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Citrus bergamia powder: Antioxidant, antimicrobial and anti-inflammatory properties

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Abstract: Bergamot whole-fruit powder was analyzed for total polyphenols, flavonoids, flavonols and ascorbic acid content and tested for in vitro and ex vivo antioxidant activity. The flavonoids profile was further characterized via HPLC-DAD-MS/MS and the most abundant flavonoids were quantified. The antimicrobial activity against potentially pathogenic bacteria and the effect on beneficial gut bacteria were determined. Lastly, we evaluated the effects of bergamot extract on endothelial alterations in LPS-stimulated human microvascular endothelial cells (HMEC-1). Our data demonstrated that bergamot powder possesses in vitro and ex vivo antioxidant activity, shows a selective inhibition against pathogenic strains and growth stimulation effects on some beneficial gut bacteria. Moreover, it protects HMEC-1 from LPS-induced activation and dysfunction and reduces the resulting endoplasmic reticulum stress. The relationship between isolated flavonoids and the protective effects are discussed. In conclusion, bergamot whole-fruit powder possesses beneficial health effects that makes it a potentially useful material for the nutraceutical industry.

Citrus bergamia powder: antioxidant, antimicrobial and anti-inflammatory properties

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Abstract

Bergamot whole-fruit powder was analyzed for total polyphenols, flavonoids, flavonols and ascorbic acid content and tested for *in vitro* and *ex vivo* antioxidant activity. The flavonoids profile was further characterized via HPLC-DAD-MS/MS and the most abundant flavonoids were quantified. The antimicrobial activity against potentially pathogenic bacteria and the effect on beneficial gut bacteria were determined. Lastly, we evaluated the effects of bergamot extract on endothelial alterations in LPS-stimulated human microvascular endothelial cells (HMEC-1). Our data demonstrated that bergamot powder possesses *in vitro* and *ex vivo* antioxidant activity, shows a selective inhibition against pathogenic strains and growth stimulation effects on some beneficial gut bacteria. Moreover, it protects HMEC-1 from LPS-induced activation and dysfunction and reduces the resulting endoplasmic reticulum stress. The relationship between isolated flavonoids and the protective effects are discussed. In conclusion, bergamot whole-fruit powder possesses beneficial health effects that makes it a potentially useful material for the nutraceutical industry.

Keywords: bergamot fruit, endothelial activation and dysfunction, LPS, HMEC-1, MIC, probiotics.

Chemical compounds studied in this article:

Brutieridin; Hesperetin 7-(2"-alpha-rhamnosyl-6"-(3""-hydroxy-3""-methylglutaryl)-beta-glucoside) (PubChem CID: 10148556)

Melitidin (PubChem CID: 101485562)

Naringin; Naringenin 7-Rhamnoglucoside) (PubChem CID: 25075)

Neoeriocitrin; Eriodictyol 7-neohesperidoside (PubChem CID: 114627)

Neohesperidin; Hesperetin 7-neohesperidoside (PubChem CID: 232990)

1. Introduction

Clinical and epidemiological evidence suggests an inverse association between the consumption of a polyphenolic rich diet and the risk of many chronic diseases (Pandey & Rizvi, 2009; Wang et al., 2014) as well as with the prevention and delay of age-related disease (Everitt et al., 2006). Besides, an improved endothelial function has been observed in both healthy people and patients with cardiovascular disease after an acute and chronic intake of rich polyphenol sources (Grassi, Lippi, Necozione, Desideri & Ferri, 2005; Schmitt & Dirsch, 2009). Among polyphenols, flavonoids exert important biological actions, including antioxidant, anti-inflammatory, anticancer, antiviral and anti-mutagenic activity (Mandalari et al., 2007; Kumar & Pandey, 2013; Yogendra Kumar, Tirpude, Maheshwari, Bansal & Misra, 2013). It has been recently reported (Tresserra-Rimbau et al., 2016) that a high flavonol intake correlates with a reduced risk of diabetes. Other polyphenols, such as catechins, provide beneficial effects on metabolic syndromes, cardiovascular and neurodegenerative diseases (Shirakami, Sakai, Kochi, Seishima & Shimizu, 2016). Besides, a recent study gave new insights in polyphenols bioavailability and in their structure-function activity (Sarkar, Mazumder, Saha & Bandyopadhyay, 2016).

Citrus fruits are rich in antioxidants including phenolic compounds such as flavanones which are negatively correlated with the risk of coronary heart and degenerative diseases (Barros, Ferreira & Genovese, 2012). Among these, *Citrus bergamia* Risso & Poiteau, commonly named bergamot, is a hybrid plant of sour lemon and orange, belonging to the Rutaceae family, growing in restricted areas due to peculiar soil and climate requirements. Italy is the main world producer of bergamot, which is cultivated in a very small coastal strip in Southern Italy, in the Reggio Calabria province (Risitano et al., 2014). Herein, its peel is used to extract a valuable essential oil widely employed in pharmaceutical, cosmetic, and food industries (Mandalari et al., 2006; Pernice et al., 2009), whereas its juice, obtained from the endocarp of the fruit, is not usually consumed as a beverage due to its bitter taste (Gattuso et al., 2006; Pernice et al., 2009). During bergamot oil extraction about 50-65% of peel, as well as mesocarp and juice, remains as primary by-product, and, because of its

fermentability, it has to be treated before being discarded (Mandalari et al., 2006; Mandalari et al., 2007; Trombetta et al., 2010; Barros et al., 2012).

Recently, bergamot derivatives have raised a great interest, because of their beneficial effects on human health (Trombetta et al., 2010; Graziano et al., 2012; Celia et al., 2013; Delle Monache et al., 2013; Kang et al., 2013; Russo et al., 2013; Cosentino et al., 2014; Risitano et al., 2014). The majority of these studies, as recently reviewed by Mannucci et al. (2017), focused on bergamot essential oil and juice while a few others investigated the composition and the activity of peel extracts (Mandalari et al., 2006; Trombetta et al., 2010; Graziano et al., 2012). It has been recently reported that bergamot juice can reduce plasma lipids in humans (Toth et al., 2016); besides, the bergamot-polyphenolic fraction can lead to substantial reduction of liver steatosis in patients with metabolic syndrome (Gliozzi, Maiuolo, Oppedisano & Mollace, 2016). Conversely, data on the biological effects of bergamot whole-fruit are not described in literature.

The protective effects of bergamot fruit are mainly related to its flavonoid content which exert anticancer, antimicrobial, antioxidant, and anti-inflammatory activities (Celia et al., 2013; Delle Monache et al., 2013; Risitano et al., 2014). Due to the inhibition of cancer cell proliferation, the bergamot flavonoid fraction has been considered as an anti-cancer drug (Visalli et al., 2016).

Regarding the antimicrobial activity of dietary polyphenols, it is mainly related to the polyphenol structure, the dosage assayed (Hervet-Hernández, Pintado, Rotger & Goñi, 2009) and it also depends on the tested strain. Some authors have described activity only against Gram-negative bacteria (Mandalari et al., 2007), whereas others also against Gram-positive strains (Fisher & Philips, 2006). The majority of dietary polyphenols is not absorbed in the small intestine and can interact with the colonic microbiota (Dueñas et al., 2015). The bioavailability of polyphenols and their interactions with the gut microbiota have been described by several authors (Marotti et al., 2011; Cardona, Andrés-Lacueva, Tulipani, Tinahones & Queipo-Ortuño, 2013; Di Gioia et al., 2014b; Marín, Miguélez, Villar & Lombó, 2015), showing that polyphenols and their metabolites may modulate the microbiota composition exerting antimicrobial activities against gut pathogenic

bacteria and stimulating the beneficial ones. However, to the best of our knowledge, the effects of bergamot whole-fruit on selected gut beneficial bacteria is not available in literature.

The consumption of citrus fruits has been associated to a lower risk of acute coronary events and stroke (Morand et al., 2011). Furthermore, much of the activity of citrus flavonoids appears to impact on blood and microvascular endothelial cells (Benavente-García & Castillo, 2008; Trombetta et al., 2010). Recently, it has been reported that the flavonoid-rich fraction of the bergamot juice decreases the intestinal ischemia/reperfusion injury development by a mechanism involving both NF-κB and MAP kinases pathways (Impellizzeri et al., 2016). Moreover, the anti-inflammatory properties of bergamot extracts in several experimental models have been extensively described by Ferlazzo et al. (2016a). Among these, a recent research demonstrates that bergamot juice inhibited the nuclear translocation of NF-κB in HepG2 cells, thus preventing its activation (Ferlazzo et al., 2016b).

Recent evidence suggests that the endoplasmic reticulum (ER) stress is involved in the induction of inflammatory response and contributes to the pathogenesis of several chronic inflammatory diseases (Hotamisligil, 2010; Gotoh, Endo & Oike, 2011; Kolattukudy & Niu, 2012). Increasing evidence suggests an extensive crosstalk between the inflammatory pathway and the ER stress response (Zhang & Kaufman, 2008). The effects of *Citrus* fruit on the ER stress has not been studied yet.

Thus, based on the growing findings concerning the biological activity of bergamot derivatives, the aim of this study was to characterize and evaluate the antioxidant capacity and the antimicrobial activity of an extract of *C. bergamia* powder obtained from lyophilized whole-fruits. Moreover, for the first time we evaluated the protective effect of this bergamot whole-fruit extract in response to lipopolysaccharides (LPS)-induced endothelial activation and dysfunction and ER stress, in human microvascular endothelial cells (HMEC-1).

2. Materials and Methods

2.1 Chemicals and reagents

All standards and reagents were of analytical grade. Methanol, acetic acid, sodium carbonate, sodium idrosside, metaphosphoric acid, Folin-Ciocalteu reagent, catechin hydrate, gallic acid, quercetin dihydrate, ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), potassium persulfate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), fluorescein sodium salt and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Fluka-Sigma-Aldrich, Inc. (St. Louis, MO). Sodium nitrite and aluminum chloride were purchased from Carlo Erba (Milan, IT), phosphate buffer saline (PBS), ethanol and sulfuric acid were purchased from VWR (Radnor, PA), while hydrochloric acid was purchased from Merck (Readington, NJ).

2.2 Plant material and extraction

Bergamot (*Citrus bergamia* Risso & Poiteau, Rutaceae) cultivar "Fantastico" fruits were collected in January from plants growing in Reggio Calabria province (Italy) and stored at – 20°C.

The whole-fruit was lyophilized and, after that, a powder of the fruit was obtained. This lyophilized powder was kindly supplied by Dr. Giuseppina Longo, Farmanatura Lab. (Bovalino Superiore, Reggio Calabria, Italy). Briefly, 1g of bergamot lyophilized powder was extracted in 10 ml of 70% ethanol through an overnight incubation at room temperature. Bergamot extracts were centrifuged 10 minutes at 2300 xg at 4°C (Jouan CR3i centrifuge, Newport Pagnell, UK) and the supernatants were collected, filtered (0.2µm VWR International PBI, Milan, IT), and kept at 4°C in the dark until use.

2.3 Phytochemical characterization

The total phenolic content was determined by the Folin-Ciocalteu colorimetric method (Singleton, Ortoger & Lamuela-Ravendo, 1999) and expressed as mg of gallic acid equivalents (GAE)/g dry weight (DW). The total flavonoid concentration was quantified using the aluminum chloride colorimetric method (Kim, Chun, Kim, Moon & Lee, 2003) and expressed as mg catechin equivalent (CE)/g DW. The total flavonols were measured according to the method described by Romani, Mancini, Tatti, and Vincieri (1996) and expressed as mg quercetin equivalent (QE)/g DW. The ascorbic acid content was measured by the UV-HPLC method described by Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2007), using a reverse-phase SUPELCOSIL™ LC-18-DB (5μm) stainless steel column (4.6mm x 250mm), as stationary phase, and 0.01% sulfuric acid (pH 2.6), as mobile phase. The ascorbic acid content was expressed as mg ascorbic acid equivalent (AAE)/100 g DW.

2.4 Instrumentation and HPLC-MS conditions

An Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with an autosampler and a diode array detector was used. An Agilent 6320 Ion Trap mass spectrometer equipped with an electrospray interface was online coupled and operated in positive and negative ionization mode using the following conditions: dry temperature, 350 °C; mass range, m/z 90–2200 Da; dry gas flow rate, 12 L min^{-1}; nebulization pressure, 40 psi.
Samples of bergamot extracts, prepared as described in Section 2.2 (100 mg mL^{-1} of 70% ethanol), were used. Separation was run on a Zorbax Eclipse XDB-C18 (4.6 x 150 mm, 5 μm particle diameter, Agilent Technologies, Santa Clara, CA, US) column, using the following mobile phases: (A) water/ACN/acetic acid (94:5:1, v/v) and (B) ACN/water/acetic acid (95:4:1, v/v/v) at a flow rate 0.6 mL min^{-1} with the following gradient: 0 min, 10% B; 10 min, 45% B; 15 min, 45% B; 20 min, 80% B; 25 min, 10% B. The injection volume was 10 μL. The detection wavelength was set at 280 nm and the UV–Vis spectra were acquired from 190 to 550 nm. For the quantification of the

main compounds present on the bergamot extract (neoeriocitrin, naringin, neohesperidin, melitidin and brutieridin) different linear concentrations of each standard were prepared in the range of 0.1-0.8 mg/mL and the calibration curve of each compound was obtained plotting the peak area of the standards against their concentration.

2.5 In vitro antioxidant activity

2.5.1 Oxygen Radical Absorbance Capacity (ORAC) Assay

The antioxidant capacity was quantified using the oxygen radical absorbance capacity (ORAC) assay with some modifications (Gabriele et al., 2015). AAPH was used as peroxyl radical generator and fluorescein as a probe. The fluorescence decay was evaluated at 485 nm excitation and 514 nm emission using a Victor^{TM} X3 Multilabel Plate Reader (Waltham, MA, US). Trolox was used as antioxidant standard. Results were expressed as ORAC units (µmol Trolox equivalents/100 g DW).

2.5.2 DPPH radical scavenging activity

The radical scavenging activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as described by Boudjou, Oomah, Zaidi, and Hosseinian (2013). The absorbance was recorded at 517 nm and the antiradical activity (ARA) was expressed as percentage of DPPH inhibition using the following equation: ARA = $[1-(A_S/A_C)] \times 100$, where A_S is the absorbance of the sample and A_c is the absorbance of control. The extract concentration corresponding to 50% of DPPH inhibition (EC_{50}) was measured according to Guimarães et al. (2010).

2.5.3 ABTS radical scavenging activity

The ABTS radical scavenging activity was determined according to the method described by Re et al. (1999). The ABTS radical cation ($ABTS^{\bullet+}$) was generated by oxidation of ABTS with potassium persulfate and reduced by hydrogen-donating antioxidants. Trolox was used as

antioxidant standard and the absorbance was recorded at 734 nm. The percentage of $ABTS^{•+}$ inhibition was measured and plotted as a function of bergamot concentration. Results were expressed as µmol Trolox equivalent/g DW antioxidant capacity (TEAC).

2.6 Ex vivo antioxidant activity

2.6.1 Preparation of erythrocytes

Human blood samples from healthy volunteers were collected in ethylenediaminetetraacetic acid (EDTA)-treated tubes and centrifuged for 10 min at 2300 xg at 4°C. Plasma and buffy coat were discarded and erythrocytes were washed twice with PBS pH 7.4.

2.6.2 Cellular antioxidant activity (CAA-RBC) assay in red blood cells

The antioxidant activity of bergamot extract was evaluated in an *ex vivo* erythrocytes system as described by Frassinetti, Gabriele, Caltavuturo, Longo and Pucci (2015). Quercetin was used as standard and the fluorescence was read at 485 nm excitation and 535 nm emission by using a Victor™ X3 Multilabel Plate Reader (Waltham, MA, US). Each value was express according to the Wolfe and Liu (2007) formula: CAA unit = $100-(\int SA / \int CA) \times 100$, where $\int SA$ is the integrated area of the sample curve and $\int CA$ is the integrated area of the control curve.

2.6.3 Erythrocytes oxidative hemolysis

Hemolysis of human erythrocytes was generated by thermal decomposition of AAPH in peroxyl radicals as described by Mikstacka, Rimando, and Ignatowicz (2010). The erythrocytes oxidative hemolysis was spectrophotometrically evaluated at 540 nm as released hemoglobin in the supernatant. Each value was expressed as hemolysis percentage with respect to control (AAPH-treated erythrocytes).

2.7 Antimicrobial activity

2.7.1 Bacterial media

Nutrient Broth (NB), Nutrient Agar (NA), Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA), de Man Rogosa Sharpe (MRS) medium, Mc Farland standard 0.5 were purchased from Oxoid (Basingstone, UK).

2.7.2 Bacterial strains and growth conditions

The pathogenic bacterial strains used in this study were supplied from American Type Culture Collection (ATCC). The antimicrobial activity of bergamot extract was tested on three Gram negative bacteria, specifically *Escherichia coli* (ATCC 25922), *Salmonella enterica* ser. *Typhimurium* (ATCC 14028), and *Enterobacter aerogenes* (ATCC 13048), and on two Gram positive bacteria, *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 25923). The above cultures were grown on NB and MHB and incubated overnight at 37°C under aerobic conditions.

Eight strains of human origin (four bifidobacteria and four lactic acid bacteria) were also used. *Lactobacillus paracasei* MB395, *Lactobacillus johnsonii* MB92, *Lactobacillus plantarum* MB91, *Bifidobacterium breve* B632, *Bifidobacterium pseudocatenulatum* B1279, *Bifidobacterium bifidum* B2009, and *Bifidobacterium adolescentis* MB16 were obtained from the Bologna University Scardovi Collection of Bifidobacteria available at the Department of Agricultural Sciences (University of Bologna), while *Lactobacillus reuteri* DSM 20016 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *Lactobacillus* strains were grown on MRS medium and incubated anaerobically at 37°C for 24 hours. Anaerobic conditions were created in a capped jar using an anaerobic atmosphere generation system (Anaerocult A, Merck, Darmstadt, Germany). *Bifidobacteriun* strains were cultivated in Tryptone, Phytone, and Yeast extract (TPY) broth (containing tryptone, 10.0 g/L, soy peptone, 5.0 g/L, glucose, 10.0 g/L,

yeast extract, 2.5 g/L, K_2HPO_4, 1.5 g/L, $MgCl_2.6H_2O$, 0.5 g/L, Cystein-HCl, 0.5 g/L, Tween 80, 0.5 g/L, pH 6.5) and incubated anaerobically at 37 °C for 24 hours.

2.7.3 Inhibition assay-Minimum inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) of the bergamot extract against selected bacteria was determined according to Delgado Adámez, Gamero Samino, Valdés Sánchez and González-Gómez (2012), with some modifications. Bergamot extract was diluted in sterile water to obtain a 1000 µg/ml solution. Then dilutions were made in water from this solution to 10 µg/ml (i.e. 1000, 700, 500, 250, 125, 100, 50, and 10 µg/ml).

The tested pathogenic microorganisms were cultured in MHB at 37°C for 16 hours. After that, the cultures were diluted to match the turbidity of 0.5 Mc Farland standard. Further dilutions in sterile MHB were made to obtain the working suspension corresponding to about 1-5 x 10^5 CFU/ml. An aliquot of 50 µl of bacterial suspensions was added to a sterile 96-well plate containing 100 µl of MHB. Lastly, 100 µl of extract dilutions were added. A positive control (containing only bacterial inoculum) was included on each microplate. The plates were incubated at 37°C for 24 hours in aerobic conditions. Afterwards, the optical density (O.D.) at 600 nm was detected using a microplate reader (Eti-System fast reader Sorin Biomedica, Modena, Italy). The MIC was defined as the lowest concentration of bergamot extract able to inhibit the microorganisms growth.

The MIC was also determined for *Lactobacillus* and *Bifidobacterium* strains previously listed. The procedure was the same as described above for pathogenic bacteria except that the media used were MRS broth and TPY medium for *Lactobacillus* and *Bifidobacterium* strains, respectively. Furthermore, microplates were incubated in anaerobic conditions at 37°C for 24 hours.

2.8 Human microvascular endothelial cell cultures

The HMEC-1(Human Microvascular Endothelial Cell) line was obtained from the Centre of Disease Control (Atlanta, USA). All reagents, media and medium supplements for cell culture were purchased from Sigma-Aldrich (St. Louis, MO).

Cells were grown in medium 199 (M199) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml epidermal growth factor (EGF), and 1 µg/ml hydrocortisone at 37°C in a humidified 5% CO_2 incubator. The medium was replaced every 3 days and all treatments were carried out on confluent cultures using M199 without phenol red, containing antibiotics and EGF.

After 1 hour pre-treatment with or without 50 µg/ml of bergamot extract, HMEC-1 were stimulated for 6 hours with or without 0.1 ng/ml of lipopolysaccharides (LPS from *Escherichia coli* serotype O55:B5). Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

2.9 Quantitative Real-Time PCR

Total RNA was isolated from HMEC-1 using the RNeasy Mini Kit (Qiagen, NL) and reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, CA). Quantitative Real-Time PCR was performed using the SsoFastTM EvaGreen® Supermix (Bio-Rad, CA) in the StepOnePlusTM Real-Time PCR System (ABI Applied Biosystems, Foster City, CA). Gene primers were designed using Beacon Designer Software (Premier Biosoft International, USA) and were: IL-6 (forward 5'-AAAGCAGCAAAGAGGCAC-3', reverse 5'-TTCACCAGGCAAGTCTCC-3'), ICAM-1 (forward 5'-ACCGTGAATGTGCTCTCC-3', reverse 5'-TCTTGATCTTCCGCTGGC-3'), CHOP (forward 5'-GAGAGTGTTCAAGAAGGAAGTGTA-3', reverse 5'-CCCGAAGGAGAAAGGCAAT-3'), ET-1 (forward 5'-GCAGAAACACACAGTCACAT-3', reverse 5'-TCAGACACAAACACTCCCTTA-3'), and β-actin (forward 5'-GAGATGCGTTGTTACAGGAAG-3', reverse 5'-TGGACTTGGGAGAGGACT-3'), used as

housekeeping. Samples were assayed in triplicate and the gene expression was calculated by the $2^{-\Delta\Delta CT}$ relative quantification method.

2.10 Cellular Reactive Oxygen Species (ROS) determination

The cellular reactive oxygen species (ROS) were detected after treatments using the 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a cell permeable dye useful to measure the redox state of cells. After diffusion into viable cells, DCFH-DA was firstly deacetylated by cellular esterases to a non fluorescent compound (DCFH), then oxidized by ROS activity to DCF, a highly fluorescent compound. Fluorescence was detected at 485 nm excitation and 535 nm emission using a Victor™ X3 Multilabel Plate Reader (Waltham, MA).

2.11 Nitrite (NO_{2-}) determination

Nitrite levels were used as an indicator of NO production and were quantified in culture media using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Michigan, USA). The optical density was read at 540 nm using a microplate reader (Eti-System fast reader Sorin Biomedica, Modena, Italy). Nitrite concentrations were expressed as percentage with respect to control.

2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 6.00 for Windows (GraphPad software, San Diego, CA). Assays were carried out in triplicate and the results were expressed as mean values ± standard deviation (SD). Differences between samples were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. A *p*-value lower than 0.05 is considered statistically significant.

3. Results and discussion

3.1 Bergamot phytochemical profile

Lyophilized powder of bergamot whole-fruits was analyzed by spectrophotometric methods for total polyphenol, flavonoid and flavonol, while ascorbic acid was quantified by UV-HPLC method. Hydroalcoholic bergamot extract contained 17.44±0.40 mg GAE/g DW of polyphenols, 16.74±0.27 mg CE/g DW of flavonoids, 3.91±0.37 mg QE/g DW of flavonols, and 66.93±0.05 mg AAE/100 g DW of ascorbic acid. These values are similar to those described for other *citrus* fruits (Zhang et al., 2014; Barros et al., 2012).

3.2. Flavonoid analysis by HPLC-DAD-MS/MS

The flavonoid profile of the bergamot extract was characterized via HPLC-DAD-MS/MS. Figure 1 shows the chromatogram at 280 nm of the extract with the main peaks numbered. The identity of 12 peaks could be established by the UV and MS spectra evaluation. The *m/z* value of each detected peak, its main MS/MS fragments and the corresponding tentative identification is reported in Table 1, as well as the relative peak area. The compounds defined by peak 6, 9, 10, 15 and 16, corresponding to the flavanones neoeriocitrin (4.2±0.76 mg/g DW), naringin (7.0±1.5 mg/g DW), neohesperidin (5.4±0.64 mg/g DW), melitidin (1.2±0.24 mg/g DW) and brutieridin (2.9±0.1 mg/g) were present in the highest amount in the extract, in good agreement with the data of flavonoid content in bergamot juice (Gattuso et al., 2006; Miceli et al., 2007; Sommella et al., 2013) and peel (Mandalari et al., 2006) reported in literature.

Table 1. Flavonoid profile in bergamot fruit extract and quantification

The antioxidant and anti-inflammatory activity of neohesperidin, naringin and neoeriocitrin from bergamot juice has been recently described (Sommella et al., 2013). Besides, it has been suggested that the anti-inflammatory properties of hesperidin are due to its inhibition on the

synthesis and activity of pro-inflammatory mediators, mainly prostaglandin E_2 and thromboxane A_2 (Benavente-Garcia & Castillo, 2008).

Moreover, the antibacterial activity of hesperidin and its aglycone hesperetin has been recently reported (Iranshahi, Rezaee, Parhiz, Roohbakhsh & Soltani, 2015); in particular, the hesperetin aglycon from citrus fruits showed inhibitory activity against *Staphylococcus aureus* and *Helicobacter pylori* (Marín et al., 2015).

3.2 In vitro and ex vivo antioxidant activities

Several methods have been developed to evaluate the *in vitro* antioxidant properties of fruits and vegetables. In this study, we investigated the *in vitro* antioxidant capacity and the radical scavenging activity of hydroalcoholic bergamot extract using the ORAC, the DPPH, and the ABTS assay.

As listed in Table 2, bergamot extract showed 950±0.37 ORAC units per gram of powder, comparable to values obtained from whole-lemon powder (Garcìa-Salas et al., 2013), and a DPPH inhibition activity (EC_{50}= 720±70 µg/ml) greater than the values reported by Trombetta et al. (2010) for two bergamot peel extracts. Moreover, bergamot extract showed a higher percentage of ABTS•+ inhibition (89.76±0.29%, corresponding to 136.3±5 µmol TE/g DW) compared to other citrus fruits (Zhang et al., 2014).

Table 2. Antioxidant capacity and antiradical scavenging activity of hydroalcoholic bergamot extract. Assays were carried out in triplicate and the results were expressed as mean values ± SD.

	ORAC (µmol TE/g DW)	**DPPH (EC_{50}= µg/ml)**	**ABTS (µmol TE/g DW)**
Bergamot extract	950±0.37	720±70	136.3±5

We also evaluated the antioxidant properties of bergamot extract in human erythrocytes under oxidative condition using the CAA-RBC assay and the hemolysis test. Erythrocytes represent a good *ex vivo* model system to assess the antioxidant activity of natural bioactive compounds (Honzel et al., 2008), allowing to get a better insight of their biological radical scavenging activity.

In this study, human erythrocytes were exposed to a peroxyl radical generator, the AAPH, after 1 hour pre-treatment with 200 μg/mL bergamot extract. As shown in Figure 2, bergamot pre-treated erythrocytes exhibited a significantly higher cellular antioxidant activity (CAA unit=30±0.78) compared to untreated cells (CAA=0; $^{**}p<0.01$), but lower than the quercetin (CAA= 94.45±4.44) used as reference standard.

Besides, bergamot extract was tested on human erythrocytes to evaluate the capability to counteract the oxidative hemolysis induced by peroxyl radicals produced by AAPH thermal decomposition. As shown in Figure 3, bergamot pre-treated cells exhibited a strong anti-hemolytic effect (85% hemolysis inhibition) compared to AAPH-treated cells ($^{***}p<0.001$), with a hemolysis inhibition comparable to the highest concentration of Trolox (50μM) used as reference standard.

3.3 Bergamot extract effects on pathogenic and beneficial bacterial growth

The antimicrobial activity against selected enteric bacterial strains was measured evaluating the O.D. at 600 nm in the presence of increasing doses of bergamot extract. The MIC values were listed in Table 3. The antimicrobial activities were compared to standard antibiotics, specifically gentamycin and vancomycin, used as positive control.

Table 3

Bergamot extract exhibited antibacterial action against all potentially pathogenic bacteria tested. The most sensitive Gram-negative microorganisms were *E. coli* ATCC 25922 and *S. typhimurium* ATCC 14028 showing MIC values of 500 μg/mL, whereas *E. aerogenes* ATCC 13048

was inhibited at 1000 µg/mL. Comparing the two Gram-positive bacteria, *S. aureus* ATCC 25923 was more sensitive than *E. faecalis ATCC 29212* with MIC values of 500 and 700 µg/mL, respectively. The inhibitory effect was similar for Gram-positive and Gram-negative strains.

The antimicrobial activity of polyphenols occurring in vegetable foods has been extensively investigated against a wide range of microorganisms (Daglia, 2012).

The antimicrobial activity of bergamot extract may be particularly related to its high content of neoeriocitrin, neohesperidin and hesperetin flavanones. This is in agreement with the results reported by Iranshahi et al. (2015). Additionally, Mandalari et al (2007) demonstrated the antimicrobial activity of pure bergamot flavonoids neohesperidin, hesperetin, neoeriocitrin, with minimum inhibitory concentrations ranging from 200 to 800 µg/mL (Mandalari et al., 2007).

As shown in Table 4, bergamot extract did not exert any marked antimicrobial activity against gut beneficial bacteria belonging to the *Bifidobacterium* and *Lactobacillus* genera. The *B. breve* B632 strain was not inhibited at all; on the contrary, it was slightly stimulated by amounts higher than 100 µg/mL. *B. pseudocatenulatum* B1279 growth was stimulated by concentrations lower than 250 µg/mL, whereas it was slightly inhibited at 500 µg/mL. *B. bifidum* B2009 growth was not affected up to 125 µg/mL, then a slight decrease of growth was observed without reaching inhibitory values. Growth of the *Lactobacillus* strains was not affected by the bergamot extract, except for a slight inhibition for the *L. reuteri* strains at the highest concentration assayed. On the contrary, a slight increase in cell growth was recorded for *L. plantarum* strains.

Table 4

The selective inhibition against potential pathogenic strains is important considering that the maintenance of a balanced gut microbiota is crucial for host health, whereas microbial imbalances are associated with metabolic disorders and/or disease status (Di Gioia, Aloisio, Mazzola & Biavati, 2014a). Besides, the presence of abundant beneficial bacteria, such as bifidobacteria and

lactobacilli, in the gut might provide protection against incoming of enteric pathogens (Jankowska, Laubitz, Antushevich, Zabielski & Grzesiuk, 2008; Montier et al., 2012; Symonds et al., 2012). Indeed, beneficial bacteria are able to compete for nutrients with enteric pathogens, to strongly adhere to the intestinal mucosa, thus preventing pathogen adhesion, and to stimulate the development of both humoral and cellular mucosal immune system (Tremaroli & Backhed, 2012).

The reason for the different growth response of *Lactobacillus* and *Bifidobacterium* strains with respect to the other assayed bacteria has not been specifically studied in this work. However, it is known that several *Bibifidobacteria* and *Lactobacillus* strains are capable of metabolizing some functional compounds, including flavonoids, releasing the sugar moiety to which they are attached in fruits and vegetables. The released sugar can act as additional growth substrate for the bacterial strain and the aglycone thus obtained can be absorbed by epithelial gut cells exerting its beneficial effects on the host. This bacterial transformation is thus essential for flavonoids absorption, bioavailability, and functional properties (Rossi, Amaretti, Roncaglia, Leonardi & Raimondi, 2010; Marotti et al., 2011; Jou, Tsai, Tu & Wu, 2013; Di Gioia et al., 2014b; Marín et al., 2015).

3.4 Bergamot extract effects in LPS-stimulated HMEC-1

Several studies have been focused on the phytochemical composition and the healthy properties of bergamot derivatives, showing an important polyphenol content and relevant biological effects in terms of antioxidant, anti-inflammatory, antitumor and antimicrobial activity (Mandalari et al., 2007; Navarra, Mannucci, Delbò & Calapai, 2015). The majority of these studies is focused on the bergamot essential oil while a few others on the bergamot juice (Trombetta et al., 2010; Celia et al., 2013; Delle Monache et al., 2013; Kang et al., 2013; Russo et al., 2013; Cosentino et al., 2014; Risitano et al., 2014). Anyway, although some studies have tested the antioxidant activity and bioactive compounds of a lyophilized pulp and peel of citrus fruits (Mandalari et al., 2006; Barros et al., 2012), as well of a whole-lemon powder (Garcìa-Salas et al.,

2013), to the best of our knowledge specific data on the biological effects of a powder from the bergamot whole-fruit are not available.

Herein, the potential protective effect of a lyophilized powder got from bergamot whole-fruit against LPS-induced endothelial activation and dysfunction was evaluated in human microvascular endothelial cells (HMEC-1). In order to identify the optimal treatment condition and detect possible cytotoxic effects we firstly performed a toxicity curve using 0-200 μg/mL as a range of concentrations for bergamot extract and 0-100 ng/mL as a range for LPS treatment. Cellular treatment effects were evaluated in terms of cell viability and assessed using the MTT assay (data not shown). Besides, we used the lowest LPS concentration able to induce a significant IL-6 gene induction, a pro-inflammatory cytokine. Specifically, we investigated in HMEC-1 the effects of 6 hours exposure to 0.1 ng/mL LPS, following 1 hour pre-treatment with 50 μg/mL bergamot extract.

To investigate the bergamot extract properties we assessed, by quantitative Real-Time PCR, its probable inhibitory effect on the modulation of genes involved in inflammation, endothelial dysfunction and ER stress.

Specifically, we analyzed the gene expression of the pro-inflammatory cytokine IL-6 (interleukin-6), the cell surface glycoprotein ICAM-1 (intercellular adhesion molecule-1), the endothelium-derived contracting factor ET-1 (endothelin-1), and the ER stress-responsive transcription factor C/EBP homologous protein (CHOP), an apoptotic transcriptional factor induced in response to ER stress.

As shown in Figures 4 A-D, exposure of HMEC-1 to 0.1 ng/mL LPS resulted in a significant up-regulation of IL-6, ICAM-1, ET-1 and CHOP compared to unexposed control cells (***$p<0.001$ vs CNT). Otherwise, bergamot pre-treatment exerted a significant inhibitory effect on the LPS-induced gene expression reducing IL-6 ($^{\#\#\#}p<0.001$ vs LPS), ET-1 ($^{\#}p<0.05$ vs LPS) and CHOP ($^{\#\#\#}p<0.001$ vs LPS) gene fold increase with overlapped values to the control levels (Figures 4A, 4C and 4D). However, although significantly reduced with respect to LPS-treated cells ($^{\#\#}p<0.01$ vs LPS), ICAM-1 gene expression resulted significantly higher in bergamot pre-treated cells than

control cells (*p<0.05 vs CNT) (Figure 4B). Lastly, we observed a slight but significant induction of IL-6 and ICAM-1 gene expression in response to bergamot treatment alone (**p<0.01 vs CNT) (Figures 4A and 4B).

3.5 Endothelial ROS production and nitric oxide (NO) bioavailability

Dysfunction of the endothelium represents an important early event in the pathogenesis of atherosclerosis that contribute to plaque initiation and progression, but it can also contribute to the onset of several cardiovascular diseases, including hypertension, diabetes, and coronary artery disease (Endermann & Schiffrin, 2004). Endothelial dysfunction is a systemic process that occurs in response to chronic inflammation, ischemia and reperfusion, and other risk factors (Ramzy et al., 2006).

It is well known that an increased ROS production and a diminished bioavailability of nitric oxide (NO), the most important vasodilator, are frequently implicated in microvascular dysfunction associated with inflammatory response (Kvietys & Granger, 2012). However, a normalization of vascular function to a physiological condition is possible through the restoration of ROS and NO balance (Kvietys & Granger, 2012).

Oxidative stress has been associated with a pro-inflammatory state of the vessel wall. Besides, ROS are known to quench NO with the formation of peroxynitrite, a cytotoxic oxidant, which contributes to LDL oxidation and to "uncoupling" of endothelial NO synthase (eNOS) that, when occurs, leads to exaggerated ROS level with deleterious effects on endothelial and vascular function (Endermann & Schiffrin, 2004).

Several studies have shown beneficial effects of enriched-polyphenols diet on the cardiovascular system that can strongly influence the incidence of cardiovascular diseases and get a better prognosis (Schini-Kerth, Auger, Kim, Etienne-Selloum & Chataigneau, 2010). In particular, both experimental and clinical studies indicated that polyphenols improve the endothelial function

through the stimulation of NO endothelial production, which contributes to control and maintain the vascular tone (Schini-Kerth et al., 2010).

Besides, as described by Rizza et al. (2011), the citrus flavonoid hesperetin exerted vasculoprotective actions increasing NO production from bovine aortic endothelial cells through eNOS activation. Moreover, clinical data on overweight healthy volunteers demonstrated that the orange juice consumption tended to increase NO plasma concentration (Morand et al., 2011). Recent studies also reported that bergamot flavonoids neoeriocitrin, naringin and neohesperidin exerted protective effect against inflammatory damages by reducing pro-inflammatory mediators as NO and iNOS. Furthermore, the anti-inflammatory effect of neohesperidin in LPS-induced inflammation have also been studied (Sommella et al., 2014).

Herein, we evaluated the effect of a whole-bergamot extract on LPS-induced ROS production using the DCFH-DA, a cell-permeable dye useful to measure the redox state of a cell. Moreover, we investigated by a colorimetric assay whether whole-bergamot extract modulate NO production from human microvascular endothelial cells. Our results showed a significantly higher level of ROS following LPS exposure (*$p<0.05$ compared to CNT) (Figure 5), which was significantly decreased (#$p<0.05$ compared to LPS 0.1 ng/ml) and normalized to a baseline level in the bergamot pre-treated cells then exposed to LPS (Figure 4). Moreover, compared to CNT, treatment with bergamot extract alone did not induce any change in intracellular ROS production (Figure 5).

Furthermore, our results showed a significant reduction of NO bioavailability following LPS exposure (-30.7%) with respect to untreated cells, which was reversed by bergamot pre-treatment resulting in increased NO level (+15.3%). Differently, the highest concentration of NO was found following bergamot extract treatment alone (+44.2%). The effect of bergamot extract on NO production may be related to both compounds neoeriocitrin and neohesperidin.

These preliminary data suggest that pre-treatment with bergamot whole-fruit powder can prevent and protect human microvascular endothelial cells from LPS-induced endothelial activation

and dysfunction. This protective effect is probably linked to the high content of flavanone constituents as neoeriocitrin and neohesperidin that exhibit also good radical scavenging and anti-inflammatory activity.

4. Conclusions

On the whole, the results obtained in this work indicate that the bergamot extract is rich in beneficial phytochemical compounds, possesses *in vitro* and *ex vivo* antioxidant activity, shows a selective inhibition against potentially pathogenic strains and a growth stimulation effect on some beneficial gut bacteria. Moreover, this extract is able to prevent and protect human microvascular endothelial cells from LPS-induced activation and dysfunction and to reduce the level of CHOP induced in response to ER stress. Therefore, bergamot peel and pulp, which can also be considered an agro-industrial by-product of the bergamot essential oil extraction, can be used to obtain a product possessing health properties exploitable by the pharmaceutical and/or nutraceutical industry.

Declaration of Interest Statement

The authors declare that they have no conflicts of interest.

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Figure captions

Figure 1. Flavonoid profile of bergamot extract analized via HPLC-DAD-MS/MS: chromagram at 280nm with the main peaks numbered. NI, not identified.

1: Vicenin-2; **2**: NI; **3**:Lucenin-2,4'-methyl ether; **4**: Rhoifolin 4'-glucoside; **5**: Quercetin-3-O-rutinoside (Rutin); **6**: Eriodictyol 7-O-neohesperidoside (Neoeriocitrin); **7**: NI; **8**: Narirutin; 9: Naringin; **10**: Hesperetin-7-O-neohesperidoside (Neohesperidin); **11**: Apigenin 7-O-neohesperidoside (Rhoifolin); **12**:Diosmetin 7-O-neohesperidoside (Neodiosmin); **13**: NI; **14**: NI; **15**: Melitidin; **16**: Hesperetin 7-[2''-α-rhamnosyl-6''-[3''''-hydroxy-3''''-methylglutaryl]-β-glucoside] (Brutieridin).

Figure 2. Effects of bergamot extract on the cellular antioxidant activity (CAA) in human erythrocytes. Quercetin was used as reference standard. Assays were carried out in triplicate and the results were expressed as mean values ± SD. * significantly different from untreated cells (CAA=0): ** $p \leq 0.01$; *** $p \leq 0.001$. One-way ANOVA with Bonferroni's multiple comparison test.

Figure 3. Effects of bergamot extract on AAPH-induced oxidative hemolysis in human erythrocyte. Trolox (10 and 50 µM) was used as reference standard. Assays were carried out in triplicate and the results were expressed as mean values ± SD. * significantly different from CNT (AAPH-treated cells): ** $p \leq 0.01$; *** $p \leq 0.001$. One-way ANOVA with Bonferroni's multiple comparison test.

Figure 4. Quantitative Real-Time PCR analysis of IL-6 (A), ICAM-1 (B), ET-1 (C) and CHOP (D) gene expression in 6 hours LPS-exposed HMEC-1, following 1 hour pre-treatment with or without 50 µg/ml bergamot extract. Experiments were carried out in triplicate and the results were expressed as gene expression fold increase with respect to control. * significantly different from

control (CNT): $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. # significantly different from LPS 0.1 ng/ml: $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$. One-way ANOVA with Bonferroni multiple comparison test.

Figure 5. Cellular ROS production (DCFH-DA assay) in 6 hours LPS-exposed HMEC-1, following 1 hour pre-treatment with or without 50 µg/ml bergamot extract. Experiments were carried out in triplicate and the results were expressed as the DCF fluorescence level with respect to control (CNT). One-way ANOVA with Bonferroni multiple comparison test. *significantly different from CNT: $^{*}p<0.05$; # significantly different from LPS 0.1 ng/ml: $^{\#}p<0.05$

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Table 1

Table 1. Tentative chemical characterization of bergamot flavonoids profile obtained via HPLC-MS analysis.

Peak	t_r	Area % of total peak area	$[M+H]^+$	$[M-H]^-$	Main fragments	Identification	Quantification** (mg/g DW)
1	11.3	1,20	595.5		577, 559, 529, 457	Vicenin-2	
2	11.4	0,71	651.3		642, 625, 561, 525, 489, 381, 363	NI	
3	12.0	0,66	625.3		607, 589, 561, 488	Lucenin-2,4'-methyl ether	
4	12.1	0,35	741.3		686, 596, 579, 434, 311, 271	Rhoifolin 4'-glucoside	
5	12.9	0,68		609.3	301	Quercetin-3-O-rutinoside (Rutin)	
6	13.3	18,85	597.4		579, 561, 543, 475, 451, 433, 331, 289	Eriodictyol 7-O-neohesperidoside (Neoeriocitrin)	4.2±0.76
7	14.3	2,10	579.4		561, 544, 525, 315, 273, 195	NI	
8	14.5	1,10	603.4*		585, 483, 331, 295, 231	Narirutin	
9	15.0	27,03	595.5		533, 449, 433, 287	Naringin	7.0±1.50

10	16.3	24,19	611.5		591, 575, 557, 539, 489, 465, 449, 345, 303	Hesperetin-7-O-neohesperidoside (Neohesperidin)	5.4±0.64
11	17.4	2,44	579.2		433, 271	Apigenin 7-O-neohesperidoside (Rhoifolin)	
12	17.8	1,34	609.2		463, 301, 286	Diosmetin 7-O-neohesperidoside (Neodiosmin)	
13	18.5	1,17		620.8	579, 561, 545, 440, 285	NI	
14	19.7	1,19	679.7		661, 548, 435, 322	NI	
15	19.9	4,91		723.9	661, 622, 580	Melitidin	1.2±0.24
16	20.5	12,08	755.8		737, 719, 701, 683, 657, 633, 615, 491, 473, 387, 303	Hesperetin 7-[2''-α-rhamnosyl-6''-[3''''-hydroxy-3''''-methylglutaryl]-β-glucoside] (Brutieridin)	2.9±0.10

NI, not identified

*Ion detected as $[M+Na]^+$

** Quantification has been performed for the 5 major peaks identified by HPLD-DAD (peak number 6, 9, 10, 15, 16).

Table 3

Table 3: minimal inhibitory concentration (MIC) of bergamot extract against selected enteric bacterial strains

Strains	Bergamot extract concentrations (µg/ml)								
	O.D. 660 nm								
	Control	10	50	100	125	250	500	700	1000
Escherichia coli ATCC 25922	0.88 ± 0.10	0.87 ± 0.05	0.95 ± 0.04	1.06 ± 0.05	0.98 ± 0.01	0.92 ± 0.2	⬇ 0.038 ±0.003	0.04 ± 0.002	0.05 ± 0.002
Salmonella typhimurium ATCC 14028	0.82 ± 0.07	0.81 ± 0.08	0.91 ± 0.02	0.92± 0.05	0.81 ± 0.08	0.53 ± 0.14	⬇0.041 ± 0.004	0.04 ± 0.01	0.05±0.01
Enterobacter aerogenes ATCC 13048	1.05 ± 0.03	0.91 ± 0.03	1.06 ± 0.09	1.18 ± 0.05	1.15 ± 0.03	1.2 ± 0.05	0.25 ± 0.05	0.16 ± 0.05	⬇0.06± 0.004
Enterococcus faecalis ATCC 29212	0.89 ± 0.08	0.95 ± 0.01	0.99 ± 0.03	1.08 ± 0.5	1.11 ± 0.02	1.06 ± 0.2	0.21 ± 0.12	⬇0.05 ± 0.01	0.07 ± 0.01
Staphylococcus aureus ATCC 25923	0.93 ± 0.09	0.88 ± 0.04	0.87 ± 0.04	0.86 ± 0.02	0.98 ± 0.05	0.56 ± 0.03	⬇0.04 ± 0.06	0.07 ± 0.04	0.06 ± 0.03

Table 4

Table 4: Growth of the *Bifidobacterium* and *Lactobacillus* strains tested in the presence of different amounts of bergamot extracts

Bacterial growth (O.D. 600 nm) after 24 hours of incubation									
	Bergamot extract concentration (µg/ml)								
Strains	control	10	50	100	125	250	500	700	1000
Bifidobacterium breve B632	0.65 ± 0.00	0.70 ± 0.03	0.68 ± 0.02	0.78 ± 0.05	0.79 ± 0.03	0.79 ± 0.05	0.76 ± 0.05	0.79 ± 0.01	0.68 ± 0.04
Bifidobacterium pseudocatenulatum B1279	0.65 ± 0.06	0.90 ± 0.04	0.82 ± 0.09	0.85 ± 0.06	0.78 ± 0.16	0.74 ± 0.05	0.58 ± 0.09	0.56 ± 0.10	0.43 ± 0.02
Bifidobacterium bifidum B2009	0.52 ± 0.01	0.54 ± 0.02	0.52 ± 0.03	0.50 ± 0.01	0.51 ± 0.02	0.38 ± 0.03	0.36 ± 0.09	0.34 ± 0.01	0.35 ± 0.03
Bifidobacterium adolescentis MB16	1.09 ±0.07	0.98 ± 0.03	0.95 ± 0.05	0.99 ± 0.14	0.90 ± 0.01	0.65 ± 0.06	0.63 ± 0.04	0.62 ± 0.00	0.60 ± 0.09
Lactobacillus paracasei MB395	0.96 ± 0.01	1.03 ± 0.01	1.00 ± 0.02	1.01 ± 0.01	1.03 ± 0.04	1.00 ± 0.02	0.97 ± 0.02	0.99 ± 0.01	0.99 ± 0.02
Lactobacillus reuteri DSM20016	0.96 ± 0.03	0.76 ± 0.02	0.74 ± 0.03	0.77 ± 0.01	0.88 ± 0.01	0.86 ± 0.03	0.84 ± 0.03	0.83 ± 0.02	0.67 ± 0.05
Lactobacillus johnsonii MB92	1.14 ± 0.08	1.13 ± 0.02	1.11 ± 0.03	1.15 ± 0.04	1.14 ± 0.01	1.18 ± 0.02	1.14 ± 0.04	1.15 ± 0.11	1.14 ± 0.04
Lactobacillus plantarum MB91	1.15 ± 0.03	1.11 ± 0.01	1.16 ± 0.01	1.18 ± 0.03	1.27 ± 0.02	1.23 ± 0.03	1.26 ± 0.06	1.36 ± 0.05	1.36 ± 0.05

Figure 1
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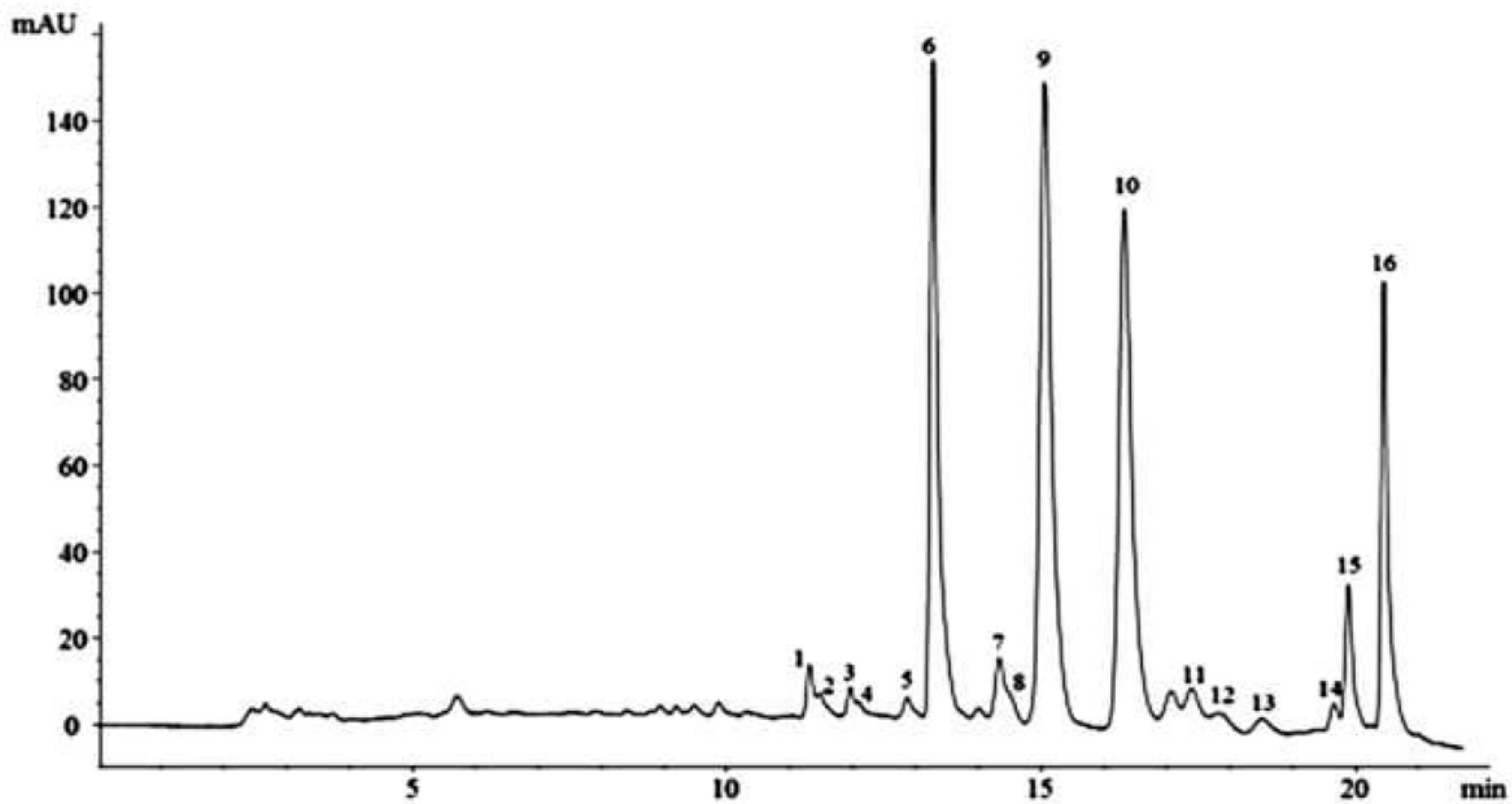

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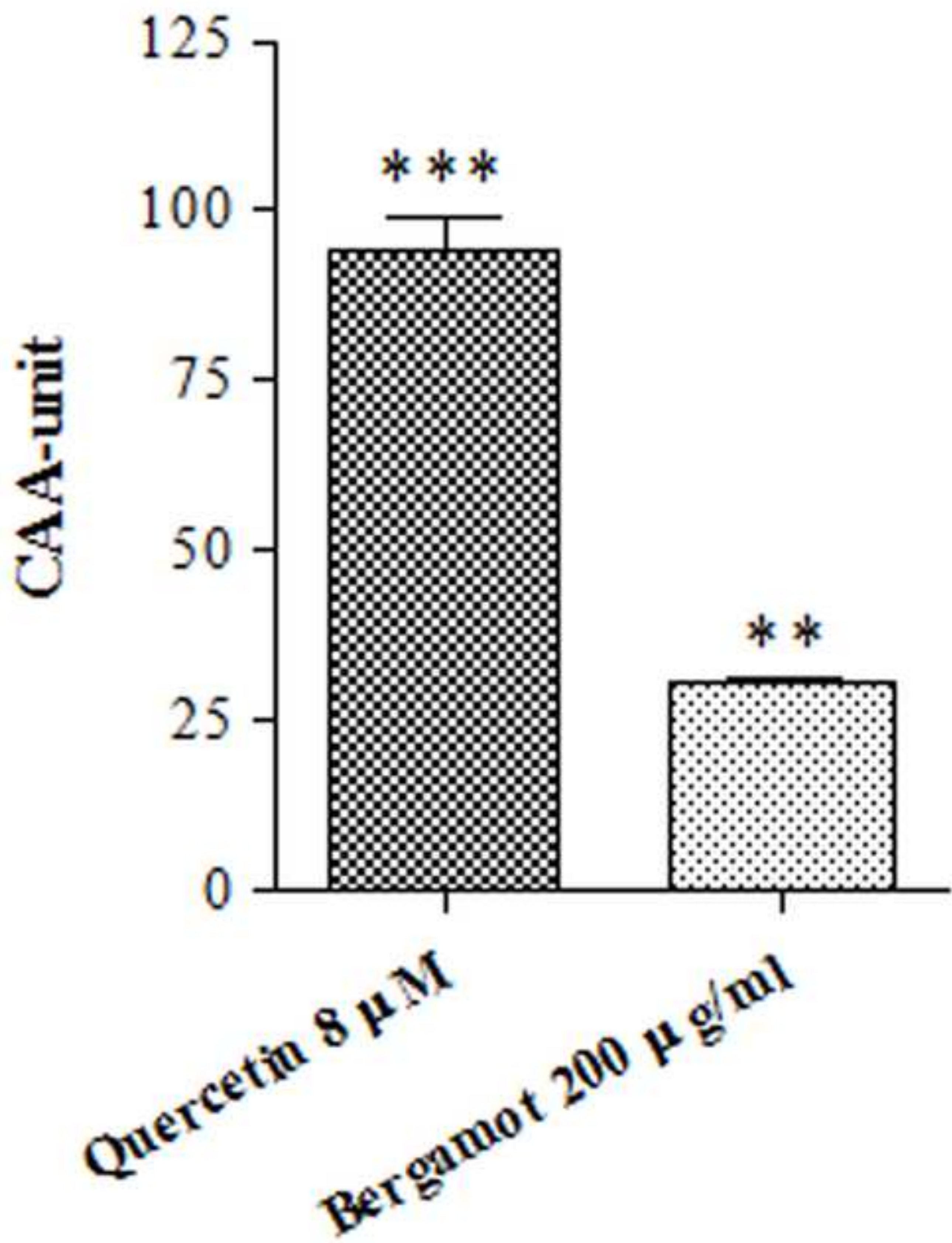

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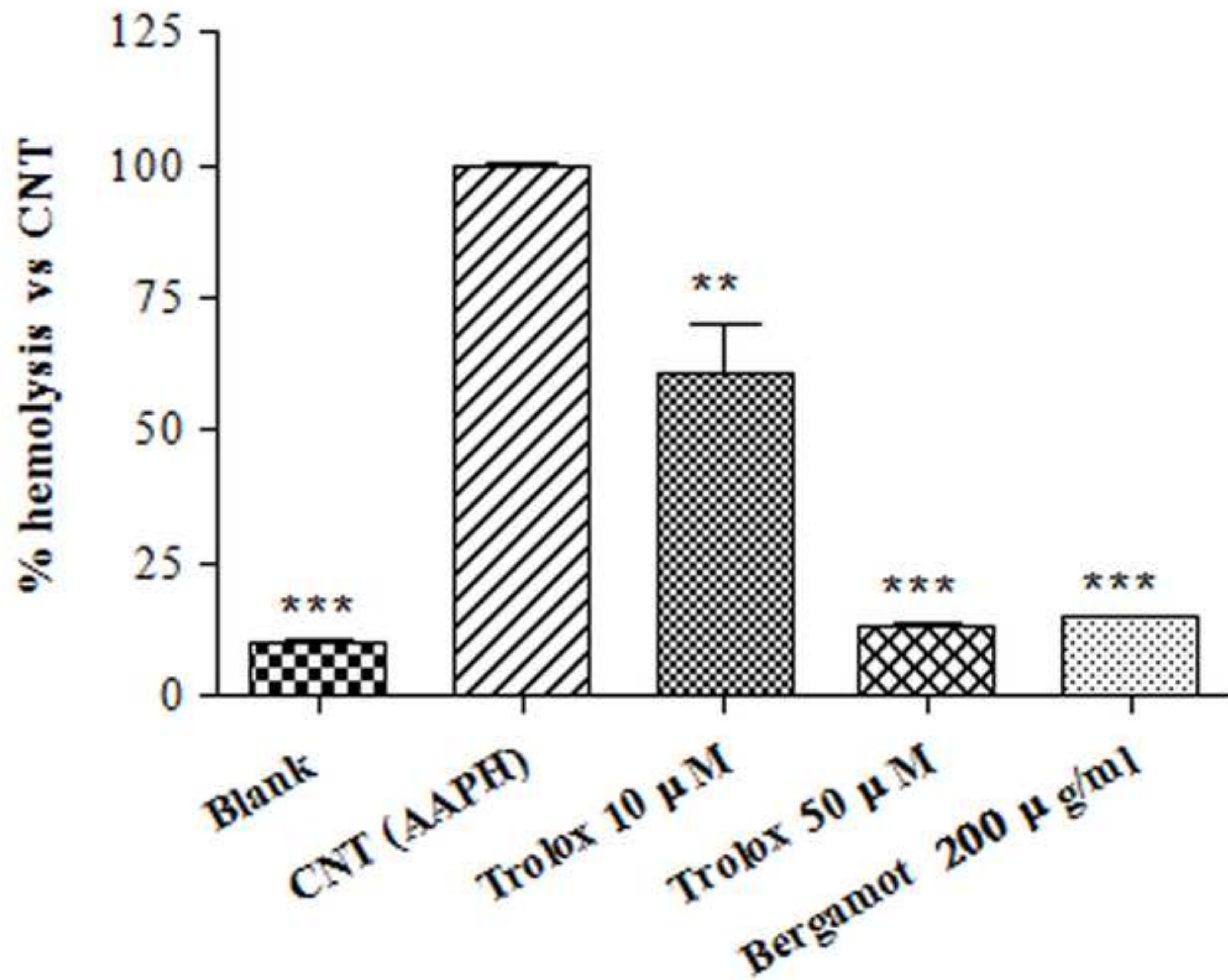

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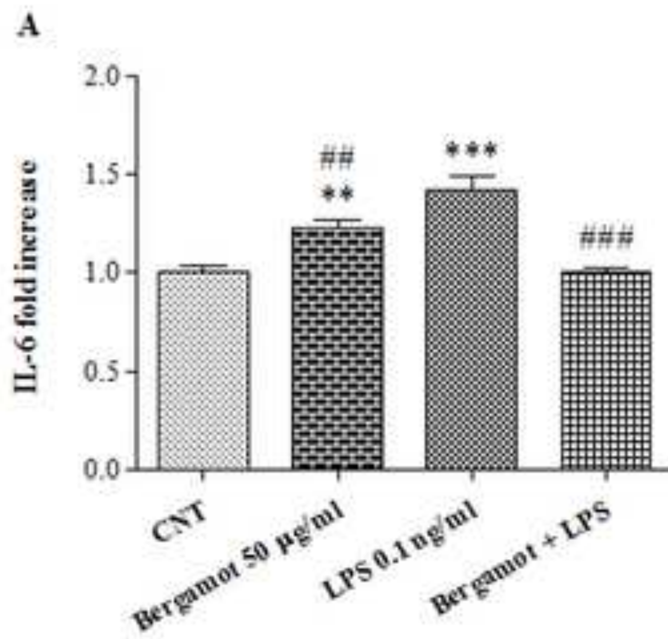

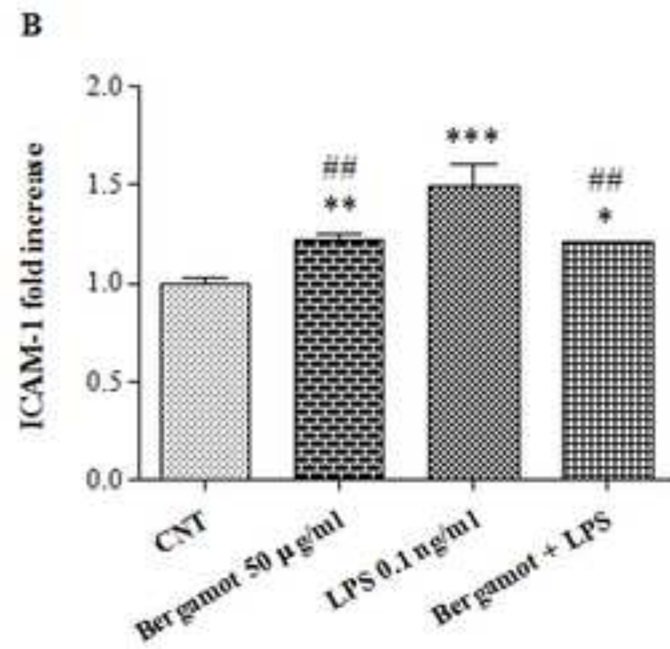

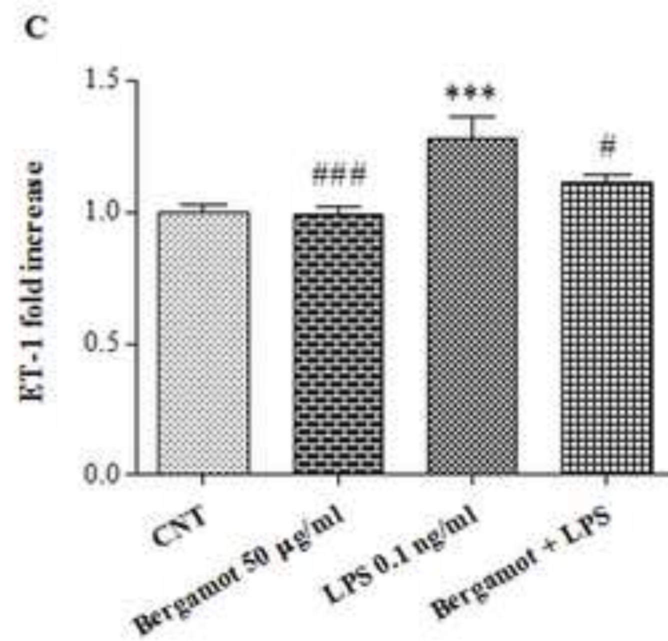

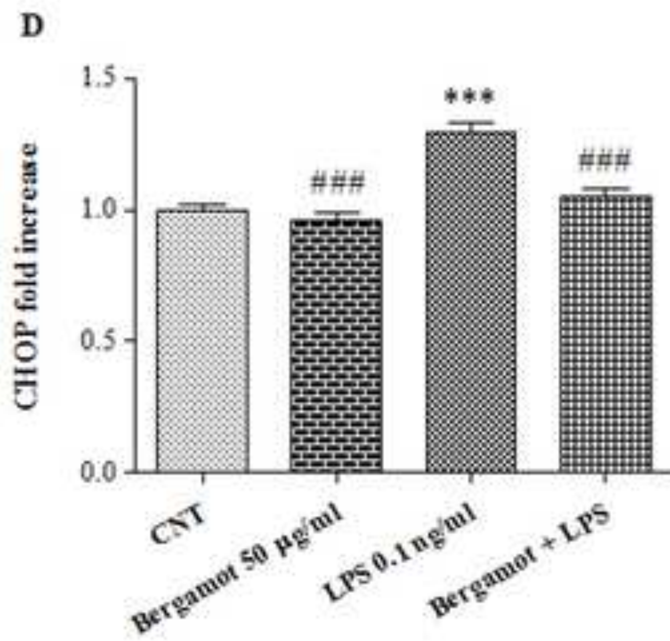

Figure 5

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