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Anti-inflammatory activities of Italian Chestnut and Eucalyptus honeys on murine RAW 264.7 macrophages

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ABSTRACT

The aim of this study was to evaluate for the first time the anti-inflammatory effects of chestnut (*Castanea sativa*) and Eucalyptus (*Eucalyptus occidentalis*) honeys collected in Italy on murine RAW 264.7 macrophages stressed with the bacterial endotoxin lipopolysaccharide (LPS). Pre-treatment with the two honeys markedly decreased apoptosis and stimulated wound healing in macrophages stressed with LPS, where they also reduced the intracellular levels of ROS and nitrite. Additionally, treatment with LPS decreased the levels of glutathione and suppressed the antioxidant enzyme activities, which were significantly ($p < 0.05$) increased in the presence of the two honeys, through the modulation of Nrf2 expression; in LPS-stressed macrophages, Chestnut or Eucalyptus honeys protected against inflammation by regulating the main inflammatory biomarkers, including TNF-α, IL-10 and iNOS, through the decrease of NF-κB expression. Finally, they improved mitochondrial respiration and the main related parameters.

1. Introduction

Inflammation is the key mechanism of the immune system to the protect human body against different harmful factors, including physical (i.e., burns, trauma, physical injury), chemical (i.e., toxins, irritants) and biological (i.e., microorganisms and their fragments) agents (Afrin et al., 2018a; 2018b; Gasparrini et al., 2018). Once initiated, the inflammatory process activates several types of immune cells and a series of signaling pathways that induce, in a very coordinated manner, the neutralization of the detrimental agent. Together with neutrophils, macrophages are key mediators of the inflammatory response, since they are essential for the initiation, maintenance and resolution of inflammation through the implementation of phagocytosis or the production of cytokines, such as the pro-inflammatory tumor necrosis

Abbreviations: CEq, catechin equivalents; CH, Chestnut honey; DMEM, Dulbecco's Modified Eagle Medium; DPPH, 2,4-DNP 2,2-diPhenyl-1-picrylhydrazyl; EH, Eucalyptus honey; FRAP, Ferric Reducing Antioxidant Power; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GAEq, gallic acid equivalents; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione transferase; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MRC, Maximal respiration capacity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; Nrf2, nuclear factor E2-related factor 2; OCR, oxygen consumption rate; RSN, reactive nitrogen species; RT-PCR, real time PCR; TE, Trolox equivalents; TEAC, Trolox Equivalent Antioxidant Capacity (TEAC); TFC, total flavonoids; TNF-α, tumor necrosis factor α; TPC, total content of polyphenols.

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factor-α (TNF-α). On the other hand, the main molecular pathway activated during inflammation is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, whose downstream targets are several genes responsible for the production of cytokines, growth factors, chemokines, etc. (Afrin et al., 2018a; 2018b; Gasparrini et al., 2018). Usually, the acute inflammatory response lasts for a few days, until its complete resolution and restoration of the tissue homeostasis, but, if uncontrolled, inflammation can become chronic, leading, potentially, to tissue damage and disease development. Oxidative stress can also contribute to inflammation. Indeed, oxidative stress and inflammation are strongly associated, with inflammation that constantly produces ROS and *vice versa*, exerting, in a synergistic way, a key role in the development and progression of many chronic diseases, including cancer, obesity and cardiovascular disorders.

A fruitful strategy to decrease inflammation and oxidative stress could be the adoption of a healthy diet: not surprisingly, in the last years, many studies have started to assess the anti-inflammatory effects of plant foods and dietary bioactive compounds in order to find successful alternatives to the use of synthetic drugs. Honey is a complex mixture of sugar, organic acid, proteins, essential minerals, pigments and phenolic compounds, whose quality and quantity are strongly associated with their floral source and geographical regions (Alvarez-Suarez et al., 2010, 2016; Amessis-Ouchemoukh et al., 2021; Battino et al., 2021). It exerts multiple biological activities, ranging from antioxidant and anti-inflammatory properties to anticancer and anti-microbial effects (Alvarez-Suarez et al., 2010, 2016). Chestnut (*Castanea sativa*) and Eucalyptus (*Eucalyptus occidentalis*) honeys are collected and commercialized worldwide and have been studied mainly for their chemical and nutritional composition as well as for their quality parameters, with little research focused on their potential beneficial effects (Kolayli et al., 2016; Karabagias et al., 2018; Bobis et al., 2020). Additionally, to date, no study has assessed *in vitro* their anti-inflammatory effects. Therefore, the aim of the present work was to investigate the effects of chestnut honey (CH) and eucalyptus honey (EH) in RAW 264.7 macrophages, stressed with the bacterial endotoxin lipopolysaccharide (LPS), on cell viability, apoptosis, proliferation, antioxidant enzyme activities and mitochondrial functionality, paying particular attention to the molecular mechanisms involved in inflammation and oxidative stress.

2. Materials and methods

2.1. Materials

o-Vanillin, m-Coumaric acid, Ferulic acid, Formononetin, Apigenin, Naringenin, Glycitein, Epicatechin, Quercetin, Ellagic acid, Rosmarinic acid, Cyanidin-O-glucoside, Kaempferol-3-O-glucoside, Quercetin-3-O-glucopyranoside, Rutin, methanol, acetic acid were purchased from Sigma-Aldrich SL (Madrid, Spain). Doubly distilled deionized water was obtained from a Milli-Q purification system from Millipore (Milford, MA, USA). All reagents were of analytical grade. All reagents, consumables and chemicals for cells were purchased from Corning (Milan, Italy). ROS kit and Tali Apoptosis assay™ were bought from Invitrogen, Life Technologies. The EvaGreen 2X qPCRMaster Mix kit and the primers for real time PCR (RT-PCR) were bought from Applied Biological Materials Inc. (Canada) and Sigma-Aldrich (Milan, Italy). All other reagents and chemicals were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Evaluation of honey samples

2.2.1. Melissopalynological analysis

Chestnut and Eucalyptus honeys were collected in the Calabria region in Italy from beekeepers of the "Calabrian Beekeepers Association" (APROCAL, Italy) in September 2019. The different areas from where the samples were collected were Catanzaro, Cosenza, Crotone, Vibo Valentia and Reggio Calabria (Fig. 1). In each area, three samples were taken randomly from each apiary and stored at 4 °C until analysis. The floral origin of the honeys was evaluated according to the protocol of Louveaux et al. (1978).

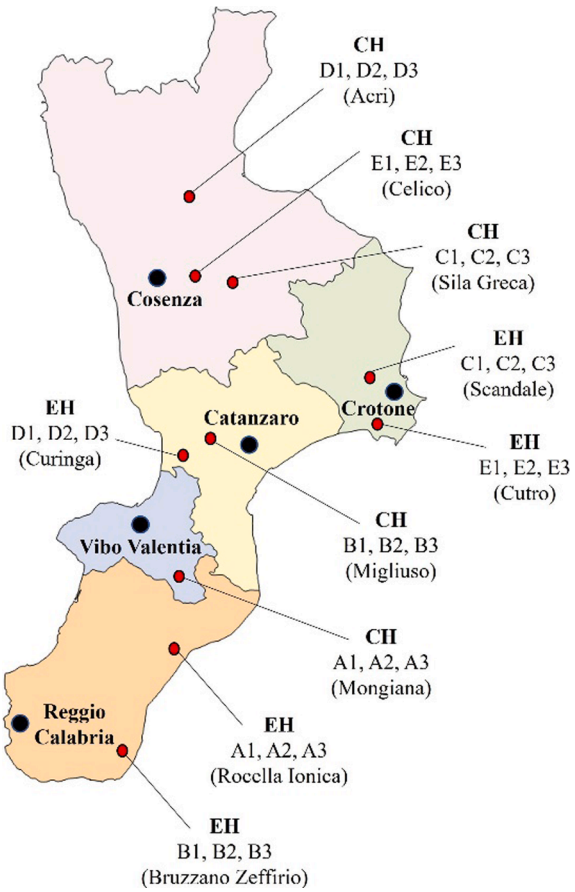

Fig. 1. Geographical origin of the different CH and EH samples collected in different areas of Calabria, in Italy.

2.2.2. Determination of total phenolics and flavonoids

The total content of polyphenols (TPC) was assessed according to the Folin-Ciocalteu method (Slinkard and Singleton, 1977), while the concentration of total flavonoids (TFC) was measured using the Aluminum chloride method (Dewanto et al., 2002). The results were expressed as mg of gallic acid equivalents (GAEq) per g of honey for TPC (mg GAEq/g) and as mg of (+)-catechin equivalents (CEq) per kg of honey (mg CEq mg/kg) for TFC, respectively.

2.2.3. Determination of total antioxidant capacity (TAC)

TAC of honey samples was measured by three different methods: Ferric Reducing Antioxidant Power (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), and 2,4-DNP 2,2-diPhenyl-1-picrylhydrazyl (DPPH) assays, as earlier reported by our group (Bompadre, Leone, Politi & Battino, 2004; Gasparrini et al., 2017, 2018). The results were expressed as μmoles of Trolox equivalents (TE) per 100 g of honey for TEAC and DPPH methods and as mmol TE/100 g of honey for FRAP assays.

2.2.4. UPLC-MS analysis

2.2.4.1. Identification of individual phenolic compounds. CH and EH MH were concentrated with a Sep-Pak C18 Plus Short SPE Cartridge (Waters S.p.A., Milan, Italy) as previously reported (Truchado, Ferreres, Bortolotti, Sabatini, & Tomás-Barberán, 2008). An UPLC-QTOF-MS/MS method was used to identify the phenolic compounds. The UPLC and

mass spectrometer conditions were the same as previously used by (Esteban-Muñoz et al., 2020). The experiments were performed on a UPLC with mass spectrometer high resolution SYNAPT G2 HDMS Q-TOF model (Waters, Mildford, USA). Conditions consisted of a full MS, and data-dependent scanning was performed in negative and positive mode with electrospray ionization (ESI). The UPLC separation was performed using an ACQUITY UPLC ™ HSS T3 2.1 × 100 mm, 1.8 mm column. The program for chromatography was set with a binary gradient consisting of (A) water with 0.5% acetic acid and (B) acetonitrile, as follows: Initial 0.0 min, 5% (B), 15.0 min, 95% (B); 15.1 min, 5% (B); and 18.0 min, 5% (B) and the flow rate was 0.4 mL/min.

The phenolic compounds were identified by comparing the molecular ions and fragments obtained with previous investigations carried out using MassLynx V4 software (Waters Laboratory Informatics, Mildford, USA).

2.2.4.2. Quantification of individual phenolic compounds. UPLC analysis was performed using a Waters ACQUITY I CLASS model chromatograph instrument (Waters, Mississauga, ON, Canada) equipped with a mass spectrometer Waters XEVO TQ-XS, with ionization performed by UniSpray (US). The same method described in (Sánchez-Hernández et al., 2021) was used. A gradient of solvent A (water) and solvent B (methanol with 0.1% [v/v] acetic acid) was used for 25 min at a flow rate of 0.4 mL/min. The gradient was programmed as follows: at 0 min 5% B, 15–15.10 min 95% B and 15.10–18 min 5% B. An Acquity UPLC HSS T3 1.8 μm column was utilised. Quantification of the phenolic compounds was accomplished by comparing the retention times of peaks and fragmentation data in samples, with those of the phenolic compound standards and measurements obtained using Multiple Reaction Monitoring.

2.3. Cell culture and analysis

2.3.1. Cell treatments

Murine RAW 264.7 macrophages were acquired from the American Type Culture Collection (ATCC-TIB71) and cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, glutamine and sodium pyruvate, supplemented with 1% penicillin–streptomycin antibiotics and with 10% heat-inactivated fetal bovine serum in Hera Cell CO_2 incubator (Thermo scientific, Milan, Italy) at 37 °C with 5% CO_2. Cells were used between the 4th and 10th passages.

Cells were treated with a with range of each honey (0–10 mg/mL) for 24 h, both in absence and in presence of LPS from *E.coli* at a concentration of 1 μg/mL for 24 h, according to our previous cytotoxicity studies (Gasparrini et al., 2018).

2.3.2. Cell viability

Cell viability was measured through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, after cell treatment, 30 μL of RPMI medium with 2 mg/mL of MTT salt were added, cells were incubated for 4 h and 100 μL of dimethyl sulfoxide were added to each well to dissolve the formazan crystals. Absorbance was read at 590 nm in a microplate reader (Thermo Scientific Multiskan EX, Monza, Italy) and the percentage of viable cells was measured compared to untreated cells.

2.3.3. Determination of apoptosis

Apoptosis rate was assessed by the Tali™ Apoptosis Assay Kit–Annexin V Alexa Fluor® 488 (Invitrogen™, Life Techonoliges, Milan, Italy) as explained in our previous publication (Afrin et al., 2018a). 1.5×10^5 cells/well were seeded into a 6 wells plate and treated according to the MTT results with (i) DMEM (ctrl group), (ii) CH at 1 mg/mL for 24 h, (iii) EH at 1 mg/mL for 24 h, (iv) LPS at 1 μg/mL for 24 h, (v) CH at 1 mg/mL for 24 h and then with LPS at 1 μg/mL for 24 h, and (vi) EH at 1 mg/mL for 24 h and then with LPS at 1 μg/mL for 24 h. After centrifugation at 1500 rpm at 4 °C for 15 min, cells were treated with 100 μL of Annexin binding buffer and 5 μL of Annexin V Alexa Fluor® 488. After 20 min of incubation at room temperature and after cell centrifugation, 1 μL of Tali™ propidium iodide was added. The samples were examined by the Tali™ Image-Based Cytometer (Invitrogen™, Life Techonoliges, Milan, Italy), reporting the values of live, apoptotic and dead cells. Results were expressed as the fold change compared to control.

2.3.4. Wound healing

Wounding assay was performed following the protocol of Alvarez-Suarez et al. (2016). A linear wound was produced in confluent cells by scratching the bottom of the plate with a 200 μL sterile pipette tip. Macrophages were washed twice with PBS and then treated as previously described, with media containing 2% serum. The photographs of wounds were taken through a light microscope LeitzFluovert FU (Leica Microsystems) at zero time and after the different periods of incubation. Cells were then fixed with methanol and stained with 0.2% methylene blue stain; the wound areas were measured with the NIH Image J software and results were expressed as the percentage of wound closure after each treatment.

2.3.5. Determination of intracellular levels of ROS and nitrite

Intracellular ROS generation was determined through the CellROX® Oxidative Stress kit (Invitrogen™, Life Techonoliges, Milan, Italy) with a Tali® Image-Based cytometer (Invitrogen™, Life Technologies, Milan, Italy), as explained in detail in our previous work (Gasparrini et al., 2018). Results were expressed as the fold change compared to control.

Nitrite levels were assessed by the Griess method (Pekarova et al., 2009). After cell seeding in a T75 flask at a density of 1×10^6 and treated for 24 h with different treatments as previously specified, cell supernatants were added with an equal volume of Griess reagent for 10 min in the dark at room temperature. Absorbance was measured at 540 nm on a microplate reader (Thermo Scientific Multiskan EX). Each experiment was carried out in three replicates and the results were expressed as μM of nitrite.

2.3.6. Evaluation of GSH levels and antioxidant enzyme activities

Cells were treated as previously described and cellular lysates were obtained with RIPA buffer (Sigma-Aldrich, Milan, Italy) for the determination of glutathione (GSH) and the activities of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione transferase (GST), as explained earlier (Gasparrini et al., 2017, 2018). The results of GSH were expressed as mM, the results of GPx, GST and GR were expressed as mM/min.

2.3.7. Determination of mitochondrial respiration

The XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica MA, USA) was used to measure in real-time the oxygen consumption rate (OCR) in cells incubated with the different treatments (Afrin et al., 2018a). Briefly, after seeding 3×10^4 cells for 16 h in the XF-24 plate and after treating them as previously indicated, the medium was replaced with 450 μL/well of XF-24 running media (Seahorse Bioscience, Billerica MA, USA), without serum and the plate was pre-incubated for 20 min at 37 °C in the XF Prep Station incubator (Seahorse Bioscience, Billerica MA, USA) in the absence of CO_2. Then, after an OCR baseline measurement, three injections of four compounds that affect bioenergetics were made: 55 μL of oligomycin (1 μg/mL), 61 μL of 2,4-Dinitrophenol (2,4 DNP) (1 mM), and 68 μL of antimycin A/rotenone (10 μM/1 μM) at injection in port C. Each treatment was performed in three replicates and the results were expressed as pmol of O_2 consumed per 10^5 cells per minute (pmol O_2/10^5 cells/min).

Moreover, basal OCR, maximal respiration capacity (MRC), ATP production (ATP), were calculated as previously described (Afrin et al., 2018b).

2.3.8. Real-time PCR analysis

After RAW 264.7 macrophages treatment, the total RNA content of the cells was determined with a PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), and its purity and concentration were measured by a microplate spectrophotometer system (BioTek Synergy HT, Winooski, USA). In-One RT MasterMix kit (Applied Biological Materials Inc. Canada) was used for synthesizing cDNA (Applied Biological Materials Inc. Canada) from 75 ng RNA and RT-PCR amplification (Corbett Life Science, Rotor-Gene 3000, Mortlake, Australia) was performed with EvaGreen 2X qPCRMasterMix (EvaGreen 2XqPCRMasterMix kit, Applied Biological Materials Inc. Canada) of forward and reverse primers (**Table S1**). For the quantitative normalization, glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used and the results were calculated by the $2^{-\Delta\Delta ct}$ method. The final results were expressed as the fold change compared to control.

2.4. Statistical analysis

Results were expressed as the mean values ± standard deviation of three independent repetitions. The statistical analysis was done with STATISTICA software (Statsoft Inc., Tulsa, OK, USA) and one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) post hoc test ($p < 0.05$) were used for the significant differences, symbolized by letters.

3. Results and discussion

3.1. Phytochemical composition and total antioxidant capacity of honey

To assess the phytochemical composition of CH and EH samples, TPC and TFC were measured. As shown in Table 1, significant differences ($p < 0.05$) were observed among the different samples for both TPC and TFC.Regarding TPC, for CH, the honeys with the highest values were E3, A1 and E1, while for EH the highest values were found in D2, D1 and D3. Concerning TFC, E2 was the CH sample with the highest contents of flavonoids, followed by E3 and E1 samples, while for EH the highest values were obtained in A2, A1 and D2 samples (Table 1).

The results obtained in the present study are comparable to those found for CH and EH collected in Portugal (Karabagias et al., 2018) and in Spain (Escuredo, Rodríguez-Flores, Rojo-Martínez & Seijo, 2019), and for EH collected in Algeria (Homrani et al., 2020), in Ecuador (García-Tenesaca et al., 2017) and in Italy (Di Petrillo et al., 2017). Similar results were also found for other honeys with different botanical origins, including those collected in Cuba (Alvarez-Suarez et al., 2010), Algeria (Khalil et al., 2012) and in New Zealand (Afrin et al., 2017); moreover, the values reported in our study were higher compared to those obtained for cardoon, carob, citrus, lavender, pennyroyal, sunflower and thyme honeys (Aazza, Lyoussi, Antunes, & Miguel, 2013).

The TAC of CH and EH was quantified with three different methods (Table 1): CH samples had lower TEAC, FRAP and DPPH activities compared to EH samples. In detail, the CH sample with the highest TAC was E3, while in EH the highest values of TEAC and FRAP were found in A3 and in A2 samples.

Our data were in accordance with the values obtained for CH by Kolayli et.al. (2016), by León-Ruiz et al. (2013), by Sahin (2016) and by Can et al. (2015), while for EH similar values were reported by Rodriguez et al. (Rodríguez, Mendoza, Iturriga & Castaño-Tostado, 2012) and García-Tenesaca et al. (2017). Overall, our CH and EH samples had similar TAC than other types of honey, including cactus, orange, bell, heater, pine and caujiniquil flower honeys (Rodríguez, Mendoza, Iturriga & Castaño-Tostado, 2012; Can et al., 2015), and higher than those reported for acacia, astragalus, clover, lavender, lime and rhododendron honeys (Can et al., 2015).

On the basis of these results, we identified two samples with the optimum combination of phenolic contents and TAC for cell treatments: E3 for chestnut honey and A3 for eucalyptus honey.

Table 1
Total polyphenol and flavonoid content and total antioxidant capacity of Chestnut and Eucalyptus honey.

Samples	TPC mg GAEq/g	TFC mg CEq /kg	TAC		
			TEAC μmol TE/100 g	FRAP mmol TE/100 g	DPPH μmol TE/100 g
Chestnut honey					
A1	0.73 ± 0.01 a	24.41 ± 3.75 abcd	83.21 ± 8.68 cde	0.24 ± 0.00 bc	59.51 ± 4.82 bcde
A2	0.61 ± 0.02 cde	24.02 ± 0.88 bcd	85.09 ± 8.94 cde	0.25 ± 0.00b	69.17 ± 8.05 abc
A3	0.59 ± 0.01 defg	26.14 ± 2.18 abcd	94.57 ± 4.76 abc	0.23 ± 0.00 cd	23.16 ± 9.99f
B1	0.58 ± 0.01 efg	22.87 ± 0.66 bcd	45.36 ± 2.03 g	0.25 ± 0.00b	60.65 ± 15.46 bc
B2	0.56 ± 0.00 ghi	24.60 ± 1.20 abcd	55.36 ± 1.535 fg	0.24 ± 0.00 bc	61.22 ± 7.43 cd
B3	0.57 ± 0.00 fgh	22.48 ± 0.57 cd	54.21 ± 0.93 fg	0.25 ± 0.00b	81.10 ± 13.23 ab
C1	0.61 ± 0.00 cde	21.91 ± 1.15 d	58.12 ± 7.28f	0.22 ± 0.00 d	72.58 ± 14.89 ab
C2	0.60 ± 0.00 def	23.25 ± 1.85 bcd	80.96 ± 0.48 de	0.23 ± 0.00 d	43.61 ± 3.93 cdef
C3	0.62 ± 0.01 bc	23.06 ± 1.73 bcd	74.31 ± 1.30 e	0.23 ± 0.00 d	31.68 ± 14.19 def
D1	0.54 ± 0.0 hi	22.48 ± 2.51 cd	85.50 ± 2.84 cde	0.21 ± 0.00 e	19.19 ± 4.29f
D2	0.53 ± 0.01 i	21.33 ± 1.52 d	94.68 ± 1.12 abc	0.20 ± 0.00 e	29.41 ± 3.55 ef
D3	0.52 ± 0.00 i	20.66 ± 1.01 d	89.06 ± 0.56 bcd	0.20 ± 0.01 e	16.35 ± 6.45f
E1	0.66 ± 0.01b	27.87 ± 2.60 abc	98.61 ± 0.64 ab	0.25 ± 0.00b	53.83 ± 6.89 bcde
E2	0.65 ± 0.02 bc	30.17 ± 3.28 a	83.78 ± 1.24 cde	0.23 ± 0.01 cd	60.08 ± 11.09 bcd
E3	0.73 ± 0.03 a	28.64 ± 0.33 ab	105.46 ± 1.21 a	0.27 ± 0.00 a	94.16 ± 2.60 a
Eucalyptus honey					
A1	0.50 ± 0.00c	30.37 ± 0.88 a	105.86 ± 0.84 a	0.32 ± 0.00b	157.78 ± 6.45 bc
A2	0.52 ± 0.01b	30.56 ± 0.10 a	102.88 ± 1.08b	0.31 ± 0.01b	208.90 ± 11.35 a
A3	0.53 ± 0.01b	27.48 ± 0.33 abc	106.49 ± 0.54 a	0.34 ± 0.00 a	182.77 ± 8.41 ab
B1	0.49 ± 0.01c	23.06 ± 0.58 cd	89.48 ± 0.69c	0.27 ± 0.01 cd	95.27 ± 10.37 efg
B2	0.45 ± 0.00 e	25.56 ± 1.45 bcd	87.91 ± 0.76 cd	0.26 ± 0.01 def	102.12 ± 3.41 efg
B3	0.45 ± 0.01 e	24.98 ± 2.02 bde	87.16 ± 0.74 cd	0.27 ± 0.01 d	85.64 ± 1.97 fg
C1	0.43 ± 0.01f	22.48 ± 1.53 d	76.41 ± 0.43 h	0.25 ± 0.01 ef	105.52 ± 10.36 efg
C2	0.46 ± 0.01 e	23.05 ± 2.31 cd	83.60 ± 0.32f	0.24 ± 0.01f	193.57 ± 31.99 a
C3	0.40 ± 0.00 g	24.21 ± 1.15 bcd	79.79 ± 0.75 g	0.26 ± 0.01 def	119.72 ± 7.68 def
D1	0.57 ± 0.00 a	26.71 ± 1.20 abcd	68.93 ± 1.62 i	0.26 ± 0.00 def	104.39 ± 12.09 efg
D2	0.58 ± 0.01 a	28.64 ± 1.85 ab	47.75 ± 0.88 j	0.26 ± 0.01 de	94.73 ± 9.61 efg
D3	0.57 ± 0.01 a	27.29 ± 2.85 abc	77.96 ± 0.53 gh	0.25 ± 0.01 ef	83.37 ± 7.81 g
E1	0.46 ± 0.00 e	24.02 ± 2.18 bcd	87.58 ± 0.31 cd	0.26 ± 0.01 de	125.40 ± 10.96 cde
E2	0.48 ± 0.00 d	25.55 ± 1.33 bcd	86.53 ± 0.41 de	0.29 ± 0.01c	115.18 ± 5.48 defg
E3	0.47 ± 0.01 d	23.44 ± 0.66 cd	84.45 ± 0.61 ef	0.28 ± 0.01 cd	143.01 ± 6.14 cd

CEq: (+)-catechin equivalents; DPPH: Diphenyl-1-picrylhydrazyl assay; FRAP: ferric reducing antioxidant power assay; GAEq: gallic acid equivalents; TE: Trolox equivalents; TEAC: Trolox equivalent antioxidant capacity assay; TFC: total flavonoid content; TPC: total polyphenol content. Data are presented as mean ± SD of three independent experiments. Different superscripts letter for each column indicated significant differences ($p < 0.05$).

3.2. Identification and quantification of individual phenolic compounds in chestnut and eucalyptus honey

The phenolic compounds identified and quantified by UPLC-MS analysis in sample E3 of CH and in sample A3 of EH are shown in Table 2. Two main families of polyphenols were found in both honeys: flavonoids, such as apigenin, naringenin, epicatechin, quercetin, cyanidin 3-O-glucoside, kaempferol-3-O-glucoside, quercetin-3-o-glucopyranoside and rutin, and phenolic acids, such as m-coumaric acid, ferulic acid and ellagic acid (Table 2); interestingly, in both honeys, the phenylpropanoid *ortho*-vanillin was also detected, a compound that usually confers flavor and aroma to malt and to buckwheat honey (Zhou, Wintersteen & Cadwallader, 2002).

Among flavonoids, naringerin and quercetin were the main components for CH followed by quercetin-3-O-glucopyranoside and cyanidin-3-O-glucoside (0.3%), while naringerin and cyanidin 3-O-glucoside were the main compounds in EH, followed by apigenin and quercetin. Regarding phenolic acids, ellagic acid and ferulic acid were the most representative in both CH and EH samples, followed by m-coumaric acid. Other studies reported similar phenolic profiles for chestnut honey collected in Spain and Turkey (Can et al., 2015; Combarros-Fuertes et al., 2019) and for eucalyptus honey collected in Australia, Brazil, India, Lithuania and Spain (Bobis et al., 2020).

3.3. CH and EH modulated apoptosis and promoted migration ability in LPS-stimulated macrophages

Since the use of synthetic anti-inflammatory drugs is usually correlated to several side effects, in the last years attention has been paid towards natural compounds, including honey, which could be an effective healthy alternative. First of all, we investigated the possible cytotoxic effects of CH and EH on murine RAW 264.7 macrophages, by treating cells with a wide range of honey concentrations (0–10 mg/mL) for 24 h using the MTT assay. After 24 h of treatment, both CH and EH

Table 2
Retention time, MS spectra (MS, [M - H]-; MS^2 [M - H]-) and MRM parameters obtained from the identification of the main phenolic acids, flavonoids and phenylpropanoids found in Chestnut and Eucalyptus honeys.

Proposed Compound	Molecular Formula	R_t (min)	Ms [M−H]$^-$ *m/z*	Cone voltage (V)	MS^2 [M−H]$^-$ *m/z*	Collision Energy (eV)	Chestnut honey (ng/mL)	Eucalyptus honey (ng/mL)
Flavonoids								
Apigenin	$C_{15}H_{10}O_5$	7.27	269.08	22	116.55	32	–	24.5 ± 2.0
					150.56	24		
					106.41	28		
					208.72	12		
Cyanidin 3-O-glucoside	$C_{21}H_{21}O_{11}^+$	5.04	447.10	58	284.70	20	0.8 ± 0.1	144.1 ± 4.8
					255.14	38		
					146.67	34		
					210.89	52		
Epicatechin	$C_{15}H_{14}O_6$	3.61	289.11	24	202.78	20	0.3 ± 0.1	–
					108.44	22		
					122.51	28		
					202.07	14		
Kaempferol-3-O-glucoside	$C_{21}H_{19}O_{11}$	5.26	447.21	2	284.09	24	–	1.7 ± 0.1
					254.88	36		
					226.82	40		
					182.50	64		
Naringenin	$C_{15}H_{12}O_5$	7.50	271.10	6	150.63	18	190.1 ± 2.5	387.3 ± 14.2
					118.52	26		
					106.47	22		
					176.65	16		
Quercetin	$C_{15}H_{10}O_7$	6.51	301.07	8	150.57	18	40.0 ± 0.7	24.9 ± 3.4
					178.67	18		
					106.47	28		
					120.55	22		
Quercetin-3-O-glucopyranoside	$C_{21}H_{20}O_{12}$	4.50	463.19	2	300.10	26	3.2 ± 0.3	1.1 ± 0.1
					270.89	40		
					254.84	40		
					242.84	46		
Rutin	$C_{27}H_{30}O_{16}$	4.33	609.31	2	300.12	38	–	0.1 ± 0.1
					270.92	54		
					254.87	62		
					242.87	58		
Phenolic acids								
m-Coumaric acid	$C_9H_8O_3$	4.55	162.95	2	90.45	20	10.0 ± 0.6	40.3 ± 0.4
					61.23	8		
					74.28	14		
					90.33	18		
Ellagic acid	$C_{14}H_6O_8$	4.58	301.10	12	144.59	36	25.0 ± 0.5	278.8 ± 4.7
					228.86	26		
					184.79	26		
					206.80	14		
Ferulic acid	$C_{10}H_{10}O_4$	5.02	192.96	2	133.51	16	15.9 ± 0.7	73.9 ± 6.9
					177.64	12		
					58.28	14		
					116.43	8		
Phenylpropanoids								
o-Vanillin	$C_8H_8O_3$	5.82	150.95	48	135.47	12	18.6 ± 1.5	401.4 ± 15.3
					58.28	12		
					91.42	16		

Values are expressed as mean ± standard deviation (SD). Each sample was analyzed in triplicate. Rt: retention time of samples.

did not affect viability at concentrations lower than 1 mg/mL, while higher concentrations decreased cell viability in a dose-dependent manner (**Supplementary Fig. S1A, S1B**). Similar results were found when macrophages were pretreated for 24 h with CH or EH and then stressed with 1 μg/mL of LPS for 24 h (**Supplementary Fig. S1C, S1D**). Therefore, we selected, for all subsequent analyses, the concentration of 1 mg/mL for each honey, that corresponds to 94.2% and 92.3% of viability for CH and EH, respectively.

Several studies have observed that LPS is able to induce apoptosis in different cell types (Lv et al., 2017; Cheng et al., 2020), including macrophages, where its deleterious effects are usually associated with different processes, especially the secretion of pro-inflammatory factors, such as nitric oxide (NO) (George, Ramasamy, Sirajudeen & Manickam, 2019) and TNF-α; these factors, once produced, are able to activate, in turn, different pathways involved in the early and late apoptotic process (Xaus et al., 2000). In addition, in different inflammatory conditions, apoptosis activated in macrophages can lead to some serious consequences, such as the development of the atherosclerotic plaque, that represents the initial phase of atherosclerosis (Seimon & Tabas, 2009). It is now confirmed that several natural compounds are able to inhibit this process of programmed cell death; for this reason, we assessed the effect of CH and EH in LPS-stimulated macrophages using the Tali image cytometer. As shown in Fig. 2A, the stimulation of cells with LPS decreased cell viability by 2.79 times compared to control cells.

On the contrary, after treatment with LPS the number of apoptotic cells increased significantly ($p < 0.05$), specifically 2.06 times compared to the control. Pretreatment with CH and EH protected RAW 264.7 macrophages from the apoptotic process: the use of CH lowered the percentage of apoptotic cells by 1.27 times ($p < 0.05$), while EH decreased the percentage of apoptotic cells by 1.94 times ($p < 0.05$), compared to cells stressed only with LPS. The results obtained in our study are in line with others published in the literature, that highlighted the ability of LPS to increase the rate of apoptosis in macrophages and specifically in the RAW 2647.7 cell line (George, Ramasamy, Sirajudeen & Manickam, 2019; Seminara, Ruvolo & Murad, 2007). In parallel, it has also been observed that several food matrices or isolated dietary compounds were able to lower the number of cells in apoptosis, such as Manuka honey, which decreased not only apoptosis in a concentration-dependent manner but also cell mortality (Afrin et al., 2018b) and, among others, the same effect was found with the radix of *Achyranthis bidentatae* (Wang et al., 2020), with polysaccharides isolated from Echinacea (Zhang et al., 2020) and with koumine (Yuan et al., 2016).

Macrophages are involved in the process of wound repair, where they contribute also to the attenuation of the associated inflammation, characterized by an excessive accumulation of ROS that are responsible for a lower rate of wound repair (Demyanenko et al., 2017; Hesketh, Sahin, West & Murray, 2017). The ability to promote a rapid and better healing of wounds with a cicatrizing effect, together with the antibacterial and anti-inflammatory properties, and the modulation of cytokine production and cell proliferation, is one of the most studied among the multiple biological properties of honey (Alvarez-Suarez et al., 2016). These different activities that honey performs on wounds are due to different components present in this matrix, such as the presence of phenolic compounds, vitamins and hydrogen peroxide content and to its chemical-physical characteristics, such as low water activity and its high acidity (Cianciosi et al., 2020). For this reason in this study, the effect of pretreatment with the two types of honey on wound closure capacity in LPS-stimulated macrophages was investigated (Fig. 2B). The treatment of macrophages with LPS had a statistically significant effect ($p < 0.05$) on wound closure capacity compared to unstressed macrophages: indeed, closure in the control cells was 50.98% compared to time 0, but when macrophages were stimulated with LPS closure was only 19.81%. Pretreatment of cells with the two honeys led to a statistically similar value ($p < 0.05$) of control, with a closure of 44.51% when pretreated with CH and of 48.76% with EH. It can also be noted that the treatment of unstressed macrophages with the two types of honey stimulated cell proliferation, obtaining a wound closure rate of 63.63% with CH and 74.84% with EH.

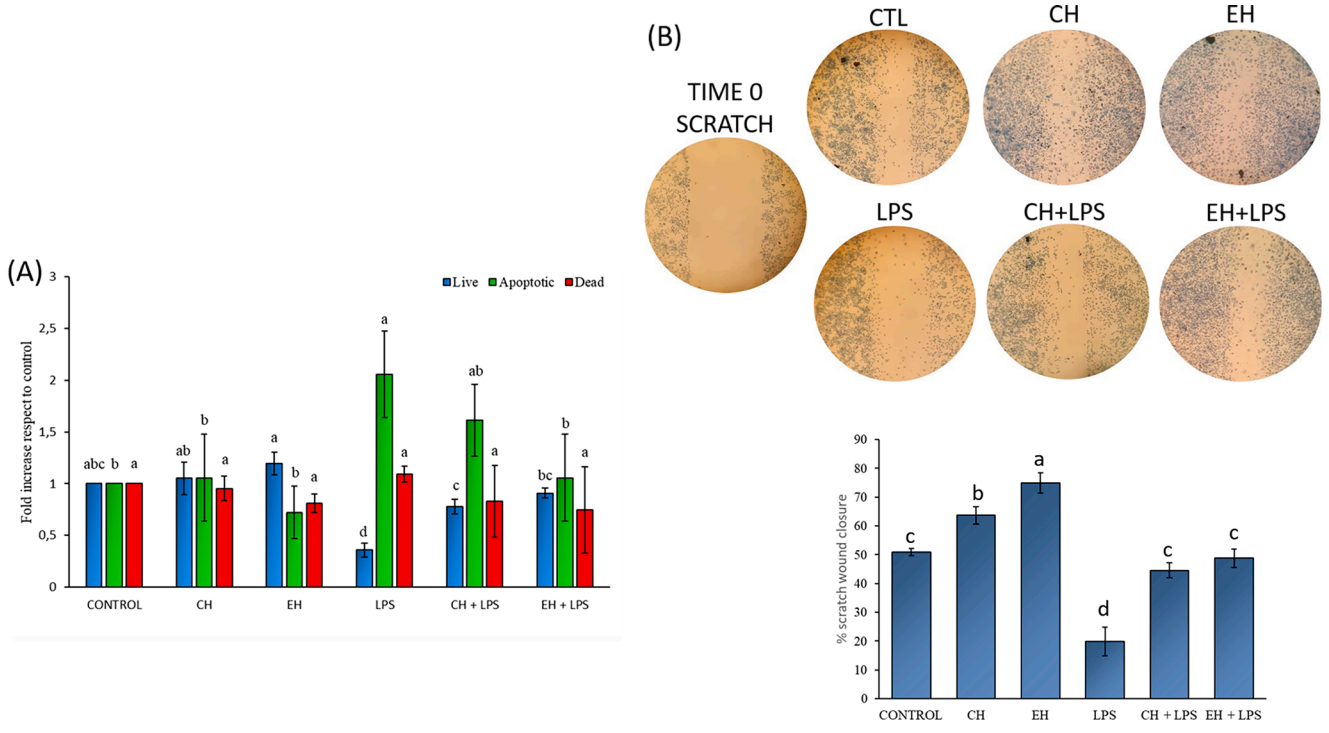

Fig. 2. Effects of CH and EH on RAW 264.7 macrophage apoptosis and migration ability. RAW 264.7 macrophages were treated with CH and EH for 24 h, with LPS for 24 h or pretreated with CH or EH for 24 h and then stressed with LPS for 24 h. (A) Histogram representing live, apoptotic and dead cells (fold increase compared to the control). (B) The percentages of wound closure were analyzed by ImageJ comparing with the time 0 scratch (in the image a representative one). Values are expressed as mean ± SD (n = 3). Different letters in the same column indicate significant differences ($p < 0.05$).

Our results agree with those that demonstrated, in the same cell line, a marked wound closure activity by promoting cell migration and wound repair, as was found for honeys with different botanical origins, such as the Manuka one (Afrin et al., 2018) or for other natural extracts, such as the polyphenolic extracts from strawberry and blackberry fruits (Van de Velde et al., 2019) and grape seed extracts (Carullo et al., 2020).

3.4. CH and EH treatments decreased the levels of ROS and nitrite in LPS-stimulated cells

Macrophages release ROS in response to phagocytosis events or following the stimulation by various exogenous or endogenous agents (Forman & Torres, 2001). Since macrophages are directly involved in the inflammatory process, being both secretors of pro-inflammatory molecules (Shapouri-Moghaddam et al., 2018) and responsible for the phagocytosis of necrotic cells during the resolution of inflammation (Westman, Grinstein & Marques, 2020), investigating the role that ROS have in influencing these responses could help to improve the anti-inflammatory processes. In the present study, the effect on the production of ROS at the intracellular level of CH and EH on RAW 264.7 macrophages was analyzed (Fig. 3A).

The stimulation of macrophages with LPS increased ROS levels by 1.43 ± 0.23 times compared to control cells. The treatment of macrophages with CH did not lead to statistically significant changes ($p < 0.05$) compared to the control, while EH lowered the intracellular levels of ROS by 1.21 ± 0.23 fold ($p < 0.05$). Interestingly, the pretreatment of cells with the two different types of honey led to a lower intracellular accumulation of ROS after the stressful stimulus with LPS: a decrease in ROS levels of 1.17 times was noted with pretreatment with CH and of 1.44 times with EH followed by the LPS treatment, compared to cells subjected to LPS alone.

Similar results were found with a pretreatment with Manuka honey, that decreased intracellular ROS accumulation in a dose-dependent manner in LPS-stressed cells (Gasparrini et al., 2018). Similarly, in LPS-stimulated RAW 264.7 macrophages, other natural compounds were able to lower the ROS levels, i.e. an extract of *Terminalia bellirica* (Gaertn.) Roxb, (Tanaka et al., 2018), Andean berry (*Vaccinium floribundum*) juice (Arango-Varela, Luzardo-Ocampo, Maldonado-Celis & Campos-Vega, 2020) and anthocyanins of *Trifolium pratense* (Red Clover) (Lee, Brownmiller, Lee & Kang, 2020). Even single compounds, such as 8,8′-Bieckol, a fluorotannin isolated from edible brown algae, exerted the same effect even if a dose-dependent effect was not observed (Yang, Jung, Lee & Choi, 2014).

NO is a signal molecule, produced by a family of nitric oxide synthases (NOS), and is involved in different biological processes, including inflammation, neurotransmission, immune response, cell proliferation, cell cycle, apoptosis and senescence; its effects strictly depend on its concentration and on diverse conditions, including the presence of other radicals (Napoli et al., 2013). During the inflammatory processes, besides ROS, macrophages produce and release also massive amounts of NO, thanks to the activity of inducible NOS (iNOS), as a defense mechanism against invading pathogens or tissue damage: for example, it contributes to neutralizing invading microorganisms, including various viruses (Xue, Yan, Zhang, & Xiong, 2018). However, NO has a dual role in immune-inflammation, since its excessive production has been correlated with a chronic status of inflammation in some inflammatory diseases, including, for example, rheumatoid arthritis, ulcerative colitis and asthma (Sharma, Al-Omran, & Parvathy, 2007; Xue, Yan, Zhang, & Xiong, 2018); consequently, the decrease of NO production can be a target for the development of new anti-inflammatory agents. To examine the effect of CH and EH on NO production, we measured the levels of nitrites, by using the Griess reagent. As shown in Fig. 3B, cells treated exclusively with LPS had a significant ($p < 0.05$) increase of nitrite levels, compared to control cells, while pre-treatment with CH or EH, before the stress with LPS, restored values similar to those of the control group; interestingly, macrophages treated with CH or EH alone showed the lowest values, highlighting the ability of these honeys in decreasing nitrite accumulation.

Similar results were found with other types of honey, that were able to counteract, in murine RAW 264.7 macrophages stressed with LPS, the levels of nitrite, including Manuka honey (Alvarez-Suarez et al., 2016), Gelam honey (Kassim et al., 2012), safflower honey (Sun et al., 2020)

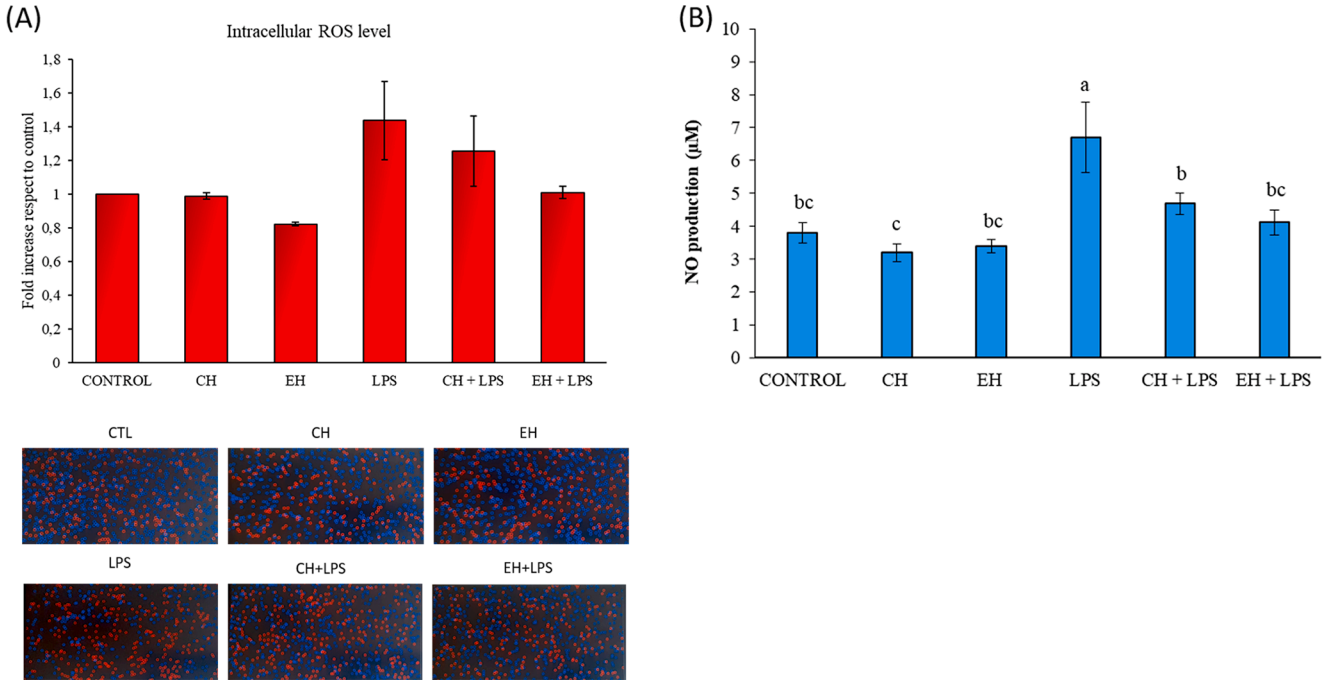

Fig. 3. Effects of CH and EH on intracellular ROS and nitrite levels. RAW 264.7 macrophages were treated with CH and EH for 24 h, with LPS for 24 h or pre-treated with CH or EH for 24 h and then stressed with LPS for 24 h. (A) Histogram representing intracellular ROS (fold increase compared to the control). Representative images of intracellular ROS quantification are shown (stressed cells appear red, unstressed cells appear blue). (B) Nitrite levels were determined by using the Griess method. Values are expressed as mean ± SD (n = 3). Different letters in the same column indicate significant differences ($p < 0.05$).

and *Mimosa scabrella* Bentham honeydew honey (Silva et al., 2020). Likewise, *Acalypha australis L.* (Kim et al., 2020a), *Echinacea* (Zhai et al., 2009) and *Heracleum moellendorffii* roots (Kim et al., 2019) extracts, as well as single compounds, including chrysoeriol (Wu et al., 2020), ellagic acid, gallic acid and punicalagin isolated from *Punica granatum* (BenSaad et al., 2017) and osthole (Fan et al., 2019) were able to decrease the production of NO in LPS-stimulated macrophages, confirming the anti-inflammatory capability of different natural compounds by modulating the intracellular levels of ROS and NO.

3.5. CH and EH modulated the activities of key biomarkers of oxidative stress in LPS-stimulated macrophages

The adequate production of ROS and NO is essential not only for a correct inflammatory response (Mittal et al., 2014), but also for maintaining the cells in a healthy state: it is widely known, that the excessive production of ROS and NO are, directly or indirectly, responsible for different cellular damage due to the oxidation of macromolecules (Bergamini, Gambetti, Dondi & Cervellati, 2004) and that their accumulation at intracellular level is one of the main contributing causes of the onset of different pathologies, including those correlated with a chronic state of inflammation (Mittal et al., 2014). GSH is one of the most important indicators of oxidative/nitrative stress in cells, acting directly or as a cofactor: on the one hand, it can react directly with ROS or reactive nitrogen species (RSN), decreasing the cellular oxidative and nitrative damage, on the other hand it is the coenzyme of different enzymes, including GST, GPx and GR (Pompella et al., 2003). Therefore, to further evaluate the efficacy of CH and EH in protecting against

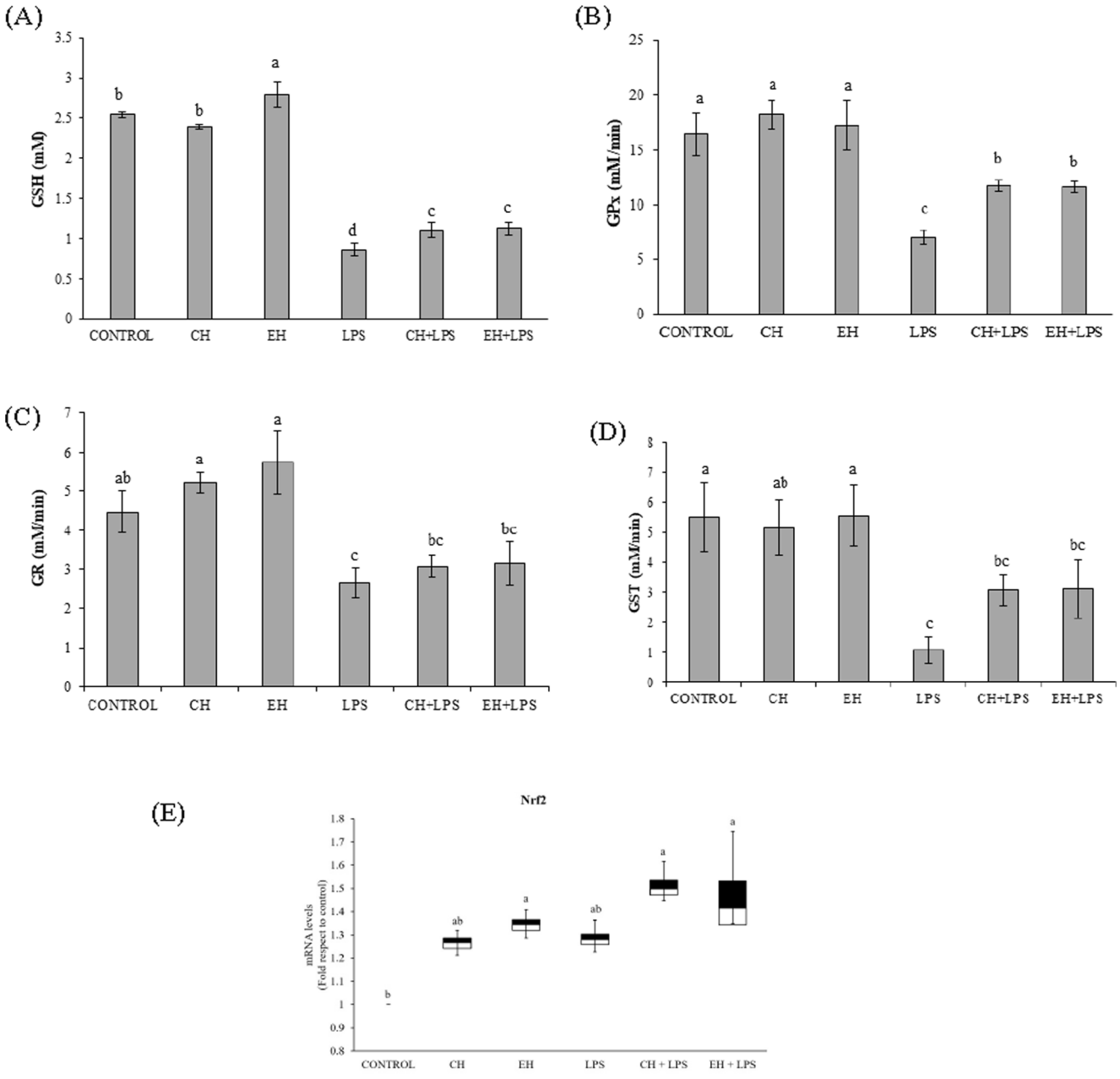

Fig. 4. Effects of CH and EH on GSH, antioxidant enzyme activities and on mRNA levels of Nrf2. RAW 264.7 macrophages were treated with CH and EH for 24 h, with LPS for 24 h or pre-treated with CH or EH for 24 h and then stressed with LPS for 24 h. (A-D) Results are expressed as mM/min, (E) The mRNA expression of Nrf2 was analyzed by RT-PCR. GADPH was utilized as a loading control. Results are expressed as a fold change in comparison with control (without treatment). Values are expressed as mean ± SD (n = 3). Different letters in the same column indicate significant differences ($p < 0.05$).

oxidative damage, we assessed the levels of GSH and the activity of GSH-related enzymes in cells treated as previously described. Similar or even increased GSH levels were observed after CH and EH treatment in murine RAW 264.7 macrophages, while the lowest ($p < 0.05$) levels were found in LPS-treated cells, compared to the control group (Fig. 4A). Interestingly, pre-treatment with CH or EH protected the LPS-induced stressed cells by increasing the levels of GSH, compared to LPS-treated cells (Fig. 4A).

Our results are consistent with previous data that highlighted increased levels of GSH after treatment with other types of honeys, such as Manuka (Gasparrini et al., 2018), with broccoli (Ke, Shyu & Wu, 2021) and eucalyptus leaf (Li et al., 2020) extracts, or with isolated compounds, including bromosulfophthalein (Cui et al., 2020) or sulfated polysaccharide isolated from *Turbinaria ornata* (Bhardwaj et al., 2020) in stressed RAW 264.7 macrophages.

In the last years, many studies found that natural compounds are able to protect stressed cells from oxidative damage, not only by neutralizing the ROS and RSN, but also by increasing the endogenous expression and/or activity of the main antioxidant enzymes, in order to restore the normal redox state of the cell (Battino et al., 2018). For these reasons we evaluated the activities of the main GSH-related enzymes and the mRNA expression of nuclear factor E2-related factor 2 (Nrf2), the key factor that regulates the cellular antioxidant defense system. Regarding the GSH-related enzymes, the activity of GR, GPx and GST were slightly increased after CH or EH treatment, compared to the control group, while, when cells were treated with LPS, a significant ($p < 0.05$) reduction was observed (Fig. 4B, C and D). Additionally, CH or EH were able to protect cells from LPS-induced damage by improving the activity of these enzymes, especially for GPx. To the best of our knowledge only few reports have evaluated the effects of food extracts or isolated compounds on the activities of antioxidant enzymes in RAW 264.7 macrophages stressed with LPS, obtaining similar results: among these matrices, Manuka honey (Gasparrini et al., 2018), strawberry extract (Gasparrini et al., 2017), Horchata (Guevara et al., 2019), *Ruta chalepensis* L. extract (Kacem et al., 2015), curcumin (Lin et al., 2019), resveratrol (Luo et al., 2016) were the most studied.

Finally, Nrf2 is a transcription factor that regulates the expression, at the transcriptional levels, of various antioxidant and detoxification enzymes able to protect against oxidative stress, also related to an inflammatory condition (Sánchez-Migallón et al., 2020; Ansary & Cianciosi, 2020). In this study, the expression levels of this transcription factor were found to be statistically up-regulated ($p < 0.05$) after the treatment of macrophages with honeys alone, specifically by 1.26 times with CH and 1.34 times with EH compared to the control group; also stimulation with LPS increased the expression of Nrf2, by 1.28 times, compared to controls (Fig. 4E). Interestingly, the cotreatment of CH or EH with LPS exerted a synergist effects by increasing, in a more marked way, the expression of this transcription factor, 1.50 times with CH and 1.35 with EH (Fig. 4E). An up-regulation of Nrf2, both in absence or in presence of LPS stimulation, also occurred with the treatment of RAW 264.7 macrophages with other natural compounds, such as Manuka honey (Gasparrini et al., 2018), *Heracleum moellendorffii* roots (Kim et al., 2019) and dihydroquercetin (Lei et al., 2020).

3.6. CH and EH modulated mRNA expression of key biomarkers of inflammation in LPS-stimulated macrophages

Macrophages are strictly correlated with the onset and progression of many inflammatory diseases, including aging-related disorders, atherosclerosis and cancer. The evaluation of the anti-inflammatory effects of CH and EH in LPS-treated macrophages on different molecular pathways involved in inflammation was analyzed through the mRNA expression.

Regarding iNOS, which is an inducer of several cytokines, including NF-κB and TNF-α and an indispensable mediator of the immune system (Cinelli, Do, Miley & Silverman, 2020), macrophages stimulated with LPS showed a 2.68 fold ($p < 0.05$) up-regulation in their levels compared to the control. Pretreatment with CH and EH led to a statistically significant reduction ($p < 0.05$) of iNOS expression compared to cells treated with LPS, respectively of 1.45 and 1.43 times (Fig. 5). Similar results were obtained in LPS-stimulated RAW 264.7 macrophages pretreated with different food matrices or dietary isolated compounds, such as Manuka honey (Gasparrini et al., 2018), *Kmeria duperreana* (Pierre) Dandy Extract (So, Bach, Paik & Jung, 2020), *Acalypha australis* L. extract (Kim et al., 2020a), naucleoffieine H (Song et al., 2020) and xanthotoxin (Lee et al., 2017).

The second pro-inflammatory marker analyzed was NF-κB, which plays a key role in inflammation, stimulating the transcription of various cytokines and chemokines (Liu, Zhang, Joo & Sun, 2017). Also in this case LPS caused an up-regulation in the mRNA expression levels of this transcription factor of 3.38 ($p < 0.05$) compared to untreated macrophages. Pretreatment of cells with CH significantly lowered ($p < 0.05$) the expression levels of NF-κB, which appears to be 1.33 times less expressed than in LPS-stressed cells (Fig. 5). Similarly, the same treatment carried out with EH led to a decrease in the expression levels of this transcription factor by 1.28 times. Other natural compounds also had the same modulatory effect against these LPS-stressed cells, such as Manuka honey (Gasparrini et al., 2018), *Acalypha australis* L. (Kim et al., 2020a) and *Smilax guianensis* Vitman extracts (Kim et al., 2020b), xanthotoxin (Lee et al., 2017) and carvacol (Somensi et al., 2019).

TNF-α is a cytokine involved in inflammation, particularly in the systemic inflammation and is able to stimulate the acute phase of this process; it is mainly produced by macrophages (Parameswaran & Patial, 2010). LPS significantly increased ($p < 0.05$) the TNF-α levels which were 2.42 fold higher compared to unstressed macrophages. Again, as with the other inflammation biomarkers, the pretreatment of macrophages with both types of honey CH and EH caused a statistically significant downregulation ($p < 0.05$) of its levels of 1.37 and 1.25 times respectively compared to those stimulated with LPS alone (Fig. 5). These results are consistent with other data performed on RAW 264.7 macrophages treated, prior to LPS stimulation, with Manuka honey (Gasparrini et al., 2018), *Acalypha australis L.* (Kim et al., 2020a) and *Smilax guianensis Vitman* extracts (Kim et al., 2020b), punicalagin (Cao et al., 2019) and different flavonoids isolated from the leaves of *Rhododendron dauricum* L. (Ye et al., 2021).

Finally, IL-10 is an anti-inflammatory cytokine, capable of inhibiting the synthesis of some pro-inflammatory cytokines and enzymes, such as TNF-α, interferon-γ and iNOS (Ouyang, Rutz, Crellin, Valdez & Hymowitz, 2011; Dowling et al., 2021). In this study it was observed that its expression increased significantly ($p < 0.05$) after the stimulation of macrophages with LPS (by 1.53 compared to the control) but also in the presence of CH or EH alone (1.36 and 1.66 times respectively) or with the CH or EH treatment followed by LPS stimulation (1.61 times and 1.99 times respectively) (Fig. 5). A similar effect was also observed by Gasparrini et al. (2018) in the same cell line treated with LPS and Manuka honey, but an increase was also observed with the use of other natural compounds, that promoted an up-regulation of the gene levels of this interleukin alone or in combination with LPS, such as with kaempferol, resveratrol (Palacz-Wrobel et al., 2017) and urolithin A (Bobowska et al., 2021).

3.7. CH and EH modulated mitochondrial respiration

The electron transport chain is the main site for ROS production, since some electrons, during the mitochondrial respiration, react directly with oxygen, leading to the formation of ROS as secondary products (Afrin et al., 2018a). Dietary bioactive compounds may exert beneficial effects on mitochondria through different mechanisms, including their intrinsic antioxidant capacity, or their ability in controlling the mitochondrial membrane potential, in modulating the pathways involved in mitochondrial biogenesis or in activating different biological processes, such as apoptosis (Afrin et al., 2018a). We assessed

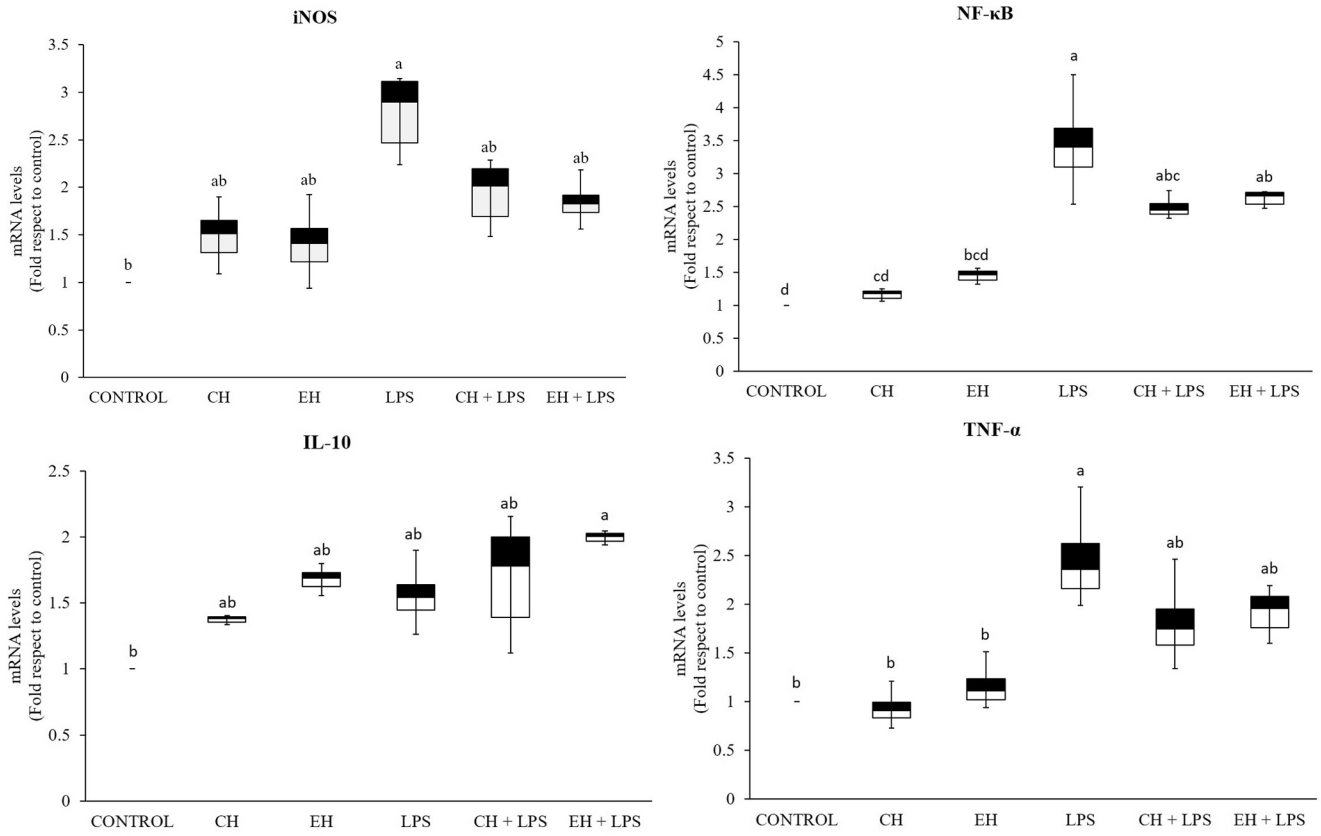

Fig. 5. Effects of CH and EH on mRNA levels of key biomarkers of inflammation. RAW 264.7 macrophages were treated with CH and EH for 24 h, with LPS for 24 h or pretreated with CH or EH for 24 h and then stressed with LPS for 24 h. The mRNA expression of iNOS, NF-κB, TNF-α and IL-10 were analyzed by RT-PCR. GADPH was utilized as a loading control. Results are expressed as a fold change in comparison with control (without treatment). All data are indicated as the mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$) between each treatment.

the capability of CH and EH to protect mitochondria from LPS-induced damage by using Agilent Seahorse XF24 Analyzer (Fig. 6).

In general, LPS treatment affected mitochondrial functionality, by decreasing all the parameters related to respiration (Fig. 6A, 6B), mainly due to the highest levels of ROS and nitrite detected in this group (Fig. 3), while pre-treatment with CH or EH, before LPS stress, exerted beneficial effects by improving the values of these factors. Specifically, LPS significantly ($p < 0.05$) decreased the basal OCR compared to the control group, while pretreatment with CH or EH, before LPS-induced stress, restored values similar to those of control group; any significant difference was instead found with CH or EH alone compared to the control group (Fig. 6). Regarding MRC, cells treated with the two honeys showed high values, especially those treated with CH, compared to the control group, while the LPS-stressed cells presented the lowest values ($p < 0.05$); the combined treatment with CH or EH and LPS increased the values of MRC compared to LPS-stressed cells. A similar trend was found also for ATP, with the lowest levels assessed in LPS-treated cells and with values similar to honey treated cells in macrophages pretreated with honey and stressed with LPS. Taken together, these data suggested that CH and EH had the ability to protect mitochondrial from LPS-induced damage, even if deeper studies are needed to identify the mechanisms involved.

Our results are in accordance with recent findings that highlighted the capacity of dietary bioactive compounds in ameliorating the mitochondrial respiration in RAW 264.7 macrophages, including genistein (Lee et al., 2019) and honey (Afrin et al., 2018), and in other *in vitro* models, as occurred with cyanidin from blueberry and cranberry (Cásedas et al., 2018) or with resveratrol and equol (Davinelli et al., 2013).

4. Conclusion

To the best of our knowledge, this is the first work to demonstrate the anti-inflammatory effects of CH and EH in murine RAW 264.7 macrophages stressed with LPS. We showed that these two Italian honeys are a relevant source of bioactive compounds with interesting antioxidant capacity. We also found that they were able to efficiently protect RAW 264.7 macrophages against LPS, by decreasing apoptosis, inducing proliferation, reducing intracellular ROS and nitrite levels, increasing antioxidant enzyme activities and improving mitochondrial functionality. These effects were mainly due to the ability of CH and EH in modulating the expression of some genes involved in inflammation and in the antioxidant system, including Nfr2, iNOS, NF-κB, TNF-α and IL10.

These results can represent the basis for additional *in vitro* and *in vivo* studies aimed at deepening the molecular mechanisms that modulate the beneficial effects of CH and EH and at identifying the main bioactive compounds responsible for their anti-inflammatory properties, especially considering that the literature on these honeys is still scarce.

Ethic statement

This research did not include any animal experiments or human subjects.

CRediT authorship contribution statement

Danila Cianciosi: Conceptualization, Investigation, Writing – original draft. **Tamara Y. Forbes-Hernandez:** Conceptualization, Investigation, Writing – original draft. **José M. Alvarez-Suarez:** Methodology. **Johura Ansary:** Investigation. **Denise Quinzi:** Software. **Adolfo Amici:**

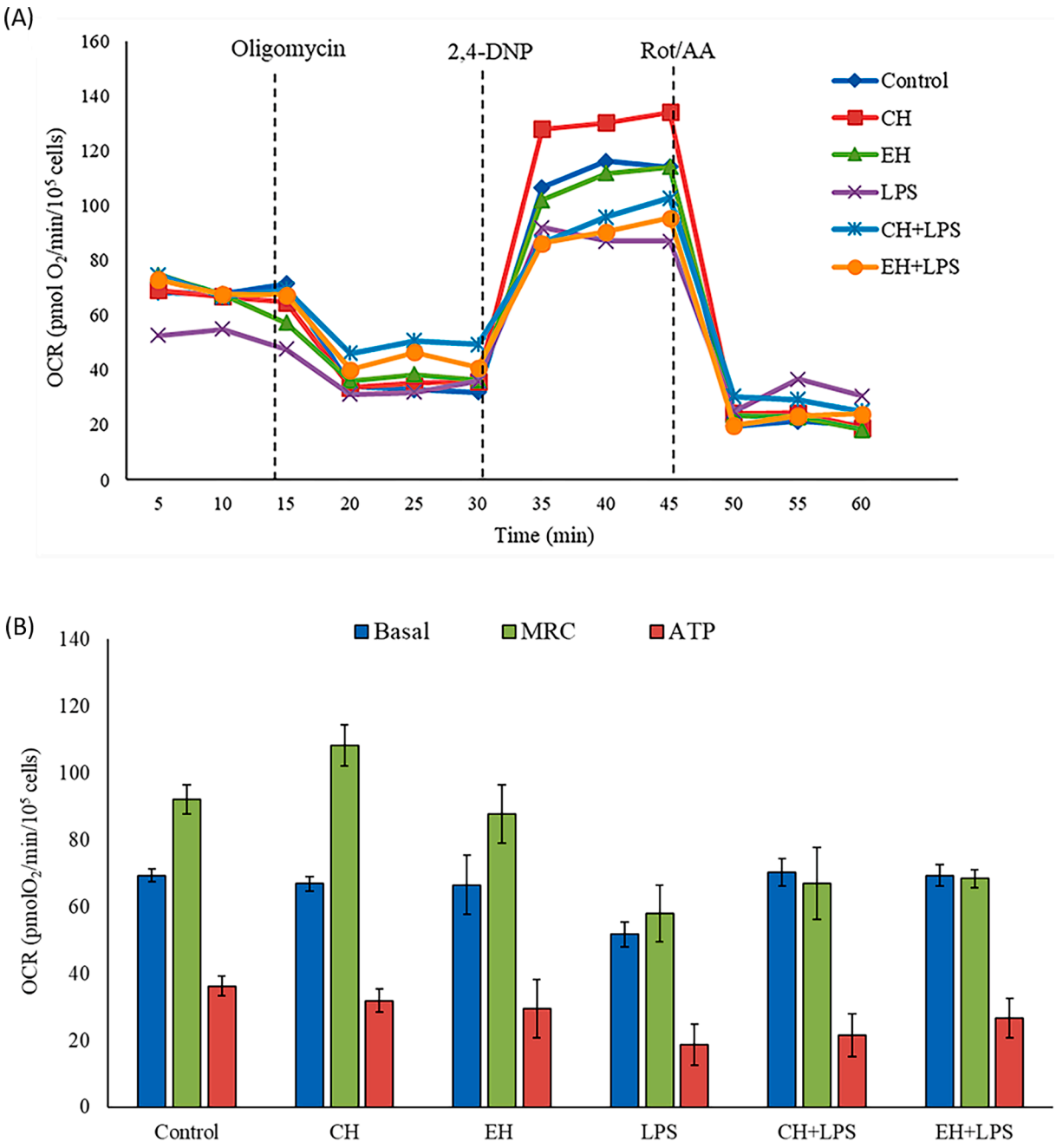

Fig. 6. Effects **of CH and EH on mitochondrial respiration.** RAW 264.7 macrophages were treated with CH and EH for 24 h, with LPS for 24 h or pretreated with CH or EH for 24 h and then stressed with LPS for 24 h. (A) OCR was determined by using the Seahorse XF-24 Extracellular Flux Analyzer after the sequential injections of oligomycin (1 μg/mL), 2,4-DNP (1 mM), and rotenone/antimycin (1 μM/10 μM). (B) Maximal respiration capacity (MRC) and ATP were calculated from the XF cell Mito stress test profile. All data are indicated as the mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$) between each treatment.

Validation. **María D. Navarro-Hortal:** Investigation. **Adelaida Esteban-Muñoz:** Formal analysis. **José Luis Quiles:** Data curation, Writing – review & editing. **Maurizio Battino:** Supervision, Visualization, Project administration. **Francesca Giampieri:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104752.

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