SUPPLEMENTARY MATERIAL

Evaluation of lipid and cholesterol-lowering effect of bioflavonoids from bergamot extract

Gabriele Ballistreri[a]*, Margherita Amenta[a], Simona Fabroni[a], Valeria Consoli[b], Salvo Grosso[b], Luca Vanella[b], Valeria Sorrenti[b] and Paolo Rapisarda[a]

[a]*Council for Agricultural Research and Economics (CREA) – Research Centre for Olive, Fruit and Citrus Crops, 95024 Acireale, Italy*

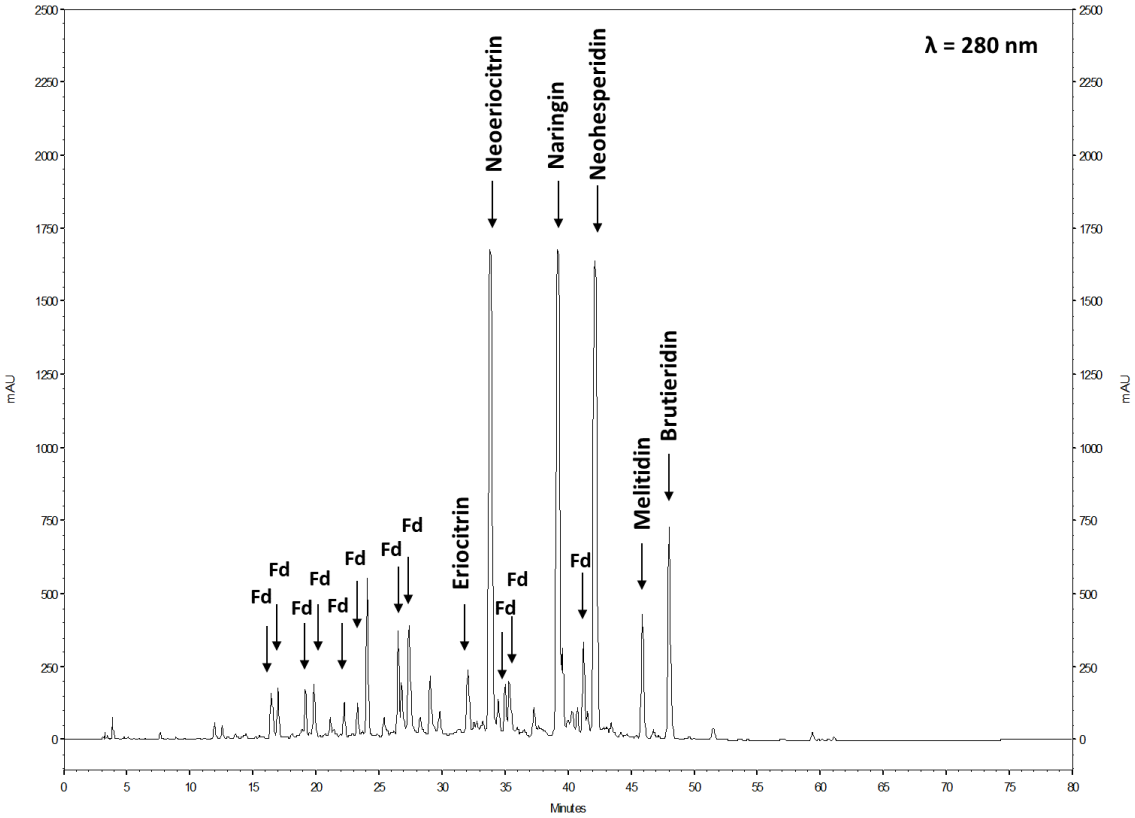
[b]*Department of Drug Science, Biochemistry Section, University of Catania, 95125 Catania, Italy*

*Corresponding Author: Gabriele Ballistreri, Council for Agricultural Research and Economics (CREA) – Research Centre for Olive, Fruit and Citrus Crops, 95024 Acireale, Italy. E-mail: gabriele.ballistreri@crea.gov.it

ABSTRACT

Several natural products have been reported to be involved in the suppression of adipogenesis. In this study, we reveal that a bergamot extract (BE) decreased the accumulation of intracellular lipids in murine pre-adipocytes 3T3-L1 cells during adipogenic differentiation. Both the inhibition of HMG-CoA reductase activity and the differentiation and proliferation of adipocytes could be used as a strategy for the treatment and prevention of obesity. The results of this study show a reduction of HMG-CoA activity and of lipid droplet accumulation in the presence of the BE, suggesting the potential of BE as an antiadipogenic agent to lower the content of cholesterol and body fat and prevent a gain in body weight. Moreover, BE as the result of high percentages of flavonoid compounds such as neoriocitrin, naringin and neohesperidin, the main flavonoids contained in BE, led to a significant inhibition of DPPH free radical, demonstrating a strong radical scavenging activity.

RESULTS AND DISCUSSION (FIGURES)



Phenolic compounds	λ_{max} (nm)	$[M–H]^-$ (*m*/*z*)	g/100 g[a]
Fd[b]	239, 282	-	5.40 ± 0.43
Eriocitrin	239, 282	595	0.80 ± 0.08
Neoeriocitrin	242, 283	595	13.15 ± 1.00
Naringin	243, 282	579	15.74 ± 0.23
Neohesperidin	242, 283	609	13.88 ± 0.68
Melitidin	242, 283	723	2.02 ± 0.11
Brutieridin	242, 283	753	3.97 ± 0.22
Total			*54.96 ± 1.45*

[a] Results are expressed as the mean ± SD.
[b] Flavanone derivative (Fd).

Figure S1. HPLC chromatogram and quantification of the flavanones of bergamot extract detected at 280 nm.

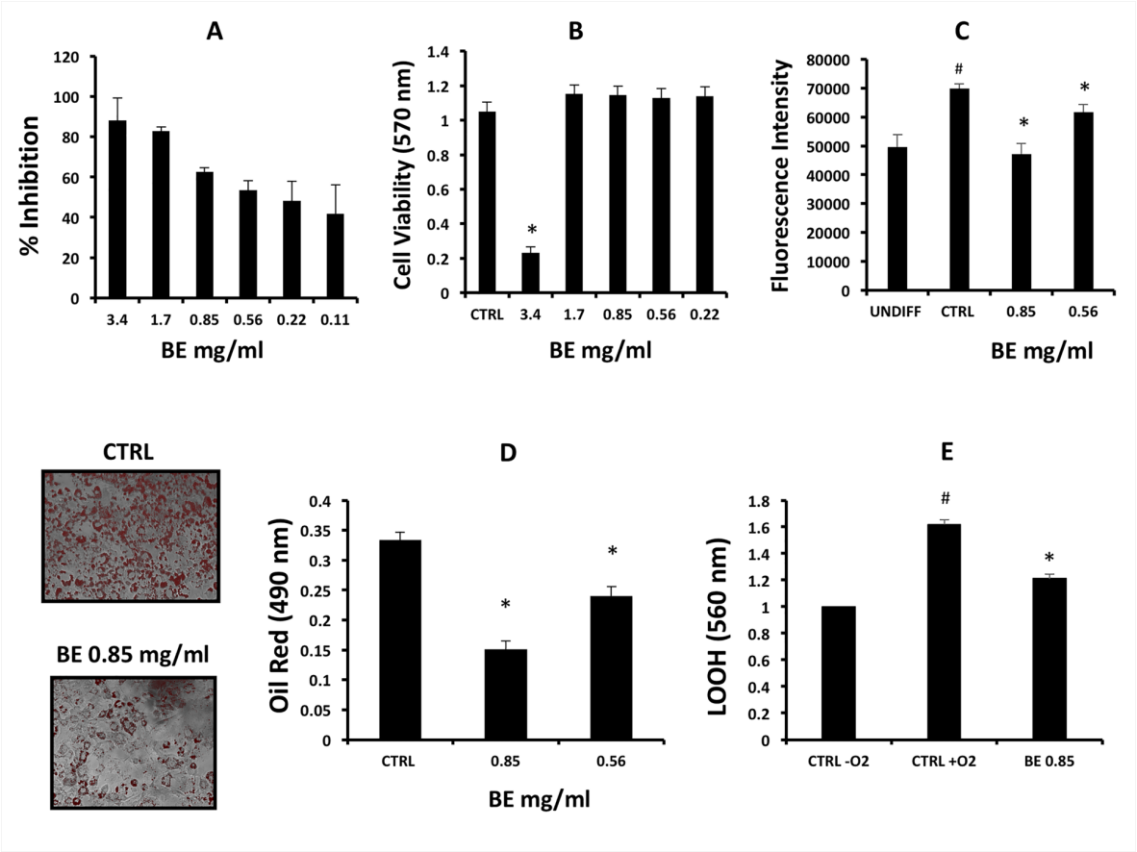
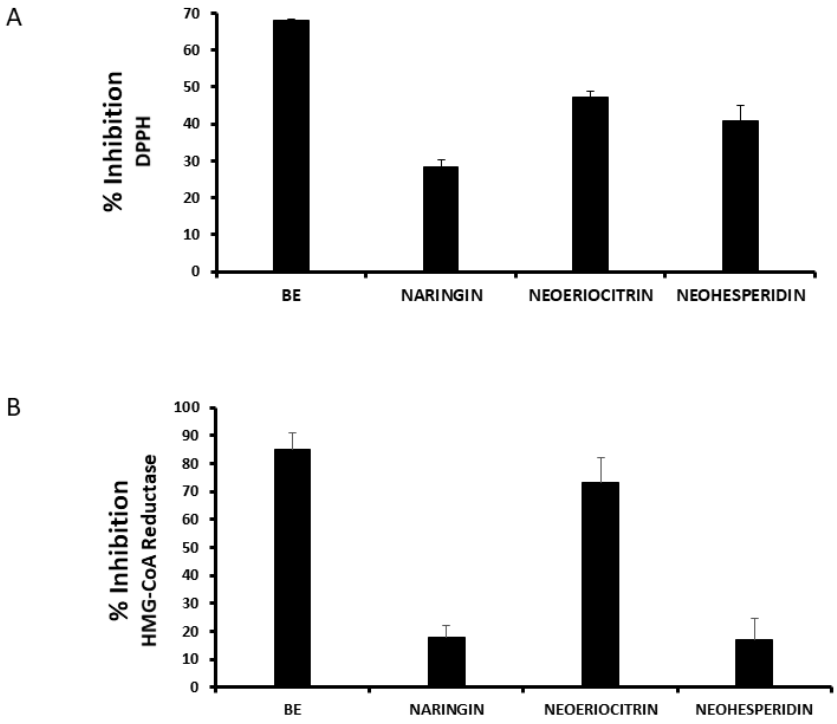


Figure S2. (A) DPPH radical scavenging activities of BE at different concentrations. Results are expressed as percentage of inhibition rate ± SD. (B) Cell viability of 3T3-L1 in absence or presence of BE at different concentrations (3.4 - 1.7 - 0.85 - 0.56 - 0.22 mg/ml). Results are expressed as the means ± SD of 4 experiments performed in triplicate. Significant vs untreated controls: *$p < 0.005$. (C) Intracellular ROS in undifferentiated (UNDIFF) and differentiated cells (CTRL) untreated and treated with BE (0.85 and 0.56 mg/ml). Values are the means ± SD of 4 experiments in triplicate. Significant vs. untreated control cells, $^{*}p < 0.05$; Significant vs. undifferentiated cells, $^{\#}p < 0.05$. (D) Representative Oil red O staining of 3T3-L1 cells in absence and in presence of BE. Lipid content was quantified at 490 nm (mean ± SD, $*p < 0.05$ versus control). (E) Effect of BE on lipid hydroperoxide levels in human plasma incubated at 37 °C for 2 h. Significant vs. oxygenated control, $^{*}p < 0.05$; Significant vs. unoxygenated control, $^{\#}p < 0.05$.



51

52 **Figure S3.** (A) DPPH radical scavenging activities of BE (0.85 mg/ml) and of different
53 concentrations of neoriocitrin, naringin and neohesperidin (0.111 mg/ml, 0.133 mg/ml
54 and 0.117 mg/ml). Results are expressed as percentage of inhibition rate ± SD. (B)
55 HMG-CoA inhibiting activities of BE (0.85 mg/ml) and of different concentrations of
56 neoriocitrin, naringin and neohesperidin (0.111 mg/ml, 0.133 mg/ml and 0.117 mg/ml).
57 Results are expressed as percentage of inhibition rate ± SD.

EXPERIMENTAL

Extraction

BERGASTAT® dry powdered extract (batch no. 19/064), manufactured by Medinutrex (Catania, Italy), standardised to contain ≥ 50.0% total flavanones (HPLC method), was used as BE for the assays. Separation and quantification of phenolic compounds were performed as previously described (Amenta et al. 2015). The extract was obtained from fruit (*fructus*) from Calabrian bergamots (*Citrus bergamia* Risso & Poiteau) (cultivars: 'Femminello', 'Castagnaro' and 'Fantastico'), harvested from November to February.

The extract was prepared from the solutions resulting by the pressing of the fruits, previously subjected to treatment with depectinizing enzymes, then decanted and filtered. The resulting solutions has been absorbed onto an adsorbent polymeric resin with a styrene-divinylbenzene brominated matrix, then the resin was washed with water and eluted with hydroalcoholic solutions. After distillation and recovery of alcohol (ethanol), the concentrated aqueous extract was subjected to spray drying process to obtain the dry powdered extract.

Inhibition of DPPH

The free radical scavenging activity of BE was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test. The reaction mixture contained 86 μM DPPH, different concentrations of extract (3.4, 1.7, 0.85, 0.56, 0.22 and 0.11 mg/ml) and of neoriocitrin, naringin and neohesperidin, (0.111 mg/ml, 0.133 mg/ml and 0.117 mg/ml) in 1 ml of ethanol. After 10 min at room temperature the absorbance at $\lambda = 517$ nm was recorded.

Cell culture and cell viability

The murine pre-adipocytes cell line 3T3-L1 was cultured in Dulbecco's modified Eagle's medium (DMEM) 4.5 g/L glucose, supplemented with 10% FBS, 1% penicillin, and streptomycin at 37 °C and 5% CO2. 3T3-L1 cells were seeded at a concentration of 2 × 105 cells per well of a 96-well, flat-bottomed 200 μl microplate. The cultures were maintained in the presence of different concentrations (3.4, 1.7, 0.85, 0.56 and 0.22 mg/ml) of Bergamot extract for 24 h. Cell viability was measured by MTT assay as previously reported (Raffaele et al. 2018; Raffaele et al. 2019a).

Measurement of ROS levels

Determination of reactive oxygen species (ROS) was performed using a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA), as previously described (Raffaele et al. 2019b). The fluorescence was measured spectrofluorometrically (excitation, λ=488 nm; emission, λ=525 nm).

Differentiation of 3T3-L1 cells

3T3-L1 cells were sub-cultured in three groups: undifferentiated, grown with standard DMEM; differentiated, grown with adipogenic medium obtained as previously described (Sorrenti et al. 2019), and differentiated with BE, grown with co-treatment of adipogenic medium and two different BE concentrations (0.85 and 0.56 mg/ml).

Oil Red O Staining

Staining solution was achieved using 0.21% oil red O in 100% isopropanol (Sigma-Aldrich, St. Louis, MO, USA). Briefly, adipocytes, grown in 24-well plate, were fixed in 10% formaldehyde, stained with oil red O for 10 min, as previously described (Raffaele et al. 2019b).

Determination of Lipid Hydroperoxide Levels in the Plasma of a Healthy Donor

Plasmatic lipid hydroperoxide levels were evaluated by oxidation of Fe^{2+} to Fe^{3+} in the presence of xylenol orange at λ = 560 nm as previously reported (Salerno et al. 2012) Plasma aliquots (500 μL) were diluted 1:1 with oxygenated and unoxygenated PBS and incubated at 37 °C for 2 h with or without BE (0.85 mg/mL) in a total volume of 1 ml. Results are expressed as fold of change respect to control - O_2 (plasma incubated with unoxygenated PBS) and represent the mean ± SD of four experimental determinations.

HMG-CoA activity

The HMG-CoA Reductase Activity Assay Kit from Abcam (Cambridge, United Kingdom) was used for measuring activity of purified HMG-CoA reductase in absence or in presence of BE (0.85 mg/mL) and neoriocitrin, naringin and neohesperidin (0.111 mg/ml, 0.133 mg/ml and 0.117 mg/ml) according to manufacturer's instructions. It is a colorimetric method based on the consumption of NADPH by the enzyme, which can be measured by the decrease of absorbance at OD = 340 nm. Results are expressed as percentage of inhibition of HMG-CoA reductase activity and represent the mean ± SD of four experimental determinations.

Statistical analysis

Statistical analyses of multiple comparisons were performed by the Fisher method. P-values lower than 0.05 were accepted as significant. Data were analyzed using either single-factor analysis of variance (ANOVA) for multiple groups, or the unpaired t-test for two groups, and the results are presented as mean ± standard deviation (SD).

REFERENCES

Amenta M, Ballistreri G, Fabroni S, Romeo FV, Spina A, Rapisarda P. 2015. Qualitative and nutraceutical aspects of lemon fruits grown on the mountainsides of the Mount Etna: a first step for a protected designation of origin or protected geographical indication application of the brand name “Limone dell’Etna”. Food Res Int. 74:250-259.

Raffaele M, Barbagallo I, Licari M, Carota G, Sferrazzo G, Spampinato M, Sorrenti V, Vanella L. 2018. N-Acetylcysteine (NAC) ameliorates lipid-related metabolic dysfunction in bone marrow stromal cells-derived adipocytes. Evid Based Complement Alternat Med. 5310961:1-9.

Raffaele M, Pittalà V, Zingales V, Barbagallo I, Salerno L, Li Volti G, Romeo G, Carota G, Sorrenti V, Vanella L. 2019a. Heme oxygenase-1 inhibition sensitizes human prostate cancer cells towards glucose deprivation and metformin-mediated cell death. Int J Mol Sci. 20(10)2593:1-16.

Raffaele M, Carota G, Sferrazzo G, Licari M, Barbagallo I, Sorrenti V, Signorelli SS, Vanella L. 2019b. Inhibition of heme oxygenase antioxidant activity exacerbates hepatic steatosis and fibrosis *in vitro*. Antioxidants. 8(277):1-12.

Salerno L, Modica MN, Romeo G, Pittalà V, Siracusa MA, Amato ME, Acquaviva R, Di Giacomo C, Sorrenti V. 2012. Novel inhibitors of nitric oxide synthase with antioxidant properties. Eur J Med Chem.49:118-126.

Sorrenti V, Randazzo CL, Caggia C, Ballistreri G, Romeo FV, Fabroni S, Timpanaro N, Raffaele M, Vanella L. 2019. Beneficial effects of pomegranate peel extract and probiotics on pre-adipocyte differentiation. Front Microbiol. 10(660):1-11.