group B) and metabolic syndrome (group C) led to a strong reduction in totChol, cLDL and a significant increase in cHDL in majority of subjects. No significant changes in the mean cholesterol parameters were recorded after 30 days maltodextrin treatment for all placebo groups (Table 1).

The significant reduction was also observed for triglyceride levels in patients with HT (Table 2). In particular, group C, comprising 59 metabolic syndrome patients, suffering from HC/HT/HG, responded very well to BPF therapy. The initial mean values such as 278 mg/ml in totChol, 188 mg/ml in cLDL and

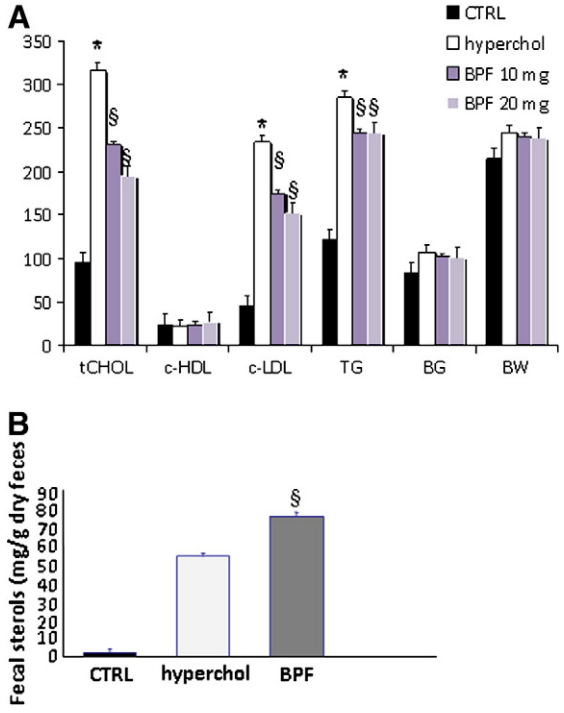


Fig. 1. Bergamot Polyphenol Fraction (BPF; 10 and 20 mg/kg/day) reduces total cholesterol (tChol), LDL cholesterol (cLDL) and tryglicerides (TG) and enhances fecal sterols excretion in Wistar rats fed a hyperlipemic diet. A. BPF was administrated daily by gavage (10 and 20 mg/kg/daily; n=10 for each dose) for 30 days in diet-induced hypercholesterolemic rats. At the day 30 the blood was drawn from penile vein and tChol, cLDL, cHDL and tryglycerides were analysed by standard methods. B. The animal feces were collected from control (CONTROL), hyperlipemic (HYPERCHOL) and BPF-treated hyperlipemic animals (BPF) (20 mg/kg/day) on the day 30 of the treatment, and sterols and bile acids were extracted with ethyl ether and analysed by gas chromatography as described in Materials and methods. Statistical analysis was performed as described in Materials and methods. §—indicates statistically significant change compared to control rats kept on high-cholesterol diet for $P<0.05$, *—indicates a statistically significant change compared to normolipemic rats for $P<0.05$.

Table 1
Percent variations in totChol, cLDL, cHDL (%Δ) in patients subjected to the 30-day BPF treatment [a].

Patients group [b]	Sub-group [b]	Nr P	BPF dose daily (mg)	totChol			cLDL			cHDL		
				% Δ ± SEM	No resp. [c]	Best 10% [d]	% Δ ± SEM	No resp.	Best 10%	% Δ ± SEM	No resp.	Best 10%
A	A1	35	500	**−20.7** ± 1.9 [f]	6	−34.6	**−23.0** ± 1.9	4	−37.2	**25.9** ± 2.3	0	50.0
	A2	37	1000	**−30.9** ± 1.5	2	−40.0	**−38.6** ± 1.5	0	−49.1	**39.0** ± 2.8 [e]	0	68.6
	APL	32	0	**−0.4** ± 0.4	32	–	**−1.7** ± 0.5	27	–	**0.5** ± 1.1	22	–
B	B1	14	500	**−21.9** ± 1.8	1	−28.3	**−25.3** ± 2.0	0	−34.6	**17.3** ± 1.4	0	26.7
	B2	14	1000	**−27.7** ± 3.4	2	−41.5	**−33.4** ± 3.9	2	−43.6	**35.8** ± 4.2 [e]	0	66.7
	BPL	14	0	**−0.5** ± 0.5	14	–	**−0.5** ± 0.7	13	–	**−1.3** ± 1.8	11	–
C	C1	20	500	**−24.7** ± 2.6	2	−41.7	**−26.8** ± 3.6	1	−53.6	**16.5** ± 1.6	0	42.9
	C2	19	1000	**−28.1** ± 2.6	1	−41.1	**−33.2** ± 3.0	1	−47.0	**29.6** ± 1.8 [e]	0	64.6
	CPL	20	0	**0.5** ± 0.5	20	–	**−0.9** ± 1.4	18	–	**2.9** ± 2.0	15	–
D	–	32	1500	**−25.0** ± 1.6	2	−39.8	**−27.6** ± 0.5	0	−32.4	**23.8** ± 1.7	0	41.1
A + B + C	–	69	500	**−21.8** ± 1.4	9	−37.8	**−24.1** ± 1.5	5	−45.0	**22.3** ± 1.3	0	48.6
	–	70	1000	**−29.4** ± 1.3	5	−40.6	**−36.0** ± 1.4 [e]	3	−47.9	**40.1** ± 1.9 [e]	0	66.4
	–	66	0	**−0.1** ± 0.3	66	–	**−1.1** ± 0.5	58	–	**1.2** ± 0.9	48	–

[a] Mean changes in blood parameters for each group or subgroup of patients were calculated by adding the changes recorded for individual patients and dividing them by the number of patients (Nr P).
[b] For division of all recruited patients in groups and subgroups see Section 2.4.1.
[c] No response—Number of patients that show a smaller than 5% reduction in totChol, cLDL or a lower than 5% increase in cHDL after 30 days treatment with BPF. Note that in some cases bigger than 5% changes were recorded in the placebo treated patients.
[d] Best 10%—mean value in the subgroup of the best 10% responders.
[e] Statistically significant difference compared to 500 mg BPF dose at $p<0.01$.
[f] Please note, that statistically significant difference at $p<0.001$ between subgroup 1 and PL was found in all groups of patients (A,B and C) for all blood measurements.

267 mg/ml in TG, dropped to 199, 126 and 158 mg/ml, respectively (Fig. 2) after the treatment with high BPF dose. The reduction in cLDL was accompanied by a dose-dependent elevation in cHDL in all patients. In the 10% subjects with the best response the effect on cHDL was very striking −64.6% (Table 1). In addition, metabolic syndrome patients presented a highly significant ($P<0.0001$) reduction in blood glucose levels (mean value of −18.9% under 500 mg BPF treatment (C1 group) and −22.4% in C2 group). No changes in glucose levels were recorded after 30 days in the placebo group (Fig. 2, Table 2). These data suggest that BPF induces complex effects on metabolic regulation. As reported in Table 1 the maximum effect for all cholesterol parameters was seen in patients taking 1000 mg BPF daily, but differences between 500 mg and 1000 mg dosage were statistically significant only for cHDL. In addition, we evaluated the efficacy of BPF depending on the metabolic disorder, but the differences were not statistically significant ($P>0.05$) (Table 1). This suggests that BPF consumption corrects common mechanisms of cholesterol metabolism similarly deregulated in all 3 groups of patients.

3.2.2. BPF as post-statin treatment

To test the responsiveness of the patients in whom the use of statins was forbidden for the appearance of side effects, we recruited 32 patients suffering from statin toxicity. These patients stopped taking statins for 2 months and then they were asked to take 3 capsules of BPF daily (1500 mg). This treatment proved to be very efficient (Fig. 3). 30/32 patients responded well and after 30 days a mean −25% reduction in tChol and −27.6% in cLDL was observed (Table 1), without re-appearance of side effects. This indicates that BPF could be considered as an alternative treatment in patients with a relevant intolerance to statins.

Neither symptoms nor hematochemical signs of toxicity were found in all patients undergoing the BPF treatment. In 6 patients treated daily with 500 mg and in 11 patients taking 1000 mg of BPF a moderate gastric pyrosis was observed. However, none of the patients taking BPF interrupted the treatment.

3.2.3. MVA urinary concentrations in patients

Twenty-four-hour urinary MVA excretion in patients undergoing BPF treatment (500/1000 mg/daily for 30 days) decreased

Table 2
Percent variations in TG and blood glucose in patients suffering from HC/HT and HC/HT/HG subjected to the 30 day BPF treatment[a].

Patients group[b]	Sub-group	Nr P	BPF dose daily (mg)	Triglycerides			Glucose		
				% Δ ± SEM	No response[c]	Best 10%[d]	% Δ ± SEM	No response	Best 10%[d]
B	B1	14	500	**−28.2** ± 3.9	2	−46.8	–	–	
	B2	14	1000	**−37.9** ± 3.3	1	−47.9	–	–	−43.6
	BPL	14	0	**0.1** ± 0.5	14	–	–	–	–
C	C1	20	500	**−32.7** ± 2.5	0	−41.6	**−18.9** ± 1.2	0	−27.1
	C2	19	1000	**−41.0** ± 2.6	1	−49.6	**−22.4** ± 1.0	0	−32.2
	CPL	20	0	**0.0** ± 0.6	19	–	**−0.5** ± 0.7	20	–

[a] Mean changes in blood parameters for each group or subgroup of patients were calculated as for Table 1.
[b] For division of all recruited patients in groups and subgroups see Section 2.4.1.
[c] No response—Number of patients that show a smaller than 5% reduction in totChol, cLDL or a lower than 5% increase in cHDL after 30 days treatment with BPF. Note that in some cases bigger than 5% changes were recorded in the placebo treated patients.
[d] Best 10%—mean value in the subgroup of the best 10% responders.

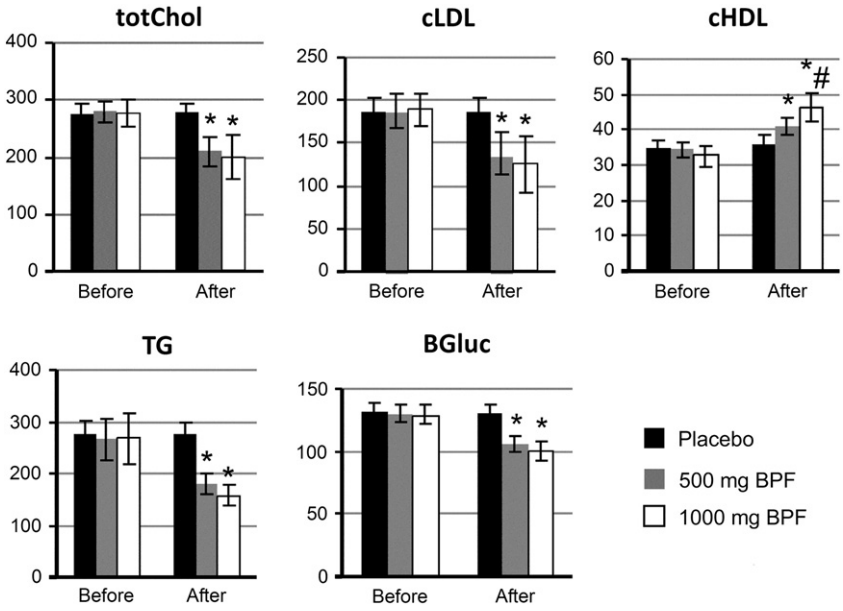


Fig. 2. Bergamot Polyphenol Fraction (BPF) reduces total cholesterol (tChol), LDL cholesterol (cLDL), tryglicerides (Tryg.) and blood glucose (Bglucose) levels in patients with metabolic syndrome (mixed hyperlipidemia and associated hyperglycemia=HC/HT/HG, group C). The graphs show mean values for indicated blood parameters from 59 patients diagnosed with metabolic syndrome before the BPF treatment with 500 mg/day (group C1, n=20) or 1000mg/day (group C2, n=19) or placebo (group CPL, n=20). The indicated patients' blood parameters expressed in mg/dl were analysed on the day 0 (Before) and day 31 (After) of the treatment. Error bars show the standard deviation (S.D.). *—indicates a statistically significant change compared to the placebo group at $P < 0.0001$; #—indicates a statistically significant change between C1 and C2 subgroups at $P < 0.05$.

($P<0.05$), from a baseline value of 2.12 ± 0.32 to 1.56 ± 0.28 and $1.34 + 0.26$ μmol/day, respectively. On discontinuation of drug therapy, urinary MVA levels increased to 1.82 ± 0.31 and $1.65 +$ 0.44 μmol/day in the first week, with no further increase seen at 4 weeks (1.88 ± 0.26 μmol/day). No changes were seen in the group of patients who received placebo over 30 day treatment (1.98 ± 0.30 μmol).

3.2.4. Reactive vasodilatation

Before starting the BPF treatment, flow-mediated vasodilation was found reduced in patients suffering from isolated HC or mixed HC/HT (groups A, B, and C), being the latter effect more pronounced in the subgroup of patients with moderate elevation of serum glucose (group C) (Fig. 4). After 30 days of BPF treatment with (5000 and 1000 mg/daily for 30 consecutive days), flow-mediated vasodilation increased significantly, whereas no changes have been observed in patients receiving placebo (Fig. 4). This suggests that BPF is able to improve the impaired endothelium-mediated vasodilation in hyperlipidemic patients with or without hyperglycemia.

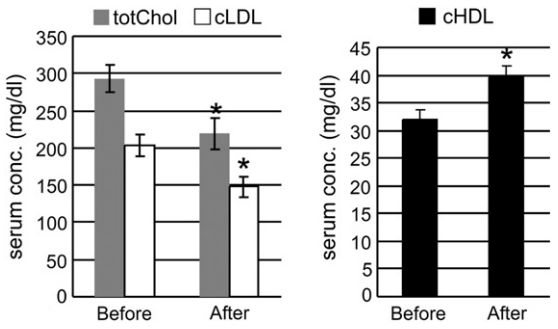


Fig. 3. Response to BPF in the statin intolerant group of patients (group D). 32 patients who were obliged to stop simvastatin therapy due to its side effects were given 1500 mg/ml BPF daily 60 days after the statin withdrawal. The indicated patients' blood parameters were recorded in mg/dl (axis Y) before and after the BPF treatment (30 days). Statistical analysis was performed as described in Materials and methods; *—indicates a statistically significant change compared to the blood parameters before the treatment at P<0.0001.

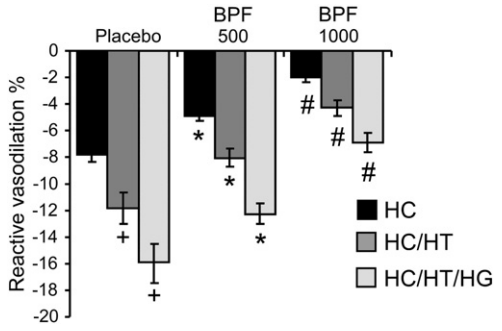


Fig. 4. The effect of BPF (500 and 1000 mg/die) on reactive vasodilation in patients suffering from isolated hypercholesterolemia (HC) or mixed hyperlipidemia (HC/HT) and associated hyperglycemia (HC/HT/HG). After 30 days of treatment with placebo or BPF (500 or 1000 mg/die), endothelial function was assessed in 3 groups of patients (14 to 30 patients per group). Flow-mediated vasodilation was measured from brachial artery diameter during reactive hyperemia. * indicates a statistically significant change compared to the respective placebo group of patients at $P < 0.05$; # indicates statistically significant changes compared to the 500 mg BPF group at $P < 0.01$; + indicates statistically significant changes compared to HC group at $P < 0.01$.

4. Discussion

Based on the data presented in this study, the BPF treatment leads to a significant reduction of coronary artery disease risk and other cardiovascular complications according to the headlines of National Cholesterol Education Programme (NCEP—ATP III) in a rat model of diet-induced hyperlipemia. This effect was also confirmed in human studies carried out in patients with pure or mixed hyperlipemia either or not associated with hyperglycaemia (metabolic syndrome).

Scientific evidence obtained with *Citrus* flavonoids and other non-nutritive constituents of *Citrus* fruits, provides some mechanistic explanations for the beneficial effects of bergamot juice. Previous data showed that *Citrus* peel extracts, rich in pectins and flavonoids, cause lowering of cholesterol levels by modulating hepatic HMG-CoA levels, possibly by binding bile acids and increasing the turnover rate of blood and liver cholesterol [19–21]. Since BJ was shown to enhance the excretion of fecal sterols in rats [15], such a mechanism may contribute to its hypolipemic and hypoglycemic effect found in patients under BPF treatment. Special contribution to the hypolipemic response seems to be related to the modulatory properties of flavonone glycoside components of BPF, in particular naringin and neohesperidin. Indeed, evidence exists that dietary hesperetin reduces hepatic TG accumulation and this is associated with the reduced activity of TG synthetic enzymes, such as phosphatidate phosphohydrolase [22]. In addition, *in vitro* studies suggest that naringenin and hesperetin decrease the availability of lipids for assembly of apoB-containing lipoproteins, an effect mediated by reduced activities of acyl CoA: cholesterol acyltransferases (ACAT) [23].

Importantly, BPF is rich in buteridine and melitidine, which are 3-hydroxy-3-methylglutaryl derivatives of hesperetin and naringenin, respectively. Given the structural similarity to HMG-CoA reductase substrate, these compounds have been proposed to possess the statin-like properties, by selective inhibition of HMG-CoA reductase [14]. In addition, the classical glycoside derivative of naringenin, which is naringin has been shown to inhibit hepatic HMG-CoA reductase [24]. Therefore it is likely that melitidine and brutieridine in concert with naringin and other flavonone glycosides might be responsible for the striking potency of BPF in reducing cholesterol levels. The direct action of BPF on HMG-CoA reductase activity is suggested by a significant mevalonate reduction in the urine of patients under BPF treatment in our study.

The oxidative stress and the inflammatory processes in the endothelium have been shown to reduce reactive NO-dependent vasodilation. Well documented antioxidant and anti-inflammatory mechanisms regulated by *Citrus* flavonoids, such as increasing superoxide dismutase and catalase activities and protecting the plasma vitamin E [25] may well attenuate overproduction of oxygen reactive species in the vascular wall thereby restoring the imbalanced endothelial function, as we observed in our patients by studying reactive vasodilation (Fig. 4).

Another potential benefit from BPF treatment is related to its hypoglycemic activity (Fig. 2). Among the few mechanistic studies on hypoglycemic effects of flavonoids, it has been shown that naringenin, similarly to other polyphenols significantly increased AMP kinase (AMPK) activity and glucose uptake in muscle cells and liver [26,27]. The hypoglycaemic activity of naringenin *in vivo* can be more complex and may depend on multilevel effects on lipid metabolism that lead to increased insulin sensitivity and glucose tolerance as shown in animal models of metabolic syndrome [28].

On the basis of our data, BPF oral supplements contribute to lowering plasma cholesterol and lipids in a rat model of diet-induced hyperlipemia and in patients, in a range of potency comparable with low dose statins. Thus BPF offers a safe alternative for patients suffering from statins toxicity. In addition, the possibility to reduce blood glucose by 15%–25% suggests a phytotherapeutic approach to control the prediabetic states in patients with metabolic syndrome.

Supplementary materials related to this article can be found online at doi:10.1016/j.fitote.2010.10.014.

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