Food Chemistry 215 (2017) 274–283

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Bioaccessible (poly)phenol metabolites from raspberry protect neural cells from oxidative stress and attenuate microglia activation

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ARTICLE INFO

*Article history:*
Received 15 May 2016
Received in revised form 21 July 2016
Accepted 21 July 2016
Available online 28 July 2016

*Keywords:*
Polyphenols
Raspberry
Bioaccessibility
Oxidative stress
Neuroprotective mechanisms
Neuroinflammation

ABSTRACT

Neuroinflammation is an integral part of the neurodegeneration process inherent to several aging dysfunctions. Within the central nervous system, microglia are the effective immune cells, responsible for neuroinflammatory responses. In this study, raspberries were subjected to *in vitro* digestion simulation to obtain the components that result from the gastrointestinal (GI) conditions, which would be bioaccessible and available for blood uptake. Both the original raspberry extract and the gastrointestinal bioaccessible (GIB) fraction protected neuronal and microglia cells against $H_2O_2$-induced oxidative stress and lipopolysaccharide (LPS)-induced inflammation, at low concentrations. Furthermore, this neuroprotective capacity was independent of intracellular ROS scavenging mechanisms. We show for the first time that raspberry metabolites present in the GIB fraction significantly inhibited microglial pro-inflammatory activation by LPS, through the inhibition of Iba1 expression, TNF-α release and NO production. Altogether, this study reveals that raspberry polyphenols may present a dietary route to the retardation or amelioration of neurodegenerative-related dysfunctions.



## 1. Introduction

The increase in life expectancy that has occurred in recent years is increasingly becoming accompanied by the intensification of neurodegenerative diseases. These diseases are characterized by an irreversible loss of brain function that may lead to cognitive deficits, dementia, movement disorders, behavior deviations and psychological disorders. Underlying pathogenic mechanisms involved in such processes include mitochondrial dysfunction, neuroinflammation, protein aggregation, defective axonal transport, excitotoxicity and oxidative stress. In the mammalian brain, reactive oxygen species (ROS) mainly superoxide anion ($^{\cdot}O_2^-$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical ($^{\cdot}OH$) are continuously being produced (Gandhi & Abramov, 2012). Generation of ROS is counteracted by effective enzymatic and non-enzymatic antioxidant systems, such as superoxide dismutase, glutathione peroxidase, catalase and glutathione. Oxidative stress may result either from an increase in ROS production, a decrease in antioxidant systems, or both, and is an important player in the aging brain and in the development of degenerative diseases (Chen, Guo, & Kong, 2012).

Neuroinflammation is mediated by glial cells and intimately entwined with the neurodegeneration process. Microglia is the major innate immune cells of the central nervous system (CNS) (Cherry, Olschowka, & O'Banion, 2014) and, in non-pathological conditions, continuously survey the surrounding environment. Upon noxious injuries, such as pathogens, endotoxins, tissue damage or dying neurons, surveilling microglial cells are activated into a pro-inflammatory M1 phenotype, also designated as the

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http://dx.doi.org/10.1016/j.foodchem.2016.07.128
0308-8146/

classical activation state (Orihuela, McPherson, & Harry, 2015). This phenotype initiates inflammatory responses mediated by the release of pro-inflammatory molecules, such as cytokines, chemokines and ROS (Cherry et al., 2014). Oxidative stress is a typical hallmark of this type of microglial activation, whether as a cause or a consequence. Microglia, as other macrophage cells, naturally have the ability to switch toward an anti-inflammatory phenotype M2, also known as the alternative activation state. This type of activation includes the inhibition of pro-inflammatory cascades, and an elevated release of neurotrophic agents (such as neuronal growth factors) into the surrounding tissues, which stimulate wound repair and debris clearance to restore tissue homeostasis after a pro-inflammatory response (Cherry et al., 2014). However, in scenarios of pathological neurodegeneration, microglia maintains a persistent activated state, with an uncontrolled release of pro-inflammatory mediators, sustaining a chronic inflammatory state, which is highly injurious to the CNS (Cherry et al., 2014). Furthermore, in neurodegenerative conditions, there is an elevated production of ROS that affects microglial polarization balance, resulting in an increased subpopulation of M1 cells. Activated microglia also release $\cdot O_2^-$ and $H_2O_2$ that impact on the surrounding neurons, leading to a vicious cycle of microglial activation and neurodegeneration (Rojo et al., 2014).

Recent studies provide evidence that consumption of (poly) phenols-rich diets, as found in fruits, may lower the risk of developing neurodegenerative diseases due to their anti-inflammatory properties (Spencer, Vafeiadou, Williams, & Vauzour, 2012). Indeed, diet may affect human health considering that it can have detrimental effects, or in contrast, being able to attenuate inflammation (Wu & Schauss, 2012). (Poly)phenols, such as flavonoids and phenolic acids, have shown to play important roles in the regulation of inflammatory processes associated with several diseases, including neurodegenerative disorders (Spencer et al., 2012). Raspberries are fruits rich in these bioactive compounds (Bobinaitė, Viškelis, & Venskutonis, 2012) and some studies with raspberry extracts have shown anti-inflammatory capacity (Jo et al., 2015), and neuroprotective effects (Chen, Su, Huang, Feng, & Nie, 2012). However, it remains unclear as to how these compounds exert beneficial effects, what concentrations are necessary, and which are the biologically active forms. In addition, when studying the potential effects of (poly)phenol-rich foods in human health, it is essential to bear in mind that the bioavailability of (poly)phenols is dependent on the modifications that ingested food suffers along the gastrointestinal (GI) tract (Manach, Williamson, Morand, Scalbert, & Remesy, 2005).

As far as we know, this study was the first aiming to assess both the neuroprotective potential and the anti-inflammatory properties of digested extracts from the red raspberry (*Rubus idaeus*). Raspberry extracts were digested *in vitro* to mimic the chemical changes occurring during human digestion and were used in concentrations relevant to circulating metabolites *in vivo*. As a model to access neuroprotective activities of the red raspberry metabolites presented in the GI bioaccessible (GIB) fraction, we used the SK-N-MC human neuronal cell line insulted with hydrogen peroxide ($H_2O_2$) to induce oxidative stress, a common model used to assess the effects produced by such insult in neuronal cells (Tavares et al., 2012). To evaluate the ability of raspberry digested metabolites to attenuate inflammation we used a N9 murine microglial cell line treated either with $H_2O_2$ as an oxidant stimulus, or lipopolysaccharide (LPS) as an inflammatory insult (Cui et al., 2002). Remarkably, the digested raspberry metabolites used at physiological levels exhibited anti-inflammatory activity not only by the reduction of Iba1 expression, a microglia activation marker (Ito et al., 1998), but also by inhibiting the release of nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α), two specific markers of the microglial classical pro-inflammatory activation (M1) (Orihuela et al., 2015).

## 2. Material and methods

### 2.1. Plant material

Raspberry (*Rubus idaeus*) cv. Himbo-Top was grown in Fataca experimental field (Odemira, Portugal). Berries were harvested at full ripeness (pH 3.00 ± 0.01, soluble solid content 9.0 ± 0.0 (°Brix), titratable acidity 13.3 g (tartaric acid)/L), frozen and then freeze-dried. Fruit extracts were prepared using a hydroethanolic solution (ethanol 50% (v/v)) as previously described (Tavares et al., 2012), prior to *in vitro* digestion.

### 2.2. In vitro digestion

Phytochemical alterations during digestion were mimicked using the *in vitro* digestion model as published (Tavares et al., 2012). Briefly, the raspberry extract was submitted to conditions that mimic gastric digestion; pH adjusted to 1.7, addition of pepsin and incubation at 37 °C with shaking at 100 rpm for 2 h. Subsequently, small intestine conditions were mimicked by the addition of pancreatin and bile salts, followed by dialysis with a cellulose tube containing $NaHCO_3$ to slowly raise the pH to that of the small intestine. After 2 h incubation at 37 °C, the solution inside the dialysis tubing was collected. (Poly)phenols of original extract and for this fraction were separated using the C18 solid phase and the recovered compounds were then dried in a Speed-Vac Concentrator to suitable phenolic concentrations. The digested fraction after SPE is designated as the gastrointestinal bio-accessible "GIB" fraction.

### 2.3. Chemical profiling of the extract

#### 2.3.1. Total phenolic measurement

Determination of total phenolic content of both the original and the GIB fraction was performed by the Folin-Ciocalteau method as adapted to a microplate reader (Tavares et al., 2012). Gallic acid was used as a standard and the results were expressed in mg of gallic acid equivalents (mg GAE).

#### 2.3.2. HPLC-MS phenolic profile determination

Digested and original raspberry extracts were dried by rotary evaporation, re-suspended in 5% (v/v) acetonitrile in water and analyzed on a LCQ-DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan), as reported previously (Tavares et al., 2013). The samples were applied to a C18 column (Synergi Hydro C18 column with polar end capping, 2.0 mm × 150 mm, Phenomonex Ltd.) and eluted over a gradient of 95:5 solvent A:B at time = 0 min to 60:40 A:B at time = 30 min at a flow rate of 200 μL/min. Solvent A was 0.1% (v/v) formic acid in ultra-pure and solvent B 0.1% (v/v) formic acid in acetonitrile. The LCQ-Deca LC–MS was fitted with an ESI (electrospray ionization) interface and analyzed the samples in positive and negative-ion mode. Before the analysis, the system was tuned by using known concentrations of cyanidin-3-glucoside (positive mode) and quercetin-3-glucoside (negative mode) in ultrapure water. Recovery of components was assessed using peak areas for specific *m/z* values generated from the resident software.

### 2.4. Cell culture and treatments

#### 2.4.1. Neurodegeneration model

Human neuroepithelioma SK-N-MC cells were obtained from the European Collection of Cell Cultures (ECACC) and cultured in Eagle's minimal essential medium (EMEM) (Sigma) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS, Gibco®),

L-glutamine (2 mmol $L^{-1}$), 1% (v/v) non-essential amino acids (NEAA) (Sigma–Aldrich® – Poole, Dorset, UK), 1 mmol $L^{-1}$ sodium pyruvate (Sigma) and containing 50 U $mL^{-1}$ penicillin and 50 μg $mL^{-1}$ streptomycin. The cells were maintained at 37 °C in humidified atmosphere of 5% (v/v) $CO_2$, and split at sub-confluence of 70–80% using 0.05% trypsin-EDTA (Gibco). Cells were then seeded into 96-well plates ($2.5 \times 10^4$ cells/well), in 10% (v/v) FBS medium and cultured for 24 h at 37 °C. Growth media was removed, cells were washed with phosphate buffer saline (PBS) and incubated with different extract concentrations [1.25, 2.5, 5, 10 μg GAE $mL^{-1}$] of both the original and the GIB fraction for 24 h in culture media with FBS reduced to 0.5% (v/v). After discarding the medium, cells were washed and treated with 300 μmol $L^{-1}$ of $H_2O_2$ dissolved in 0.5% (v/v) FBS medium for additional 24 h.

#### 2.4.2. Microglia-induced inflammation model

N9 murine microglial cells were kindly provided by Dr. Teresa Pais (Institute of Molecular Medicine, Universidade de Lisboa, Portugal). This cell line consists of a retroviral-immortalized cell line derived from mouse brain that shares many phenotypical characteristics with primary mouse microglia. This cell line has been used extensively to study microglia performance in physiological and pathological circumstances related to neurodegenerative diseases (Stansley, Post, & Hensley, 2012). Microglial N9 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI) media, supplemented with 1% (v/v) L-glutamine (Biochrom AG), 1% (v/v) penicillin/streptomycin, and 10% FBS (Gibco). Cells were maintained at 37 °C in 5% (v/v) $CO_2$ and split at sub-confluent cultures (about 60–80%) using 0.5% Trypsin-EDTA. Cells were washed with versene solution (NaCl 8 g $L^{-1}$, KCl 0.4 g $L^{-1}$, EDTA 0.2 g $L^{-1}$, phenol red 0.02 g $L^{-1}$; pH 7.4), seeded at $3 \times 10^5$ cells/well and cultured for 24 h in 24-well plate containing coverslips (13 mm diameter, positively charged, Superior Marienfeld, Germany). Then, cells were pre-incubated with 1.25 μg GAE $mL^{-1}$ of both the original and the GIB fraction during 24 h. The medium was discarded and cells were washed with PBS. New culture media either with 300 μmol $L^{-1}$ of $H_2O_2$ or 300 ng $mL^{-1}$ of LPS (Sigma–Aldrich – Poole, Dorset, UK) was added and cells were incubated for more additional 24 h. Such $H_2O_2$ and LPS moderate concentrations were previously used in other studies to test oxidative stress and neuroinflammation (Bochkov et al., 2002; Tavares et al., 2012).

### 2.5. Determination of neuronal and microglial cell viability

Prior to analysis, treated neuronal cells were washed and incubated for 3 h at 37 °C with CellTiter-Blue reagent in 0.5% (v/v) FBS medium, accordingly with manufacturer's instructions (Promega, Fitchburg, Wisconsin, United States). Fluorescence values were recorded in a Synergy HT microplate reader, from Biotek. Necrotic-like microglial cell death was assessed by monitoring the cellular uptake of the fluorescent dye propidium iodide (PI). N9 microglial cell line was cultured on coverslips and incubated with a 75 μmol $L^{-1}$ PI solution for 15 min in the absence of light. Subsequently, cells were fixed paraformaldehyde 4% (w/v) in PBS and the nuclei were stained with Hoechst 33258 dye to quantify the total number of cells. Fluorescence staining was acquired by wide-field microscopy (original magnification: 400×) using a fluorescence microscope (Axioskope, Zeiss, Germany) attached to an AxioCam HRm camera (Zeiss). PI positive cells were counted and results were expressed as a percentage per total number of cells.

### 2.6. Neuronal ROS quantification

The ability of original extracts and GIB fraction to reduce ROS levels upon neuronal stimulation with $H_2O_2$ was evaluated using an assay based on the conversion of 2′,7′-dichlorofluorescein diacetate ($H_2$DCFDA, Invitrogen) to fluorescent 2′,7′-dichlorofluorescein (DCF) as adapted by (Tavares et al., 2013). Cells pre-incubated with digested and original raspberry fruit extracts were washed and incubated with 25 μmol $L^{-1}$ $H_2$DCFDA in PBS for 30 min at 37 °C. Cells were washed with PBS and treated with $H_2O_2$ (300 μmol $L^{-1}$) in PBS for 1 h at 37 °C. Intracellular ROS generation was measured by fluorimetry (λex: 485 nm, λem: 530 nm) using a FLx800 Fluorescence Microplate Reader (Biotek) as an increase in fluorescent signal between control and $H_2O_2$-treated cells.

### 2.7. Immunocytochemistry of Iba1 marker of microglial activation

To access Iba1 in microglia, cells were cultured for 24 h in 24-well plate containing coverslips, as already described (Caldeira et al., 2014). LPS- and $H_2O_2$-treated cells were permeabilized with 0.1% (v/v) Triton in PBS for 30 min at room temperature (RT). Afterwards, the coverslips containing the cells were washed once with PBS, and blocking solution [1% (w/v) BSA, 0.4% (v/v) Triton, 4% (v/v) FBS in PBS] was added for 30 min at RT. Coverslips were incubated overnight at 4 °C with rabbit anti-Iba1 (1:100) (from Wako, Japan), then washed 3 times with PBS and incubated for 2 h at RT with Alexa488 anti-rabbit (1:1000). Fluorescence staining was acquired by wide-field microscopy (original magnification: 400×) using a fluorescence microscope (Axioskope, Zeiss, Germany) attached to an AxioCam HRm camera (Zeiss) and fluorescence intensity quantified by ImageJ software (NIH, USA).

### 2.8. Nitric oxide (NO) quantification

NO levels were estimated by measuring the concentration of nitrites ($NO_2$), the stable end product from NO metabolism, in culture media with the Griess reagent (Sigma–Aldrich – Poole, Dorset, UK) as described. N9 microglial cells were seeded ($5 \times 10^5$ cells $mL^{-1}$) in 6-well plates and pre-incubated with the GIB fraction at 1.25 μg GAE $mL^{-1}$ during 2, 4, 6 and 24 h in culture media with 0.5% (v/v) FBS, prior to LPS treatment (300 ng $mL^{-1}$) in culture media with 10% (v/v) FBS during 24 h. For each time point a control of the pre-treatment was performed by incubating cells with media with 0.5% (v/v) FBS before the LPS treatment. Cell media was removed and stored at −80 °C until nitrite analysis. For nitrite quantification, a standard curve of sodium nitrite [0–25 μmol $L^{-1}$] was prepared and absorbance data was acquired in a Synergy HT microplate reader from Biotek. Nitrite levels were normalized for total protein, extracted with RIPA buffer and quantified by Lowry protein assay.

### 2.9. Quantification of TNF-α

TNF-α release by microglia cells was assayed by ELISA according to the manufacturer's instructions (PeproTech; Princeton Business Park, Rocky Hill NJ, United States). Cells were pre-incubated with the GIB fraction at 1.25 μg GAE $mL^{-1}$ during 2, 4, 6 and 24 h in culture media with 0.5% (v/v) FBS or the media alone, prior to LPS treatment (300 ng $mL^{-1}$) in culture media with 10% (v/v) FBS during 24 h. For the standard curve, recombinant murine TNF-α (PeproTech) was diluted from 0 to 2 μg $L^{-1}$. TNF-α results were normalized for total protein, which was extracted by RIPA buffer and quantified by Lowry protein assay.

### 2.10. Statistics

Data are presented as mean values ± standard deviations (SD) of at least three independent experiments performed in three different days. Data was checked for normality by Shapiro-Wilk test and

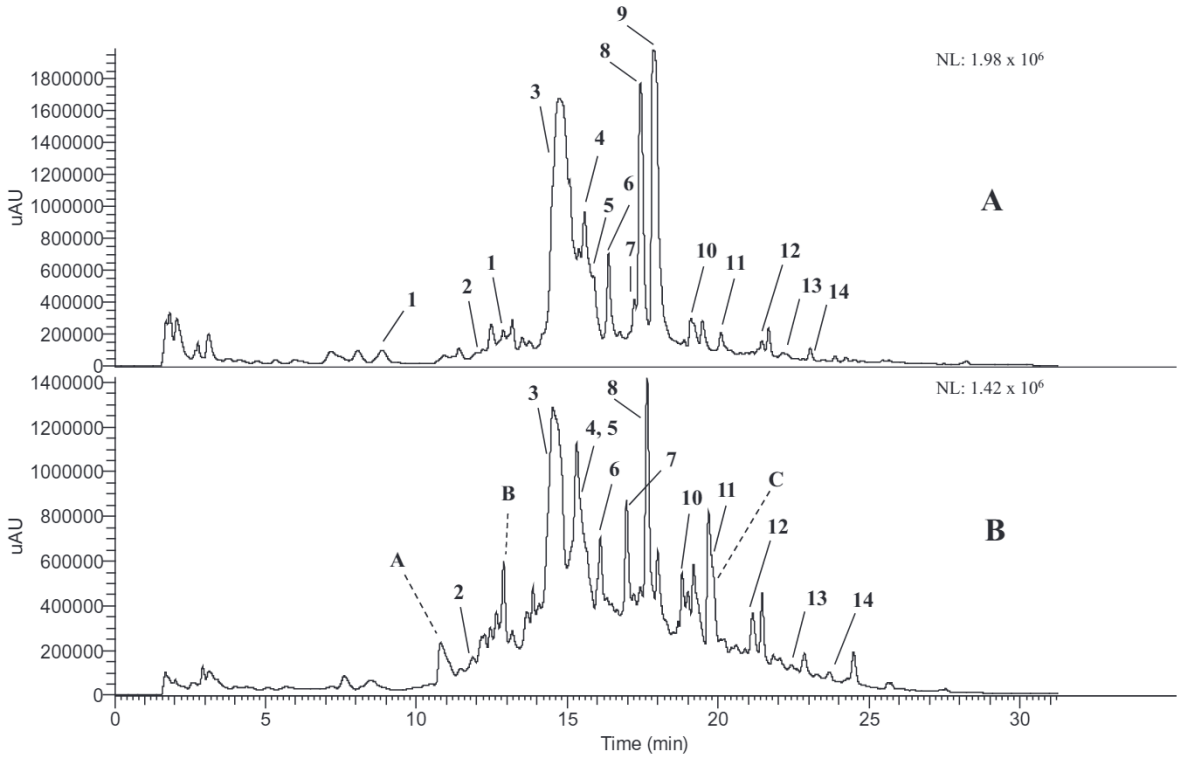


**Fig. 1.** Chemical profile of raspberry fruit extracts obtained by liquid chromatography-mass spectrometry (LC–MS). Analysis of fruit original extract (A) and the GIB fraction (B). Chromatograms are representative traces recorded at 280 nm. The full scan deflection is shown in the upper right corner of each panel. Peaks are labeled as described in Table 1.

**Table 1**
Peaks assignments, retention times and mass spectral data of major phenols present in raspberry fruit original extract and GIB fraction, with corresponding putative identifications obtained by comparison with literature. Recovery % was obtained based on the peak area of the mass spectrometer response for each m/z of GIB fraction compared with original.

| Peak | Retention time (min) | $\lambda_{max}$ (nm) | *m/z* [M−H] | $MS^2$ | Putative ID | Detected in | | Recovery (%) | Refs. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Original | GIB | | |
| 1 | 8.86; 12.88 | 260 | 783.07 | 301.17, 481.03, 275.15 | Pedunculagin-like isomer | + | – | 0 | Macedo et al. (2015) |
| 2 | 11.96 | 515 | 899.08(+) | 575.04, 557.00, 423.23, 329.15 | Cyanidin epicatechin conjugate | + | + | 63.1 | Macedo et al. (2015) |
| 3 | 14.74 | 517 | 757.15(+) | 287.18, 610.99, 269.58, 685.30 | Cyanidin-3-(2G-glucosylrutinoside) | + | + | 58.0 | Beekwilder et al. (2005) |
| 4 | 15.54 | 514 | 611.20(+) | 287.09 | Cyanidin-3-O-sophoroside | + | + | 44.8 | Macedo et al. (2015) |
| 5 | 15.65 | 514 | 595.10(+) | 287.19, 448.96 | Cyanidin-3-rutinoside | + | + | 37.8 | Beekwilder et al. (2005) |
| 6 | 16.36 | 280 | 1568.99 | 933.97, 1265.97, 631.05, 1103.84 | Sanguiin H-10 | + | + | 107.9 | Macedo et al. (2015) |
| 7 | 17.21 | 360 | 771.22 | 300.07, 591.16 | Quercetin glucosylrutinoside | + | + | 85.2 | Borges, Degeneve, Mullen, and Crozier (2010) |
| 8 | 17.43 | 280 | 1401.38 | 1251.07, 1869.00, 1567.96, 934.26, 633.30 | Lambertianin C | + | – | 0.0 | Mullen, Yokota, Lean, and Crozier (2003) |
| 9 | 17.86 | 280 | 1869.00 | 1264.70, 1401.14, 1566.00, 934.20, 633.12 | Sanguiin H-6 | + | + | 21.2 | Macedo et al. (2015) |
| 10 | 19.08 | 360 | 433.09 | 301.17, 300.17 | Ellagic acid pentoside[a] | + | + | 187.6 | Macedo et al. (2015) |
| 11 | 20.09 | 360 | 477.07 | 301.05 | Quercetin-3-glucoronide | + | + | 167.0 | Mullen, Yokota, Lean, and Crozier (2003 |
| 12 | 21.45 | 360 | 475.09 | 301.23, 300.27 | Ellagic acid 4-acetylpentoside | + | + | 94.3 | Mullen, Yokota, Lean, and Crozier (2003) |
| 13 | 23.04 | 220 | 677.25 | 617.23, 627.24, 585.27, 645.25 | Unknown | + | + | 56.4 | – |
| 14 | 23.61 | 220 | 679.36 | 517.33, 499.39 | Unknown triterpenoid | + | + | 17.8 | McDougall et al. (2016) (in press) |
| A | 13.18 | | 890.88 | 403.17 | Unknown | + | + | 144.0 | – |
| B | 10.95 | | 205.02(+), 188.19 | 146.12 | Tryptophan | – | + | n.a. | – |
| C | 19.75 | 360 | 301.15 | 179.06, 150.96 | Quercetin | + | + | 520.6 | Mullen, Yokota, Lean, and Crozier (2003) |

(+) Acquired in positive mode.
[a] Two Ellagic acid pentosides eluted as one, partly resolved peak.

homogeneity of variances by Levene test. Statistical differences were tested using unpaired one-way ANOVA with Duncan's multiple range test, and considered significant when $p < 0.05$ as recommended by (Granato, de Araújo Calado, & Jarvis, 2014). SigmaStat was used as statistical software.

## 3. Results and discussion

### 3.1. Chemical characterization of gastrointestinal bioaccessible (GIB) fraction

An *in vitro* method was used that simulates gastro-intestinal conditions that food components suffer during digestion. The components that survive digestion are gastrointestinal–bioaccessible (GIB) products that are available for uptake into the serum, which were compared for efficacy with the original raspberry extract. Only 19.2% of total phenols were recovered in the post-digestion GIB fraction, which is similar to previous studies on blackberry (Tavares et al., 2012), blueberry (Correa-Betanzo et al., 2014) and blackthorn (Pinacho, Cavero, Astiasarán, Ansorena, & Calvo, 2015), as well in other fruits. Substantial alterations in phytochemical composition were caused by digestion (Fig. 1 & Table 1). The original raspberry extracts had a (poly)phenolic composition characteristic of this fruit and was dominated by anthocyanins, ellagitannins and ellagic acid derivatives, and quercetin-type flavonols (Fig. 1 & Table 1).

Anthocyanins, which are known to be less stable at the neutral pH found in the small intestine, (McDougall, Dobson, Smith, Blake, & Stewart, 2005) showed recoveries of between 40% and 60% (Table 1). Losses may be due to degradation or binding to proteins during the digestion steps or simple partitioning across the dialysis membrane. Indeed, ~30% of the original total phenol content remained outside the dialysis tube (McDougall et al., 2005) and was not considered in this study.

The major ellagitannins showed mixed behaviours. Lambertianin C and the pedunculagin-like components at *m/z* 783 were not detected in the GIB fraction whereas sanguiin H-6 was recovered at ~20% and sanguiin H-10 showed ~100% recovery. Low recovery of ellagitannins *in vitro* after digestion has been noted previously for blackberry extracts (Tavares et al., 2012) and pure ellagitannins were shown to degrade in both acidic and alkaline conditions (Larrosa, Tomás-Barberán, & Espín, 2006). However, the trend to smaller ellagitannins has been noted before (Coates et al., 2007) and probably reflects degradation of the larger components, such as Lambertianin C to sanguiin H-6 then sanguiin H-10 through successive losses of ellagic acid groups. This would explain the apparent stability of sanguiin H-10 which is a product of degradation of both Lambertianin C and sanguiin H-6 and also the intermediate stability of sanguiin H-6 (Brown et al., 2012). The same order of stability was noted for these ellagitannin components in ileal fluids after raspberry intake (McDougall et al., 2014). The pedunculagin-like components may degrade to unidentified components.

Other components, such as the ellagic acid conjugates and quercetin derivatives showed mixed recoveries. Ellagic acid 4-acetyl

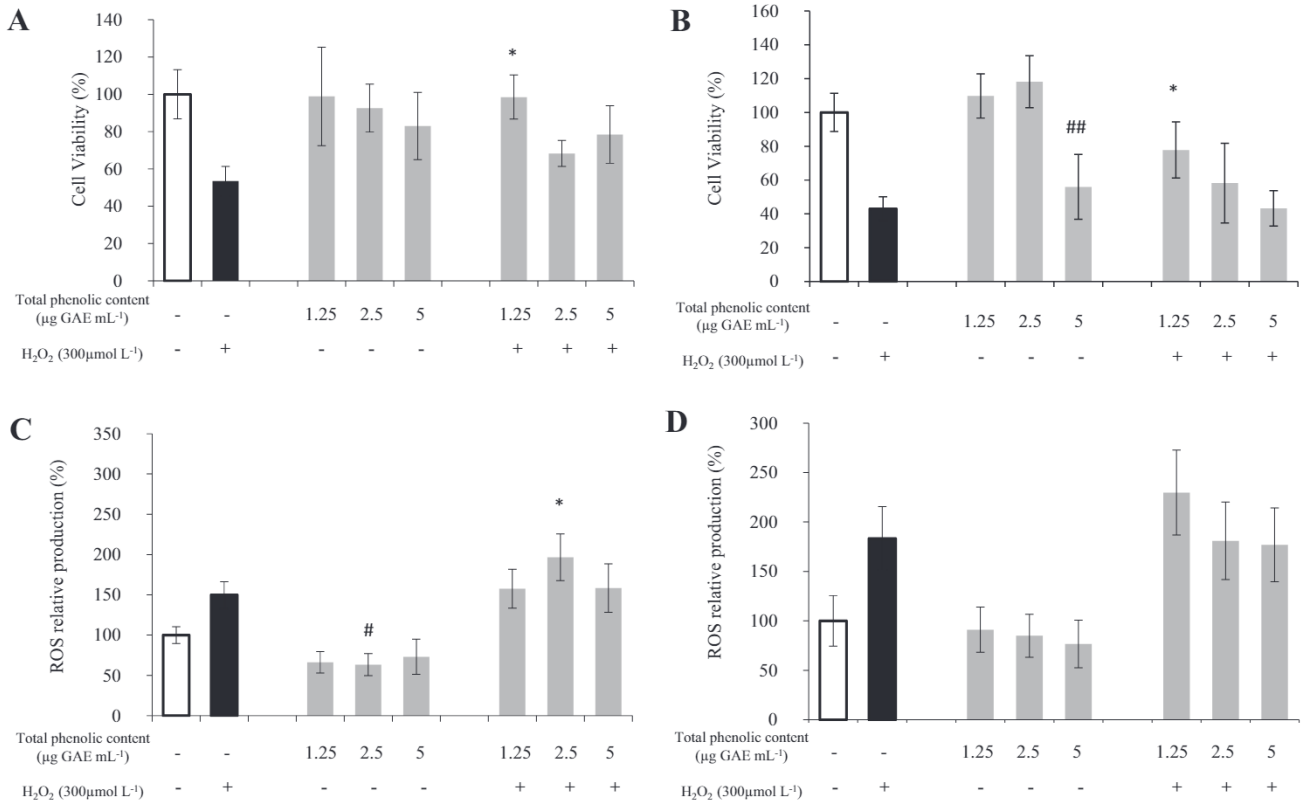


**Fig. 2.** Neuroprotection and intracellular ROS production in neuronal cells treated with original and GIB fractions of raspberry towards $H_2O_2$-mediated oxidative stress. Neuroprotection in SK-N-MC cells by pre-incubation with original extract (A) or GIB fraction of raspberry (B) for 24 h with different concentrations (0–5 μg GAE $mL^{-1}$) before treatment with 300 μmol $L^{-1}$ $H_2O_2$ for 24 h. Cell viability was assessed by CellTiter-Blue® reagent. Relative intracellular ROS production in SK-N-MC cells pre-incubated with original (C) or GIB fraction of raspberry (D) for 24 h before treatment with 300 μmol $L^{-1}$ $H_2O_2$ for 24 h. DCF was used as fluorimetric probe for intracellular ROS detection. Values are expressed as percentage relatively to control cells (white bars). Statistical differences between treatments in the absence of $H_2O_2$ and control cells (white bars) are denoted as $^{\#}p < 0.05$; $^{\#\#}p < 0.01$. Statistical differences between treatments in the presence of $H_2O_2$ and stressed cells (black bar) are denoted as $^{*}p < 0.05$. Not significant differences ($p > 0.05$) are not shown.

pentoside was recovered at ~90% whereas ellagic acid pentoside was recovered at ~200%. The two major quercetin derivatives had very different recoveries after digestion, quercetin-3-rutinoside at 50% and quercetin-3-glucuronide at 170% (Table 1). Peak 7 with $[M-H]^-$ *m/z* 771, putatively identified as a quercetin glucosyl rutinoside derivative, was recovered at ~80%. The greater than 100% recoveries of quercetin-3-glucuronide and ellagic acid pentoside reflect the relatively high stability of these components, the loss of other phenolics and the concentration inherent in the drying of samples to equal total phenol content. Indeed, relative recoveries above 100% were also described for flavonols after *in vitro* digestion of blackberry (Tavares et al., 2012) and green tea (Okello, McDougall, Kumar, & Seal, 2011). The appearance of peak A (putatively identified as tryptophan) probably resulted from concentration of this more stable component. Peak B also increased from low levels during *in vitro* digestion but its identity remains unknown. The identification of the new peak C after digestion as quercetin suggests flavonol degradation. Despite widespread ellagitannin degradation, no ellagic acid was detected in the GIB fraction, perhaps due to binding to proteins.

### 3.2. Neuroprotective effects

We assessed both the original and the GIB fraction for their neuroprotective effects to determine if digestion affected efficacy, as noted previously (Tavares et al., 2012). SK-N-MC cells were pre-treated for 24 h with either the original extract or the GIB fraction at the range of physiologically relevant concentrations (0–5 μmol $L^{-1}$), as previously indicated for circulating (poly)phenols in humans (Manach et al., 2005). No significant cytotoxic effect was observed for either the original extract or the GIB fractions, except for the highest GIB concentration (5 μg GAE $mL^{-1}$), suggesting that structure of metabolites were changed by digestion as expected and therefore its biological effects (Fig. 2A and B). As the increased $H_2O_2$ level is a hallmark of age-related diseases and more particularly of neurodegeneration (Tabner et al., 2005), cells were treated with 300 μmol $L^{-1}$ $H_2O_2$ for 24 h to mimic a chronic oxidative insult known to induce cell death. Curiously, as depicted in Fig. 2A and B, the viability of cells pre-treated either with the original extract or the GIB fraction before $H_2O_2$ exposure showed a significant protection from the insult as compared with $H_2O_2$ treated control cells, but only for the lower concentration (1.25 μg GAE $mL^{-1}$). Protection against oxidative stress by raspberry extracts were previously evidenced by studies performed in other conditions (Choi, Shim, & Kim, 2016).

Intracellular ROS production is an indicative test of the neuronal scavenging capacity to deal with exterior oxidative insults. In a neurodegenerative context, neurons lose their scavenging capacity and become vulnerable to the additional oxidative stress. Basal ROS production was significantly reduced by the original extract at 2.5 μg GAE $mL^{-1}$ concentration (Fig. 2C), but such effect was not observed with the GIB fraction (Fig. 2D). This finding suggests

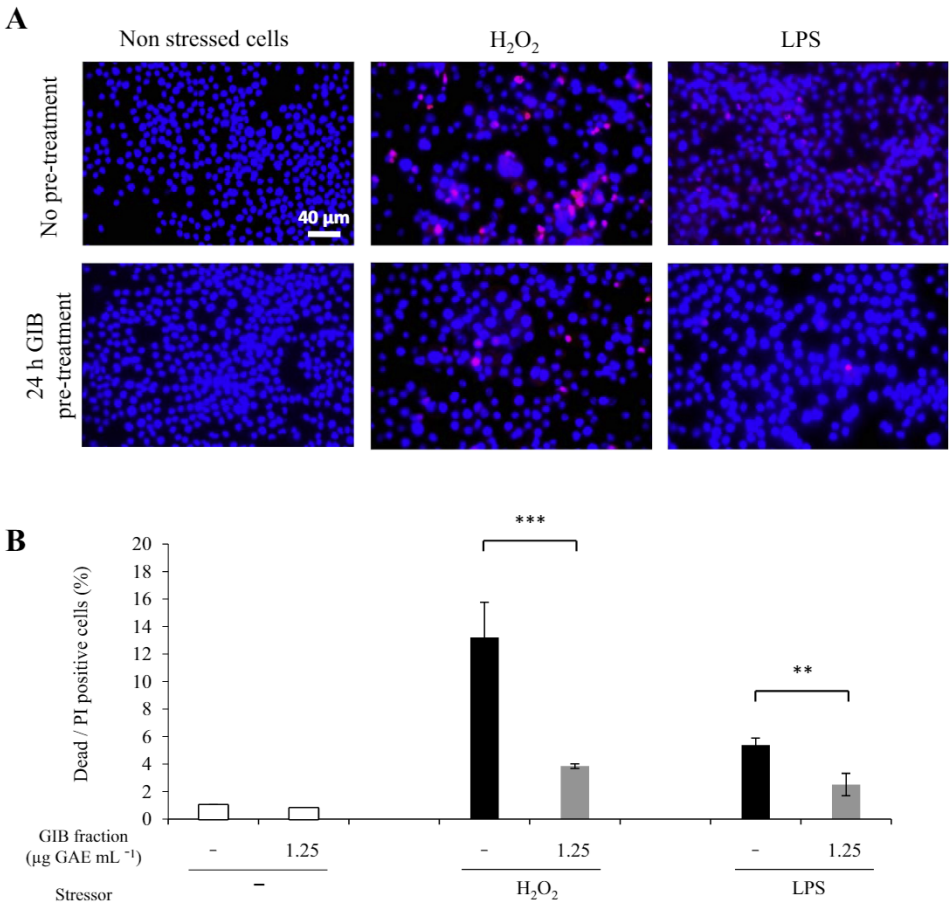


**Fig. 3.** Cytoprotective effect of the GIB fraction towards an oxidative stress ($H_2O_2$) and inflammatory insult (LPS) in N9 microglial cells. Microglial cells were pre-treated with GIB fraction of raspberry (1.25 μg GAE $mL^{-1}$) 24 h, before incubation either with $H_2O_2$ (300 μmol $L^{-1}$) or LPS (300 ng $mL^{-1}$) for additional 24 h. Cells were stained with Hoechst 33258/Propidium iodide. (A) Microscopy images; blue staining represents nuclei from live cells, red staining corresponds to necrotic cells. (B) Percentage of dead cells over total cells. Results are mean (±SD) from at least three independent experiments. Differences between treatments in relation to $H_2O_2$ or LPS control are denoted as $^{**}p < 0.01$; $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the loss of the scavenging capacity of (poly)phenols after digestion, as already described (Manach et al., 1998). Treatment with $H_2O_2$ increased intracellular ROS production and pre-treatment with neither original or GIB fraction counteracted such increase (Fig. 2C and D). Taken together, these results suggest that the increased neuronal viability toward $H_2O_2$ after a pre-treatment with both original and GIB fraction (Fig. 2A and B) is not a direct outcome produced by an enhanced intracellular ROS scavenging activity (Fig. 2C and D) and other intracellular mechanisms should be involved. Similar data were previously observed for digested extracts from blackberry (Tavares et al., 2012). Moreover, the differential effects of the original extract and GIB fraction on cellular protection clearly reinforces the importance of evaluating the physiological effects of the GIB fraction, since it mimics the metabolites that are bioaccessible to the human blood circulation. Considering the results obtained in the maintenance of neuronal cell viability by the GIB fraction upon $H_2O_2$ treatment (Fig. 2B) we decided to use in our further studies 1.25 μg GAE $mL^{-1}$. From all concentrations, this is considered the most physiological concentration in which dietary (poly)phenolic-derived metabolites are reported to be found in plasma (Manach et al., 2005). Furthermore, evaluation of mechanistic effects of these compounds is of major interest due to their potential entrance in the brain and the protective effects they exhibit against neurodegeneration (Schaffer & Halliwell, 2012; Spencer et al., 2012). An important aspect in the neurodegenerative process is the burst in inflammatory response exhibited by microglia, the resident brain immune cells. Some works have approached the attenuation of microglial activation by original (poly)phenols (Chuang et al., 2013; Rojanathammanee, Puig, & Combs, 2013), but not by its metabolites.

### 3.3. Neuroinflammatory activity

In the present study, the ability of GIB fractions to reduce neuroinflammation was evaluated by potentially preserving microglial viability and activation markers upon $H_2O_2$ or LPS stimulation. As expected, N9 microglial cell death increased significantly when exposed to both stressors (Fig. 3), although the extent of LPS-induced cell death was much less than that produced by $H_2O_2$. Pre-treatment with the GIB fraction was able to significantly prevent the loss of microglial viability in cells treated with either $H_2O_2$ or LPS (Fig. 3) by 70 ± 3.2% and 52 ± 2.4%, respectively, when compared to respective controls without GIB addition. Moderate cell death of N9 microglial cells upon the tested insults (not surpassing 15%) may derive from the sustained microglial pro-inflammatory activation along the 24 h incubation, with the release of pro-inflammatory cytokines that were shown to trigger

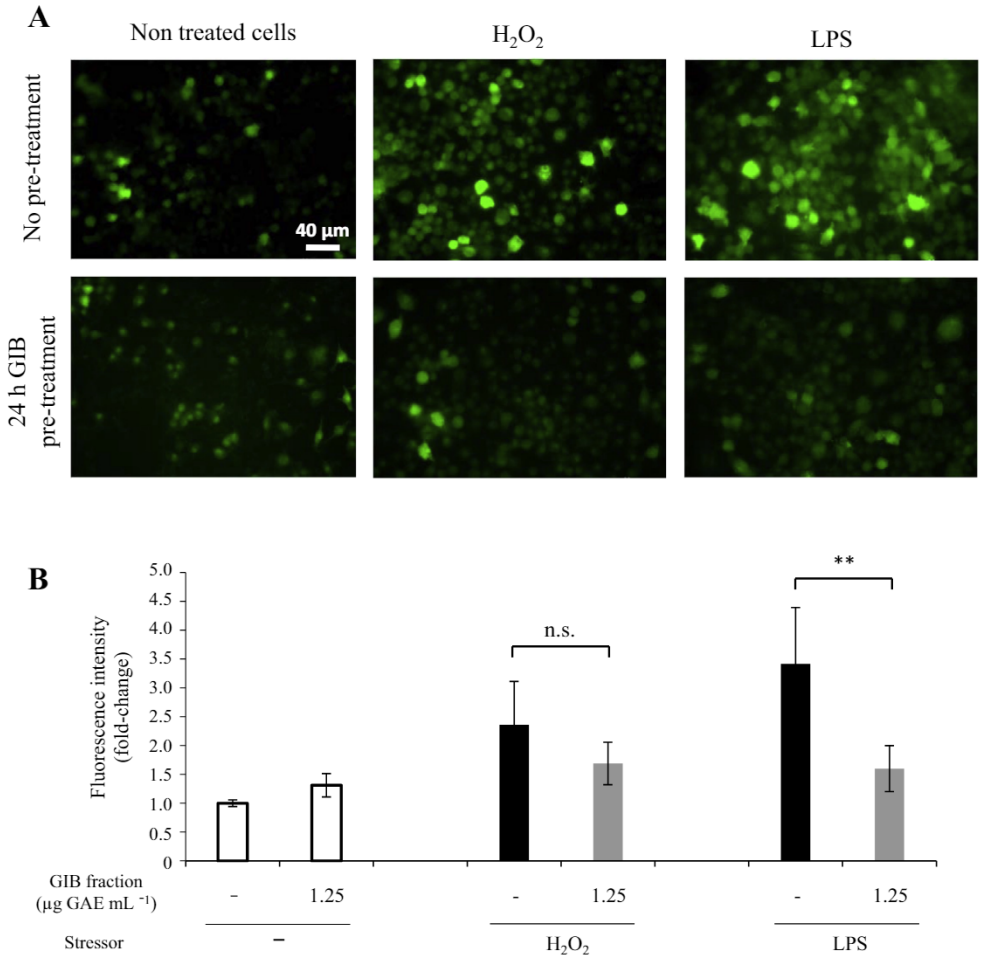


**Fig. 4.** Anti-inflammatory effect of the GIBfraction in N9 microglial cells when submitted to oxidative stress ($H_2O_2$) and inflammatory (LPS) insults. Microglial cells were pre-treated with GIB fraction of raspberry (1.25 μg GAE $mL^{-1}$) during 24 h, before incubation either with $H_2O_2$ (300 μmol $L^{-1}$) or LPS (300 ng $mL^{-1}$) for additional 24 h. Cells were immunostained for Ionized calcium binding adaptor molecule 1 (Iba1), a marker of activated microglia. (A) Representative microscope images; green staining represents activated microglia expressing Iba1. (B) Fluorescence intensity levels quantified by software. Results are mean (±SD) from at least three independent experiments. Statistical differences between treatments and the respective $H_2O_2$ or LPS control are denoted as $^{**}p < 0.01$. "n.s." means not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microglia degeneration (Silva et al., 2010). Indeed, LPS is preferentially an inducer of microglia polarization into the activated M1 state, rather than a promoter of cell demise (Orihuela et al., 2015). In our study it is evident that microglia are more resistant towards $H_2O_2$ insult than the neuronal cells, as observed when comparing data from Figs. 1 and 3. For this it may account the intrinsic capacity of microglia to generate and manage high amounts of ROS during inflammatory processes, in part by upregulation of the transcription factor Nrf2 known to restore redox homeostasis (Rojo et al., 2014). Nonetheless, the pre-treatment with the GIB fraction significantly reduced the loss of microglia viability by oxidative stress and inflammation. This clearly shows that the (poly)phenols present in the GIB fraction have capacity to protect not only neuronal cells, but also microglial cells towards $H_2O_2$ injury. As previously mentioned, the microglial activation by LPS is a well-recognized process of cell polarization into the M1 phenotype, and there is also a link between reactive oxygen species (ROS) and this pro-inflammatory phenotype, as already reviewed (Orihuela et al., 2015).

Activation was clearly noticed in our study by the increased staining of the microglia activation marker Iba1 upon cell incubation with both insults (Fig. 4). Protective effect of the GIB fraction, although less notorious in $H_2O_2$ treated cells (Fig. 4B), was markedly noticed in the LPS-induced microglial activation experiments. In this point we thought to be interesting to evaluate the effects produced by the GIB fraction in the release of NO and TNF-α, two recognized markers of M1 polarization in LPS-stimulated cells (Fig. 5). Thus, microglia cells were pre-treated with the GIB fraction during four different times (2, 4, 6 and 24 h), which reflects the time of circulation of the metabolites after either an acute consumption (2–6 h) or a chronic ingestion (24 h), prior to LPS exposure. Pre-treatment with the GIB fraction significantly reduced NO release at all times considered, when compared with the LPS-stimulated cells without GIB pre-treatment (Fig. 5A). The highest difference was observed with 6 h of pre-incubation, where a decrease of 33.2 ± 2.4% was obtained. Notably, the reduction prompted by the 24 h pre-treatment was only 17.7 ± 1.4%, which is approximately half of the NO suppressive effect observed at 6 h incubation. However, the overall levels of NO were substantially higher when the incubation with the digestion fraction was extended to 24 h, due to accumulation in the extracellular media. Interestingly, a similar pattern was observed for TNF-α release (Fig. 5B). Cells pre-treated with the GIB fraction for 2, 4 and 6 h significantly reduced TNF-α release upon LPS stimulation. In fact, the 6 h pre-treatment had the highest anti-inflammatory efficiency, lowering the TNF-α release by 43.1 ± 2.1% and effectively, abrogating the LPS effect. Differences between each time point, either for NO and TNF-α release in response to LPS, can be

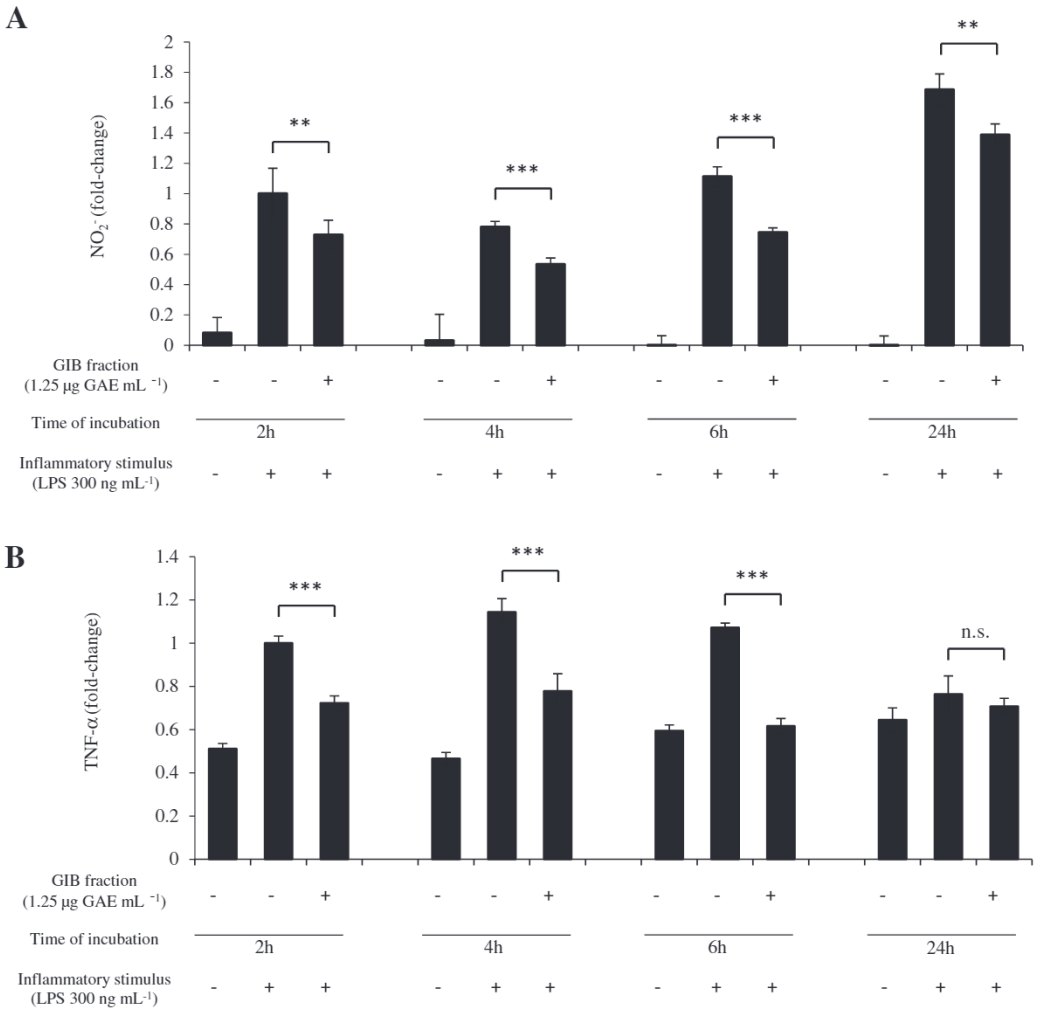


**Fig. 5.** Preventive effects of the GIB fraction over the release of NO and TNF-α by the N9 microglia cells when treated with lipopolysaccharide (LPS) and after different pre-treatment periods. Microglial cells were pre-treated with GIBfraction of raspberry (1.25 µg GAE mL$^{-1}$) during 2, 4, 6 and 24 h and then stimulated with LPS (300 ng mL$^{-1}$). NO and TNF-α release were normalized for total protein in each condition. Differences between control and stimulated cells are denoted as $^{**}p < 0.01$ and $^{***}p < 0.001$; "n.s." means not significant.

explained, at least in part, by the replacement of media with a fresh one containing low FBS concentration during the pre-treatment period. Indeed, FBS restriction mimics caloric restriction, thus leading cells to differently respond to LPS, with attenuated differences in NO and TNF-α release after GIB incubation. As recently noticed, caloric restriction reduces microglial activation (Radler, Wright, Walker, Hale, & Kent, 2015), which seems to be the case specially when FBS was limited for a longer period of time (24 h).

## 4. Conclusions

This work provides new insights about the neuroprotective and anti-inflammatory potential of raspberries in the context of neurodegenerative diseases. At physiologic concentrations, GIB components from raspberry evidenced neuronal protection against oxidative stress. More importantly, we showed for the first time that these metabolites are able to attenuate microglial pro-inflammatory activation. We also exposed the differences in (poly)phenolic composition after gastro-intestinal digestion, which influence the neuroprotective effects of the original and GIB fraction. This clearly highlights the importance of using a digestive process that mimics the human digestion to achieve greater physiological relevance. It was explicit that the observed neuroprotective effect did not rely directly on intracellular ROS scavenging mechanisms. Regarding neuroinflammatory effects, (poly)phenols present in the GIB fraction not only contributed to protect microglial cell viability against $H_2O_2$ induced stress, but also remarkably reduced Iba1 expression, which is an important microglial activation marker. Furthermore, the attenuation of NO and TNF-α release, which are two molecular players typical in the microglial M1 classical pro-inflammatory phenotype, reinforces the anti-inflammatory potential of the raspberry (poly)phenols as a preventive approach for neuroinflammation. Our study provides the framework for future animal studies to assess the attenuating effects of bioaccessible (poly)phenol metabolites from raspberry over neuroinflammation, a common denominator among the diverse list of neurodegenerative diseases and aging processes.

## Acknowledgements

This work was supported by iNOVA4Health Research Unit (LISBOA-01-0145-FEDER-007344), which is co-funded by Fundação para a Ciência e Tecnologia (FCT)/Ministério da Ciência e do Ensino Superior, through national funds, and by Fundo Europeu de Desenvolvimento Regional – FEDER (Portugal) under the PT2020 Partnership Agreement. The study was also supported by the FCT projects PTDC/SAU-FAR/118787/2010 and in part by UID/DTP/04138/2013 and the IF/01097/2013 (CNS). Moreover, the authors would also acknowledge the support of the EUBerry FP7 KBBE-2010-4 265942 (DS and CNS), BacHBerry FP7-KBBE-2013-613793 (DS, GG and CNS) and SFRH/BD/86584/2012 (IF). The authors (DS and GMD) also specially acknowledge for the support from the Scottish Government's Rural and Environment Science and Analytical Services Division; to Dr Pedro Oliveira, INIAV, Oeiras, Portugal for providing the fruits to be used in research; and to Dr. Teresa Pais, IGC, Oeiras, Portugal for the N9 cells and the support on the discussion. The authors declare no conflicts of interest.

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