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RESEARCH ARTICLE

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Effect of *Citrus bergamia* extract on lipid profile: A combined in vitro and human study

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Abstract

With the aim of characterising the hypo-lipidemic function of the Brumex™ ingredient obtained from the whole fruit of *Citrus bergamia*, a combined pre-clinical and clinical study was conducted. In the HepG2 experimental model, we first demonstrated that Brumex™ does not trigger any significant alteration in cell viability over the tested concentration range of 1–2000 μg/mL (4 and 24 h). By stimulating the phosphorylation of AMP-activated protein kinase (AMPK) at threonine 172, Brumex™ significantly reduces both cholesterol and triglyceride (TG) intracellular content of HepG2 cells and impairs the expression levels of lipid synthesis-related genes (namely, *SREBF1c*, *SREBF2*, *ACACA*, *SCD1*, *HMGCR* and *FASN*). In vitro data have been validated in a dedicated double-blind, placebo-controlled, randomised clinical trial performed in 50 healthy moderately hyper-cholesterolemic subjects, undergoing supplementation with either Brumex™ (400 mg) or placebo for 12 weeks. Clinical and blood laboratory data were evaluated at the baseline and at the end of the trial. Brumex™ positively impacted on both plasma lipid pattern and liver enzymes compared with the placebo, mainly in terms of significant reduction of total cholesterol (TC), TG, low-density lipoprotein-cholesterol (LDL-C), non-high-density lipoprotein-cholesterol (non-HDL-C), apolipoprotein B100 (ApoB), fasting plasma glucose (FPG), glutamic-oxaloacetic transaminase (GOT), glutamate pyruvate transaminase (GPT) and gamma-glutamyl-transferase (gGT).

KEYWORDS
bergamot, cholesterol, clinical trial, liver, phospho-AMPK, triglycerides

Abbreviations: ACACA, acetyl-CoA carboxylase alpha 1; AICAR, 5-Aminoimidazole-4-Carboxamide Riboside; AMPK, AMP-activated protein kinase; AMPKi, AMP-activated protein kinase inhibitor; Apo, apolipoprotein; BMI, body mass index; BW, body weight; CCK-8, Cell Counting Kit-8; CPK, creatine phosphokinase; DBP, diastolic blood pressure; FASN, fatty acid synthase; FPG, fasting plasma glucose; Fwd, forward PCR primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; gGT, gamma-glutamyl-transferase; GOT, glutamic-oxaloacetic transaminase; GPT, glutamate pyruvate transaminase; HDL, high-density lipoprotein; HDL-C, high-density lipoprotein-cholesterol; HepG2, human hepatocarcinoma cell line; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMG-flavonone, 3-hydroxy-3-methylglutaryl-flavanone; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein-cholesterol; P-AMPK, phospho-AMPK; Rvs, reverse PCR primer; SBP, systolic blood pressure; SCD1, stearoyl-CoA desaturase 1; SREBF, sterol regulatory element-binding transcription factor; TC, total cholesterol; TG, triglyceride; WC, waist circumference.

Clinical trial registration: clinicaltrial.gov NUT1-BO-2019 (approved on 09/12/2019).

1 | INTRODUCTION

Bergamot (*Citrus bergamia*, *C. bergamia*) is a Mediterranean citrus fruit typical of Calabria (southern part of Italy), that has raised increasing attention due to its rich bioactive molecules composition and unique profile made of flavonoid glycosides, including neoeriocitrin, neohesperidin, naringin and glycosylated polyphenols, such as brutieridin and melitidin (Salerno et al., 2016). Mainly bergamot juice and its derivates, originally considered a by-product of essential oil extraction used in cosmetic and food industries, have been characterised as promising sources of biomolecules with favourable health-related functions (Ferlazzo et al., 2016; Navarra et al., 2014; Toth et al., 2016). More recently, other parts of the plant-like peel extracts have been studied for their promising multiple health-related properties (Benavente-Garcia & Castillo, 2008; Mannucci et al., 2017; Navarra et al., 2015).

Several natural products, including *C. bergamia* and bergamot flavonoids, have been specifically proven to affect human adipogenesis (Di Donna et al., 2014; Mollace et al., 2012; Sakurada et al., 2011; Trombetta et al., 2010), thus envisaging their possible clinical application for those patients that experience drug-derived side effects and elect a nutraceutical-based approach for the treatment of dyslipidaemia. So far, most experimental data, focusing on lipid-related functions, are based on single bergamot biomolecules or juice, rather than on extracts obtained from the bergamot whole fruit; besides, clinical evaluations often lack a corresponding molecular counterpart that might help to identify the underlying pathway(s) responsible for the hypo-lipidemic effect. In the context of molecular characterisation of human adipogenesis, the AMP-activated protein kinase (AMPK) plays a key role (Ahmad et al., 2020; Hardie et al., 1989). It is a sensor of the intracellular energy state and its activation—through threonine 172 (Thr^{172}) phosphorylation—triggers multiple downstream pathways to control lipid homeostasis (Stein et al., 2020). Phospho-AMPK (P-AMPK) inhibits the acetyl-CoA carboxylase alpha 1 (ACACA), the rate-limiting enzyme for the de novo synthesis of lipids that—together with fatty acid synthase (FASN)—controls the fatty acid synthesis way in human tissues (Wakil & Abu-Elheiga, 2009). AMPK also interacts with and directly phosphorylates both sterol regulatory element-binding transcription factors (SREBF-1c and -2), thus triggering the inhibition of their transcriptional activity (Li et al., 2011). SREBF-1c mainly regulates the lipogenic process by activating genes involved in fatty acid and triglyceride synthesis, including the Stearoyl-CoA desaturase 1 (SCD1), whereas SREBF-2 mostly monitors cholesterol synthesis and uptake, by activating the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and the low-density lipoprotein receptor (LDLR) (Eberle et al., 2004; Osborne & Espenshade, 2009).

The stimulation of P-AMPK is therefore a powerful strategy for the effective inhibition of adipogenesis as a therapeutic option. Both metformin and AICAR (5-Aminoimidazole-4-Carboxamide Riboside) are drugs, largely used in the context of obesity and type 2 diabetes mellitus, that specifically modulate AMPK activity by Thr^{172} phosphorylation (Luo et al., 2016; Sun et al., 2007). Natural products which target AMPK signalling may thus be potential and novel options for the management of diabetes and obesity, although their potential health effect has mostly been assessed by in vitro studies and in animal models (Joshi et al., 2019), whereas data obtained from combined preclinical and clinical studies are still limited.

The aim of the present study was to elucidate the functional effect of the supplementation with the Brumex™ nutraceutical ingredient (obtained from different parts of the whole fruit of *Citrus bergamia*) in the regulation of adipogenesis, by performing an integrated preclinical (in vitro) and clinical approach. We first run a study in HepG2 human hepatocarcinoma cells to identify the possible mechanism(s) underlying its effect in human liver cells and focused on P-AMPK activation, lipid-related genes expression and intracellular lipid content. We then tested the effect supplementation of Brumex™, in terms of lipid and glucose metabolism as well as liver enzymes, in a dedicated double-blind, placebo-controlled, randomised clinical trial.

2 | MATERIALS AND METHODS

2.1 | Brumex™ extract preparation

Brumex™ is a commercial ingredient from Esserre Pharma Srl (https://www.esserrepharma.com). It is obtained from different parts of the Citrus Bergamot whole fruit (*Citrus bergamia Risso et Poiteau*, *fructus*), specifically from fresh fruits collected from November to February. Fruits are submitted to a process of separation of essential oil and then pressed to obtain fresh juice. The by-product obtained is then processed and mixed into the fresh juice. The product is then filtered and concentrated, and eventually spray-dried. This process foresees several steps of quality control to assess and monitor the content of flavonoids and furocoumarins both in fresh juice, by-products, and final extract. The finished product is a fine powder, yellow-brown coloured, with a slightly pungent smell, typical of citrus and a bitter acid taste.

2.2 | Brumex™ extract characterisation by HPLC analysis

The characterisation of the Brumex™ extract (provided by Esserre Pharma srl) was carried out by HPLC-DAD analysis (ThermoFisher Scientific, USA). The chromatographic separation of flavanones and furanocoumarines was performed on a C18 ODS Luna column (250 × 4.6 mm; 5 µm) (Phenomenex, Aschaffenburg, Germany), thermostated at 30°C. The mobile phase consisted of Water/0.3% Formic Acid (solvent A) and Acetonitrile/0.3% Formic Acid (solvent B) using the following elution gradient: 0 min 5% B, 50 min 28% B, 60 min 43% B, isocratic for 5 min. The flow rate was 1 mL/min and the injection volume was 20 µL. Data acquisition was set in the range of 190–800 nm and chromatograms were recorded at 280 and 320 nm. The HPLC profile of the Brumex™ formulation is reported in Figure S1.

2.3 | Human HepG2 cell culture and treatments

The HepG2 human hepatocellular carcinoma cells (RRID: CVCL_0027) were purchased from the European Collection of Cell Cultures (ECACC, Sigma Aldrich-St Louis, MO). The cells were cultured in 75 cm^2 flasks as a monolayer at 37°C in a humidified atmosphere containing 5% CO_2. The medium was DMEM containing 2 mM glutamine, 1% non-essential amino acids, 1000 U/mL penicillin, 10 mg/mL streptomycin and 10% foetal bovine serum (all chemicals are from Aurogene, Rome, Italy).

Brumex™ extract (0.1 m, provided by Esserre Pharma srl) was dissolved in sterile-filtered water to a stock concentration of 5 mg/mL and filtered through a 0.22 μm polyvinyl difluoride (PVDF) Primo® Syringe Filters (Euroclone, Milan, Italy). Dilutions were freshly prepared in a complete cell medium before each experiment.

For inhibition of AMPK, the InSolution™ AMPK Inhibitor (AMPKi, compound C, Merck, Damstadt, Germany) was used. Briefly, HepG2 cells were treated with 100 μM of AMPK inhibitor for 1 h, before treatment with different concentrations of Brumex™ ingredient.

2.4 | Assessment of cell viability in HepG2 cells

In cytotoxicity assays, cell viability was evaluated by the Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich); it is based on the conversion of water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] to a water-soluble formazan dye upon reduction in the presence of an electron carrier by dehydrogenases. Briefly, the HepG2 cells were plated in a 96-well cell culture plate at a density of 5×10^3 cells/well; 24 h after plating, cells were treated with Brumex™ extract (1, 10, 50, 100, 200, 500, 1000, 2000 μg/mL) in 100 μL of complete cell medium, for 4 and 24 h. Untreated cells (set as negative controls) were included in the analysis. In each experiment, negative and Brumex™-exposed cells were plated in triplicate. After treatment with the extract, 10 μL of CCK-8 solution was added to each plate well, and the plate was incubated at 37°C for 4 h. The absorbance of each well was measured at 450 nm using a Glomax® Discover Microplate Reader (Promega, Madison, WI, USA). The cell viability (%) was expressed as the ratio between the mean absorbance of the experimental (Brumex™) group and the mean absorbance of the negative control.

2.5 | Phospho-AMPK ELISA test in HepG2 cells

HepG2 cells were plated in a 6-well cell culture plate at a density of 1.5×10^6 cells/well; 24 h after plating, cells were treated with either Brumex™ extract (1000 μg/mL), Metformin (2 mM) or AICAR (2 mM) (selected as positive controls) (Sigma-Aldrich) for 4 h. Untreated cells (negative controls) were included in the analysis. For the valuation of the AMPK-inhibiting ability of the AMPKi, cells have been treated with the specific inhibitor for 1 h at the 10, 50 and 100 μM concentrations. The concentration of the phospho-AMPK (P-AMPK, phosphorylated at threonine residue 172) was determined in cells lysates by an enzyme-linked immunoassay (ELISA) (Invitrogen, cat. number KHO0651, Camarillo, CA, USA) according to the manufacturer's instructions. Cell lysates were prepared using a cell extraction buffer containing 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na4P2O7, 20 mM Na3VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate (all chemicals from Invitrogen, ThermoFisher Scientific) supplemented with protease inhibitor cocktail (Cell Signaling, Danvers, MA, USA) and 1 mM of phenylmethylsulfonyl fluoride (PMSF) (ThermoFisher Scientific), according to the manufacturer's recommendation. In the ELISA test, the absorbance at 450 nm was measured using a Glomax® Discover Microplate Reader (Promega) and transformed into the concentration of phosphorylated-AMPK according to the standard curve, and the relative amount was normalised to the untreated cells. All experiments were performed in triplicate.

2.6 | Total RNA extraction and gene expression analysis in HepG2 cells

HepG2 were plated in a 6-well cell culture plate at a density of 1×10^6 cells/well; 24 h after plating, cells were treated with Brumex™ extract at 1000 μg/mL for 4 h, given as a single agent or in combination with AMPKi. Total RNA from treated and untreated cells (negative control) was isolated using a Quick-RNA Microprep kit (Zymo Research, Irvine, CA, U.S.A) according to the manufacturer's instructions. The amount and purity of the extracted RNA was evaluated by fibre optic spectrophotometer (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA) calculating the 230/260 and the 260/280 absorbance ratios. One microgram of total RNA was reverse transcribed into cDNA using the SensiFAST™ cDNA Synthesis Kit (Bioline Meridian BioScience, Cincinnati, Ohio, US). mRNA levels were analysed by real-time PCR using a SensiFAST™ SYBR No-ROX Kit (Bioline Meridian BioScience, Cincinnati, Ohio, US). Real-time PCR was performed in a StepOnePlus™ Real-Time PCR (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). PCR primers were designed by NCBI Primer Blast-free software (https://www.ncbi.nlm.nih.gov/tools/primerblast), according to gene sequences available in the UCSC database (https://genome.ucsc.edu) and selected to amplify an exon–intron–exon region (≤250 bp) to exclude genomic contamination. PCR primers were synthesized by Bio-Fab research s.r.l. (Rome, Italy). The following human primers (forward primer, fwd; reverse primer, rvs) were used: *ACACA (acetyl-CoA carboxylase alpha 1)* gene (RefSeq NM_198834), fwd: 5′-TCGCTTTGGGGGAAATAAAGT-3′, rvs: 5′-ACCACCTACGGATAGACCGC-3′ (amplicon: 91 bp; annealing T: 59.6°C); *FASN* (*fatty acid synthase*) gene (RefSeq NM_004104.5), fwd: 5′-GGGATGAACCAGACTGCGTG-3′, rvs: 5′-CTGCACTTGGTATTCTGGGT-3′; *HMGCR* (*3-hydroxy-3-methylglutaryl-CoA reductase*) gene (RefSeq NM_000859), fwd: 5′-CACCAAGAAGACAGCCTGAA-3′, rvs: 5′-CATCCTCCACAAGACATTG-3′ (amplicon: 120 bp; annealing T:

55.8°C); *SREBF-1c* (*Sterol regulatory element-binding transcription factor-1c*) gene (RefSeq NM_001005291), fwd: 5′-CGCCTTGACAGGTGAAGTC-3′, rvs: 5′-GCCAGGGAAGTCACTGTCTT-3′ (amplicon: 125 bp; annealing T: 59.1°C); *SREBF-2* (*Sterol regulatory element-binding transcription factor-2*) gene (RefSeq NM_004599), fwd: 5′-CAGTGGGACCATTCTGAC-3′, rvs: 5′-TTCCTCAGAACGCCAGACTT-3′ (amplicon: 235 bp; annealing T: 59.5°C); *SCD1* (*stearoyl-CoA desaturase 1*) gene (RefSeq NM_005063), fwd: 5′-AGCTCATCGTCTGTGGAGC-3′, rvs: 5′- GCCACGTCGGGAATTATGAGG-3′ (amplicon: 176 bp; annealing T: 58.5°C); *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) gene (RefSeq NM_002046), fwd: 5′-GCACCGTCAAGGCTGAGAA-3′, rvs: 5′-GAGGGATCTCGCTCCTGGA-3′ (amplicon: 75 bp; annealing T: 59.8°C).

In each experiment, all reactions were run in quadruplicate and the relative abundance of the transcripts was calculated by normalizing to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, applying the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.7 | Total cholesterol and triglycerides detection in HepG2 cells

HepG2 cells were plated in a 96-well cell culture plate (1×10^4 cells/well) and incubated overnight. Twenty-four hours after plating, cells were treated with the AMPK inhibitor (AMPKi, 100 μM, 1 h), Brumex™ extract (500, 1000, 2000 μg/mL), a combination of AMPKi + Brumex™ and Metformin (2 mM, positive control) (Sigma-Aldrich) for 24 h. Untreated cells (negative control) were included in the analysis. Total cholesterol and triglycerides were detected using Cholesterol/Cholesterol Ester-Glo™ Assay and Triglyceride-Glo™ Assay (Promega), respectively. These are bioluminescent assays for rapid and sensitive measurement of cholesterol and triglycerides. At the indicated time, luminescence was measured using a Glomax® Discover Microplate Reader (Promega). Cholesterol and triglyceride concentrations were measured using a standard curve, according to the manufacturer's instructions. All experiments were performed in triplicate and expressed as fold change compared to the control (untreated cells).

2.8 | Clinical trial

The parallel arms, double-blind, placebo-controlled, randomised clinical trial was carried out in 50 overall healthy moderately hypercholesterolemic subjects in primary prevention for cardiovascular diseases, consecutively enrolled in the ambulatory service of cardiovascular disease prevention in the Medical and Surgical Sciences Department of the University of Bologna.

Inclusion criteria: age between 18 and 70, and low-density lipoprotein-cholesterol (LDL-Cholesterol) level between 115 and 190 mg/dL, confirmed in at least two sequential checks before signing the consent form.

Exclusion criteria: (i) personal history of cardiovascular disease or risk equivalents; (ii) active smoking habit; (iii) TG > 400 mg/dL and/or high-density lipoprotein-cholesterol (HDL-C) <35 mg/dL; (iv) obesity (body mass index, BMI > 30 kg/m^2); (v) assumption of lipid-lowering drugs or drugs affecting lipid metabolism and (vi) known thyroid, liver, renal or muscle diseases.

At baseline, patients were given standard behavioural and qualitative (not quantitative) dietary suggestions to correct unhealthy habits. Standard diet advice was given by an expert physician who periodically provided instruction on dietary intake recording procedures as part of a behaviour modification program and then later used the subject's food diaries for counselling. In particular, subjects were instructed to follow the general indication of a Mediterranean diet, avoiding excessive intake of dairy products and red meat-derived products during the study, maintaining overall constant dietary habits. Individuals were also generically encouraged to increase their physical activity by walking briskly for 20–30 min, 3–5 times per week or by cycling.

Treatments. After 2 weeks of diet and physical activity, patients were allocated to treatment with indistinguishable tablets containing active or placebo, in two groups defined as A (25 subjects consuming tablets from A jar) and B (25 subjects consuming tablets from B jar).

A jar contained tablets composed of 400 mg of Brumex™ as the active ingredient, microcrystalline cellulose, mono and diglycerides of fatty acids, yellow-coating agents, magnesium stearate, silicon dioxide, calcium phosphate, yellow iron oxide dye, chlorophyllin dye as excipients. B jars contained tablets composed of microcrystalline cellulose, yellow-coating agents, magnesium stearate, silicon dioxide, calcium phosphate, yellow iron oxide dye, chlorophyllin dye as excipients.

Each subject was instructed to take one tablet from the box assigned in evening, before sleeping.

The treatment has then continued for 12 weeks. Clinical and laboratory data have been obtained at the baseline and at the end of the trial. Randomisation was done using a drawing of envelopes containing randomisation codes prepared by an independent statistician and specific software. The envelopes were then further mixed and distributed to the investigators who assigned the randomisation code in a progressive way to the enrolled subjects. A copy of the code was provided only to the person responsible of performing the statistical analysis.

Throughout the study, we instructed patients to take the new product's first dose on the day after they were given the study product in a blinded box. At the same time, all unused products were retrieved for inventory. Product compliance was assessed by counting the number of product doses returned at the time of specified clinic visits.

Assessments. Body weight, waist circumference and blood pressure were measured at each visit.

All plasma parameters were obtained after 12-hour overnight fasting. Venous blood samples were drawn by a nurse in all patients between 8:00 a.m. and 9:00 a.m. Plasma used was obtained by the addition of Na_2EDTA (1 mg = mL) and centrifuged at 3000*g* for 15 minutes at 48°C. Immediately after centrifugation plasma samples were frozen and stored at −80°C for no more than 3 months. The following parameters were evaluated via standardized methods: TC, HDL-C, TG, LDL-C, apolipoprotein AI (apo A1), apoB, glucose, liver transaminases, gGT and CPK. All measurements were performed by trained personnel in the Lipid Clinic laboratory of the Medicine and

Surgery Sciences Department, at the S. Orsola-Malpighi University Hospital.

2.9 | Ethics statement

The study was fully conducted in accordance with the Declaration of Helsinki, its protocol was approved by the Ethical Committee of the University of Bologna (Ethical approval number 324/2019).

2.10 | Informed consent

An informed consent form was signed by all the participants after giving them adequate time to read and understand it and after eventual clarifications by the involved investigators before the inclusion in the study.

2.11 | Statistical analyses

In the in vitro study, data are expressed as mean ± standard deviation (SD), calculated in $N \geq 3$ independent experiments (as detailed in figure captions). Statistical analysis was performed with GraphPad PRISM software (GraphPad Software, San Diego, CA, USA). Comparison among experimental groups was performed using the Mann–Whitney U test or non-parametric ANOVA ordinary test (Kruskal–Wallis Test). Multiple comparisons post-test was carried out with the Dunn's test. A p level of < 0.05 was considered significant for all tests; the description of symbols, and correspondent p values, is provided within each figure caption.

In the clinical trial, data have been analysed using the intention to treat by means of the Statistical Package for Social Science (SPSS) 25.0, version for Windows. The sample size suggested to detect a mean difference of 5% between treatments in terms of LDL reduction, with a power of 0.90 and an alpha error of 0.05, was of at least 20 subjects per group. As per protocol, we decided a priori to check the efficacy of treatments in subjects consuming at least 90% of the tested product doses foreseen by the trial design. Normally distributed baseline characteristics of the population have been compared using Student's t-test and χ^2 test followed by Fisher's exact test for categorical variables. Between-group differences was assessed by the ANOVA followed by Tukey's post-hoc test. All data are expressed as means and SD. A p level of < 0.05 was considered significant for all tests.

3 | RESULTS

3.1 | Four and 24 h treatment with Brumex™ formulation is not cytotoxic to HepG2 cells in vitro

We first characterised the response of human HepG2 cells to the bergamot extract treatment, to identify the possible lipid-related molecular mechanism(s) underlying the effect of Brumex™ in vitro.

The profile of the *Citrus bergamia* extract used in the preclinical study was characterised by HPLC-DAD (Figure S1), showing its rich content in neoesperidin, neoeriocitrin, naringin, brutieridin and melitidin flavonoids (≥40% w/w), with a peculiar profile of the 3-hydroxy-3-methyl-glutaryl-flavanones (HMG-Flavanones, namely the brutieridin and melitidin) ≥5% w/w.

We then verified whether the treatment with increasing concentrations of Brumex™ might drive any cytotoxic effect in HepG2 cells. We selected a set of bergamot extract concentrations, according to comparable literature in vitro data (Huang et al., 2021; Maugeri et al., 2019; Trombetta et al., 2010), to cover a wide range of screening doses. To this aim, cells were treated with Brumex™ (1, 10, 50, 100, 200, 500, 1000 and 2000 μg/mL) for both 4 and 24 h of continuous exposure, and cytotoxicity was assessed at any given time and dose by CCK8-test. As reported in Figure 1, no change in cell viability was reported in HepG2 cells up to 2000 μg/mL at 4 and 24 h Brumex™ treatment, thus demonstrating that the dose–time range chosen for the in vitro characterisation is not cytotoxic, and therefore appropriate for further investigation of the biological and molecular properties of the nutraceutical formulation. We selected the 500, 1000 and 2000 μg/mL concentrations for subsequent analyses.

3.2 | Brumex™ increases the level of phospho-AMPK in HepG2 cells

To test the ability of the Brumex™ formulation to activate AMPK by specific phosphorylation, HepG2 cells were treated with 1000 μg/mL of the bergamot extract for 4 h, and the amount of P-AMPK was measured by ELISA. The assay specifically detects and quantifies the level of AMPK protein that is phosphorylated at threonine residue 172 (Thr^{172}).

Both AICAR (2 mM) and Metformin (2 mM) were used as positive controls for AMPK activation by Thr^{172} phosphorylation. As shown in Figure 2, 4 h of Brumex™ treatment induces a significant increase—though quantitatively less marked than the pharmacological positive controls—in the intracellular amount of P-AMPK, if compared to untreated cells.

3.3 | Brumex™ impairs the intracellular lipid content and the expression of lipid genes in HepG2 cells via phosphorylation of AMPK

The activation of P-AMPK is reported to inhibit intracellular lipid synthesis in liver cells (Ahmad et al., 2020). The intracellular content of both total cholesterol and triglyceride (TG) was therefore assessed in HepG2 cells undergoing 24 h treatment with different concentrations (500, 1000, 2000 μg/mL) of the bergamot extract. Metformin (2 mM, 24 h) was used as a positive control. As shown in Figure 3, Brumex™ efficiently inhibits the synthesis of both lipids; in detail, all Brumex™ tested concentrations impaired the cholesterol synthesis if compared to untreated cells, whereas the effect was less effective in terms of

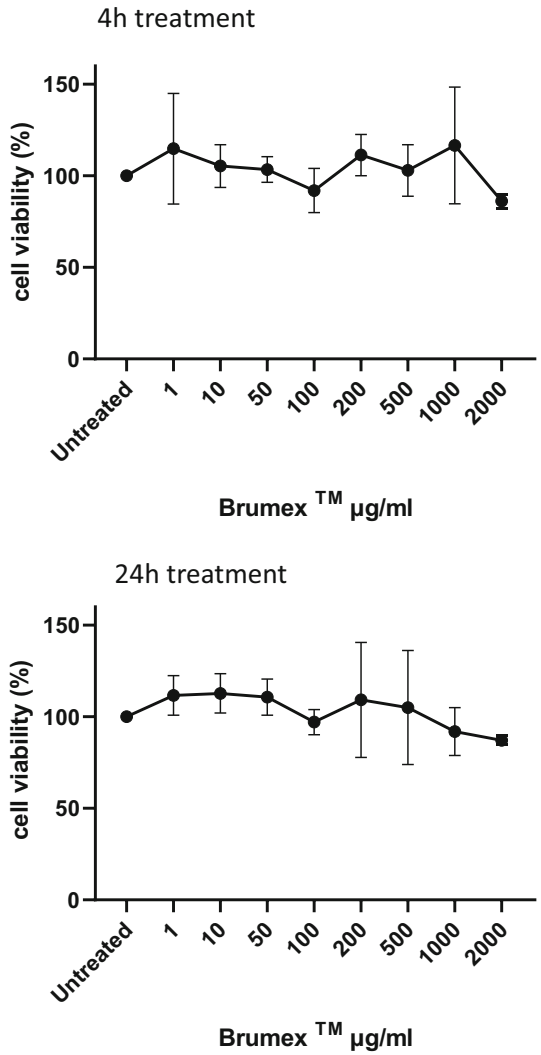

FIGURE 1 Treatment with the *Citrus bergamia* Brumex™ formulation is not cytotoxic to HepG2 cells in vitro. Evaluation of cell viability carried out in HepG2 cells by CCK-8 test, following 4 h and 24 h of continuous treatment with the Brumex™ formulation (ranging from 1 to 2000 µg/mL). Data are presented as percentage values versus untreated cells (100%) and shown as mean ± SD in $N = 3$ independent experiments.

intracellular TG content, as a significant TG reduction was observed exclusively at 1000 µg/concentration.

To test whether such inhibition was mediated by the upstream P-AMPK stimulation, a set of combined treatments of Brumex™ with the specific AMPK inhibitor (AMPKi) was carried out. The ability to impair AMPK phosphorylation was preliminarily assessed at 10, 50 and 100 µM concentrations of AMPKi (given for 1 h), and data reported in Figure S2. The concentration of 100 µM was selected for the subsequent experiments, performed in combination with the Brumex™ extract. Figure 3 shows that the administration of AMPKi can revert the bergamot-triggered cholesterol and TG depletion in HepG2 cells.

The P-AMPK-dependent lipid synthesis inhibition proceeds through the impairment of lipid synthesis-devoted genes, mostly controlled by SREBF-1 and SREBF-2 regulatory proteins (Ahmad et al., 2020). We thus measured the expression level of *SREBF-1*, *SREBF-2*, *ACACA*, *SCD1*, *HMGCR* and *FASN* genes by quantitative real-time PCR in HepG2 cells treated with the selected 1000 µg/mL Brumex™ dose, given for 4 h (Figure 4). The level of all selected genes was significantly reduced in Brumex™-treated if compared to control untreated HepG2 cells; moreover, by combining Brumex™ treatment with the specific AMPKi (100 µM, 1 h), we further proved that the bergamot-driven impairment in lipid genes expression is attributable to AMPK phosphorylation, as its inhibition can significantly revert the expression levels of all the genes analysed.

3.4 | Effect of the Brumex™ extract on plasma lipids, glucose metabolism and liver enzymes in a double-blind, placebo-controlled, randomized clinical trial

We finally evaluated the effect of Brumex™ extract on the key plasma biochemical parameters (related to lipid and glucose metabolism as well as to liver enzymes) in a double-blind, placebo-controlled, randomized clinical trial.

Enrolled patients were age- and sex-matched. The baseline characteristics of patients assigned to the two different treatments were similar, and no significant differences were observed regarding the studied parameters (Table 1).

From the randomisation visit to the end of the study, the enrolled subjects maintained a overall similar dietary pattern, without significant change in total energy, TC and total saturated fatty acid intake.

No adverse event was registered during the study and no patients withdrew from the study.

During the study, no significant change was observed as regards body weight, waist circumference, BMI, blood pressure and plasma creatine phosphokinase (CPK) level in both groups (p always > 0.05).

Brumex™ treatment had a significant impact on lipid pattern and liver enzymes compared with the placebo, while the placebo group had a mild but significant improvement in TG plasma level versus baseline only. In particular, the Brumex™ treated subjects experienced a significant 7.8 ± 1.2% decrease in TC, 7.6 ± 1.1% in low-density lipoprotein-cholesterol (LDL-C), 12.4 ± 1.5% in non-HDL-C, 13.4 ± 1.4% in TG, 8.7 ± 1.1% in apoB plasma level, while a significant increase in 4.8 ± 0.9% HDL-C and 10.2 ± 0.9% in apo AI plasma level (p always > 0.05).

Fasting plasma glucose (FPG) decreased by 3.4 ± 0.5%, GOT by 15.9 ± 1.5%, GPT by 13.6 ± 1.4%, and gGT by 32.3 ± 2.4% in Brumex™-treated subjects (p always > 0.05), only.

4 | DISCUSSION

We here demonstrate that the *Citrus bergamia* nutraceutical extract (Brumex™) specifically activates AMPK by Thr^{172} phosphorylation, drives P-AMPK-dependent cholesterol and TG depletion by reducing

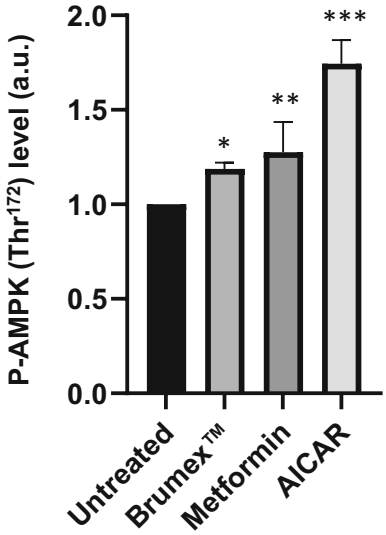

FIGURE 2 *Citrus bergamia* Brumex™ extract increases the level of phospho-AMPK (Thr^{172}) in HepG2 cells. Analysis of the intracellular content of the P-AMPK levels carried out by ELISA assay in HepG2 cells undergoing treatment with Brumex™ extract (1000 μg/mL, 4 h), Metformin (2 mM, 4 h) and AICAR (2 mM, 4 h). Data are presented as fold change value versus untreated cells and shown as mean ± SD in N = 4 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.0001.

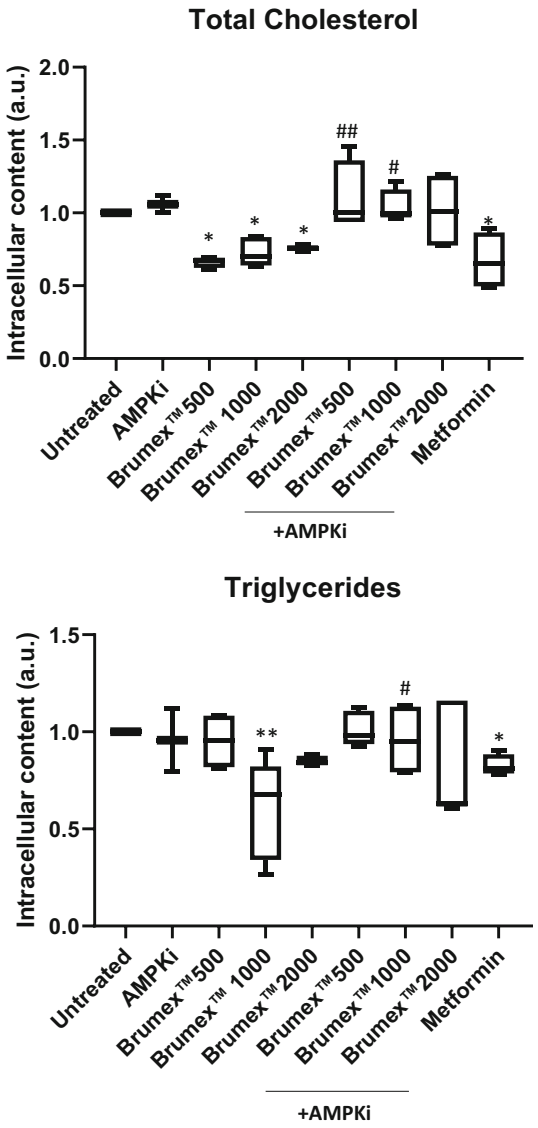

FIGURE 3 *Citrus bergamia* Brumex™ extract decreases the intracellular lipid content through activation of P-AMPK. Intracellular content of total cholesterol (upper panel) and triglycerides (lower panel) evaluated in HepG2 cells undergoing the treatments with AMPK inhibitor (AMPKi, 100 μM, 1 h), Brumex™ (500 μg/mL, 1000 μg/mL, 2000 μg/mL for 24 h), combined AMPKi + Brumex™ and Metformin drug (used as a positive control, 2 mM, 24 h). Data are presented as mean ± SD in $N = 4$ independent experiments. *$p \leq 0.05$ evaluated in either Brumex™ or Metformin groups if compared with untreated cells; #p < 0.05; ##p < 0.01 evaluated in (AMPKi + Brumex™) group if compared with single Brumex™-treated cells.

the expression of lipid synthesis-controlling genes, including *HMGCR*, in human HepG2 cells in vitro. Moreover, Brumex™ significantly ameliorates the full lipid and lipoprotein pattern, FPG and liver enzymes in healthy human subjects undergoing supplementation with Brumex™—if compared to placebo—in a dedicated double-blind, placebo-controlled, randomized clinical trial.

Although preliminary evidence on bergamot's beneficial properties—in terms of lipid-lowering capability—has been envisaged, we here provide an integrated preclinical and clinical study based on a commercial bergamot extract (Brumex™). It is peculiarly obtained from different parts of the whole fruit, mainly from bergamot juice, rather than from bergamot juice and/or based on single polyphenolic active components, as mostly demonstrated in literature data (Ferlazzo et al., 2016; Navarra et al., 2014; Toth et al., 2016). In our study, we prove the effectiveness of a whole bergamot extract that is specifically rich in neoesperidin, neoeriocitrin, naringin, brutieridin and melitidin flavonoids, with a peculiar HPLC profile of HMG-Flavanones ≥5% w/w (Figure S1). Noteworthy, the latter bio-components have been proven to exert a statin-like activity, by binding to the HMGCR enzyme (Di Donna et al., 2009; Di Donna et al., 2014), thus suggesting a possible molecular mechanism, based on HMGCR target inhibition, as we demonstrated in the in vitro part of our study.

At the molecular level, several natural products are known to suppress adipogenesis by either the activation of AMPK (via its phosphorylation) and/or the inhibition of HMGCR, as promising strategies for the treatment and prevention of obesity. Preliminary evidence has been also documented for *C. bergamia*, although most data are focused on single active bio-components. *Citrus bergamia* bioflavonoids impair adipogenesis in mature 3T3-L1 adipocytes in vitro, by acting at the HMGCR level (Ballistreri et al., 2021; Richard et al., 2013) and drive AMPK phosphorylation in both high glucose-treated and palmitate-challenged HepG2 cells (Dayarathne et al., 2021; Rajan et al., 2022), thus paving the way to the identification of such enzyme as a master regulator of the functional properties of bergamot. Thus, with the aim to characterize the possible lipid-related mechanism(s) underlying the function of Brumex™, we

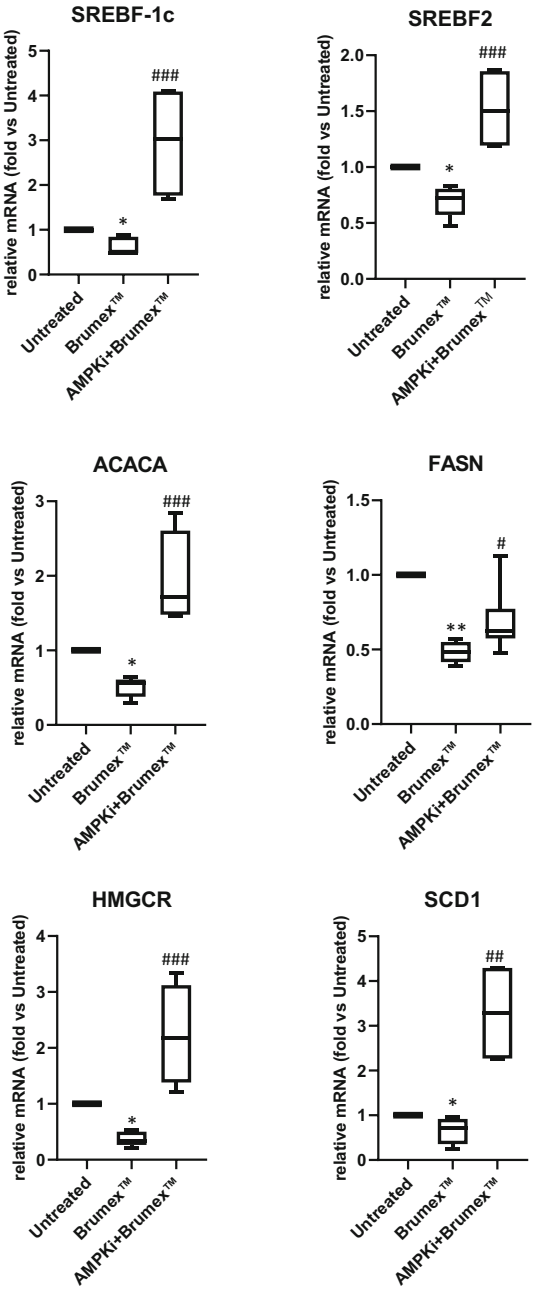

FIGURE 4 *Citrus bergamia* Brumex™ extract impairs the lipid genes expression of HepG2 cells via P-AMPK. Gene expression analysis was carried out by real-time PCR in HepG2 cells undergoing treatment with either the Brumex™ formulation (1000 μg/mL, 24 h) or the combination AMPK inhibitor (AMPKi, 100 μM, 1 h) + Brumex™. Data are presented as mean ± SD in $N = 4$ independent experiments. *$p < 0.05$, **$p < 0.01$ calculated in Brumex™ group when compared with untreated cells; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ evaluated in (AMPKi + Brumex™) group if compared with single Brumex™-treated cells.

performed an in vitro characterisation in HepG2 liver cells. These cells represent a reliable and widely used cellular model to mimic hepatic metabolism and to perform rapid and reproducible molecular screening—although they are carcinoma cells—especially when the in vitro findings are accompanied by in vivo/clinical results, like in our study. We treated HepG2 cells with a whole extract of Brumex™ and provide experimental evidence that the bergamot-based nutraceutical (characterised over a concentration window that is non-cytotoxic to liver cells in vitro, Figure 1) specifically stimulates the Thr^{175} phosphorylation of AMPK (Figure 2, Figure S2), a post-translational event specifically tuned by drugs that are routinely used in the treatment of obesity and type 2 diabetes mellitus, like AICAR and metformin (Luo et al., 2016; Sun et al., 2007).

By combining the Brumex™ with the specific AMPK inhibitor, we demonstrate that the activation of P-AMPK is directly involved in both cholesterol and TG depletion in HepG2 liver cells induced by the bergamot extract, thus proving the functional efficacy of Brumex™ formulation in the improvement of the in vitro hepatic lipid profile (Figure 3). We reported a slightly divergent response to Brumex™ between cholesterol and TG synthesis in HepG2 cells. This inconsistency might be attributable to the fact that hepatic TG intracellular contents are controlled by multiple biochemical pathways that do not always imply the upstream P-AMPK stimulation.

AMPK controls energy metabolism and regulates hepatic lipid metabolism through multiple pathways, mostly regulated through the activity of the SREBF-1c and SREBF-2 lipogenic transcription factors (Eberle et al., 2004; Horton et al., 2002; Li et al., 2011; Osborne & Espenshade, 2009; Viollet et al., 2006). We demonstrate that Brumex™ significantly reduces the expression of both SREBF transcription factors as well as their downstream lipid-related target genes, supporting the occurrence of a broad range of control on liver lipid metabolism triggered by Brumex™ (Figure 4). The ability of the extract to impair HMGCR expression represents a significant added value in the characterisation of its health benefits, as the enzyme is the primary target of statins in cholesterol-lowering therapy (Istvan & Deisenhofer, 2001).

The identification and valuation of the individual biomolecule(s), responsible for the biological effect triggered by a whole natural extract, is undoubtedly mandatory for research and clinical purpose, although it is also increasingly reported that the synergy among different bioactive molecules composing a vegetal extract might account for the overall health-related benefit (Liu, 2013). Our study is based on whole bergamot extract treatment of human cells in vitro, in line with what was recently reported by Huang et al. (2021), that also tested a whole bergamot extract in both HepG2 and Caco-2 cells. We specifically aimed at identifying the mechanism of action of the commercially available Brumex™ nutraceutical formulation, also by further verifying its functionality in human subjects. Although in vitro screening of whole natural extracts (including bergamot preparations) compared to single biomolecules, has been reported in the literature (Ferlazzo et al., 2016; Maugeri et al., 2019; Trombetta et al., 2010), we are aware that our molecular characterisation represents only a first level of screening of Brumex™ functionality, necessary for addressing further study. In particular, future evaluation will be devoted to mimic the rate of intestinal absorption and metabolisation of the Brumex™ extract in vitro, likely involving the microbiota contribute, that might affect plasma availability of the active bergamot

TABLE 1 Effect of placebo or Brumex™ intake on anthropometric, haemodynamic and laboratory parameters.

	Pre-run-in	Placebo T0	Bergamot T0	Placebo T1	Bergamot T1
Age (years)	52.6 ± 5.0	54.5 ± 4.2	54.1 ± 4.4	–	–
BW (kg)	63.4 ± 4.2	64.3 ± 5.4	63.8 ± 5.4	63.8 ± 5.4	62.7 ± 4.3
WC (cm)	88.3 ± 6.1	90.4 ± 4.4	89.6 ± 4.3	89.1 ± 4.7	87.9 ± 6.5
BMI (kg/m^2)	22.8 ± 2.2	23.7 ± 1.7	23.6 ± 1.5	23.6 ± 1.5	22.6 ± 2.3
SBP (mmHg)	134.5 ± 5.6	136.3 ± 4.8	134.9 ± 4.3	134.9 ± 4.3	135.7 ± 4.3
DBP (mmHg)	87.3 ± 2.2	88.6 ± 3.2	86.9 ± 2.4	86.9 ± 2.4	86.9 ± 3.5
TC (mg/dL)	248.3 ± 13.0	239.8 ± 11.7	237.6 ± 9.5	241.1 ± 13.5	219.1 ± 13.8*
HDL-C (mg/dL)	44.1 ± 2.7	46.5 ± 3.5	46.1 ± 2.7	44.3 ± 2.7	48.3 ± 2.8*
LDL-C (mg/dL)	161.4 ± 8.5	150.7 ± 9.9	149.1 ± 8.6	157.3 ± 8.9	137.7 ± 9.4*,§
Non-HDL-C (mg/dL)	204.7 ± 11.4	197.4 ± 10.2	195.5 ± 9.9	197.1 ± 11.8	171.5 ± 14.7*,§
TG (mg/dL)	216.8 ± 19.1	205.4 ± 13.7	198.5 ± 17.9	198.5 ± 17.9	171.8 ± 11.9*,§
ApoB (mg/dL)	146.3 ± 9.4	140.1 ± 8.5	138.4 ± 8.1	141.5 ± 7.4	126.5 ± 8.6*,§
ApoAI (mg/dL)	118.7 ± 12.4	101.9 ± 11.7	117.4 ± 13.5	118.2 ± 13.7	129.5 ± 11.7*,§
FPG (mg/dL)	88.9 ± 3.3	89.5 ± 3.5	88.2 ± 3.5	88.3 ± 3.2	85.3 ± 2.1*
GOT (mg/dL)	23.7 ± 3.8	24.5 ± 3.2	25.3 ± 3.4	25.3 ± 3.4	21.1 ± 2.3*
GPT (mg/dL)	22.0 ± 3.3	21.9 ± 2.4	22.1 ± 2.9	22.1 ± 2.9	19.3 ± 1.8*
gGT (mg/dL)	32.4 ± 2.1	34.6 ± 2.3	34.4 ± 2.4	32.7 ± 2.2	23.9 ± 3.1*
CPK (U/mL)	104.7 ± 19.2	101.3 ± 21.3	121.9 ± 15.1	111.9 ± 25.1	106.1 ± 18.9

Abbreviations: Apo, apolipoprotein; BMI, body mass index; BW, body weight; CPK, creatine phosphokinase; DBP, diastolic blood pressure; FPG, fasting plasma glucose; gGT, gamma-glutamyl-transferase; GOT, glutamic-oxaloacetic transaminase; GPT, glutamate pyruvate transaminase; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; WC, waist circumference.

*$p < 0.05$ vs. baseline; §$p < 0.05$ vs. Placebo.

biomolecules actually reaching the liver. The absorption, distribution, metabolism and excretion, particularly in the presence of food matrix, impact the bioflavonoids bioavailability, which might affect their bioactivities in vivo (Yang et al., 2022). Moreover, the molecular analysis might be extended to a more physiological liver cellular model, like the THLE-2 human primary hepatocytes (Benassi et al., 2021; Benassi et al., 2023), to provide a better connection between in vivo and in vitro experimental data.

In terms of clinical findings, we directly proved the hypo-lipidemic effect of the supplementation, as the Brumex™-treated subjects experienced a significant improvement in the whole plasma lipid and lipoprotein profile, FPG level and liver enzymes, suggesting a global positive metabolic effect, probably mediated by an improvement in insulin-resistance (Table 1). Our clinical data confirm the short-term high tolerability of Brumex™ and its potentially usefulness in the management of a large range of mildly hyper-lipidaemic subjects (Cicero et al., 2021). Overall, our findings highlight the significant lipid-related functionality of the bergamot supplementation in humans and pave the way for future studies aimed at correlating the clinical findings with the P-AMPK/HMGCR molecular pathways, and extending the functional properties of bergamot ingredient.

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Esserre Pharma Srl provided the Brumex™ ingredient used in the in vitro part of the study. The company also provided the Brumex™ and placebo tablets administered in the clinical trial.

AUTHOR CONTRIBUTIONS

Barbara Benassi and **Arrigo F. G. Cicero**: Conceptualization; conduction of thein vitroexperiments with statistical analyses. **Maria Pierdomenico**: Volunteers enrolment and follow-up. **Arrigo F. G. Cicero**, **Federica Fogacci** and **Maddalena Veronesi**: Data analysis and interpretation. **Barbara Benassi**, **Maria Pierdomenico**, **Arrigo F. G. Cicero** and **Federica Fogacci**: Writing original draft preparation. **Barbara Benassi**, **Maria Pierdomenico**, **Arrigo F. G. Cicero**, **Costanza Riccioni** and **Federica Fogacci**: Writing-review and editing. **Barbara Benassi**, **Arrigo F. G. Cicero** and **Costanza Riccioni**: Funding acquisition. **Barbara Benassi** and **Arrigo F. G. Cicero**: All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

C.R. is an employee at Esserre Pharma Srl. All other authors declare no conflict of interest. The Esserre Pharma srl had no role in the design of the study, in the collection, analyses and interpretation of the data or in the decision to publish the results.

DATA AVAILABILITY STATEMENT

All data generated and analyzed during this study are included in this article. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

PATIENT CONSENT STATEMENT

An informed consent form was signed by all the participants after giving them adequate time to read and understand it and after eventual explications by the involved investigators.

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SUPPORTING INFORMATION

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