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Evaluation of anti-inflammatory activity and fast UHPLC–DAD–IT-TOF profiling of polyphenolic compounds extracted from green lettuce (*Lactuca sativa* L.; var. Maravilla de Verano)

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ABSTRACT

Fresh cut vegetables represent a widely consumed food worldwide. Among these, lettuce (*Lactuca sativa* L.) is one of the most popular on the market. The growing interest for this "healthy" food is related to the content of bioactive compounds, especially polyphenols, that show many beneficial effects. In this study, we report the anti-inflammatory and antioxidant potential of polyphenols extracted from lettuce (var. *Maravilla de Verano*), in J774A.1 macrophages stimulated with *Escherichia coli* lipopolysaccharide (LPS). Lettuce extract significantly decreased reactive oxygen species, nitric oxide release, inducible nitric oxide synthase and cycloxygenase-2 expression. A detailed quali/quantitative profiling of the polyphenolic content was carried out, obtaining fast separation (10 min), good retention time and peak area repeatability, (RSD% 0.80 and 8.68, respectively) as well as linearity ($R^2 \geqslant 0.999$) and mass accuracy ($\leqslant 5$ ppm). Our results show the importance in the diet of this cheap and popular food for his healthy properties.

1. Introduction

A balanced diet is crucial to ensure and improve health status. Numerous observations (Block, Patterson, & Subar, 1992; Rice-Evans, Miller, & Paganga, 1997) show that a diet rich in fruits and vegetables is one of the best strategies to reduce the risk of chronic pathologies such as cancer and cardiovascular diseases (Hooper & Cassidiy, 2006). This protective effect is due to the presence in these foods of molecules with biological activity, such as polyphenols. Vegetables provide a significant source of polyphenolic compounds. Among these, green lettuce (Lactuca *sativa*) is one of the most popular vegetables, and its consumption is growing since it is considered a "healthy" food (Ferreres, Gil, Castaner, & Tomas-Barberan, 1997). Genetic, agronomical and environmental factors can influence the lettuce composition (Llorach, Martinez-Sanchez, Tomas-Barberan, Gil, & Ferreres, 2008). Particularly, the abundance of these compounds can be modified by many factors, such as light intensity, water availability, nutrient supply, pesticide, weather, as well as fertilisation, production and cultivation methods (Durazzo et al., 2014). Regarding the polyphenolic profile of lettuce the main compounds belong to two classes: hydroxycinnamic acids, mainly represented from caffeic acid derivatives and flavonols, which are usually linked to sugar moieties in position 3, such as 3-O glucosides or glucuronide form (Carazzone, Mascherpa, Gazzani, & Papetti, 2013; Santos, Oliveira, Ibanez, & Herrero, 2014). Many studies have focused the attention on the potential health effects of polyphenols, such as anticancer and antiinflammatory properties as well as the ability of reducing oxidative stress (Braca et al., 2011). Inflammation is a dynamic biological process occurring as result of chemical, physical, immunological and/or biological stimuli, and it involves an enormous expenditure of metabolic energy, damage, and destruction of host tissues, even with risk of sepsis, multiple organ failure and death. A moderate inflammation is essentially an adaptative response of human body to restore homeostasis. However, if the inflammation persist as chronic inflammation, it contributes to

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the pathogenesis various disease (e.g. type 2 diabetes, cardiovascular disease, cognitive impairment, brain atrophy and also cancer (Colotta, Allavena, Sica, Garlanda, & Mantovani, 2009). Macrophages play a major role in host defence during inflammatory and immune response however, excessive activation of these cells may cause extensive damage to tissues. It is widely know that, in response to lipopolysaccharide, a component of Gram-negative bacteria cell walls, macrophages produce and release inflammatory mediators, including cytokines, pro-inflammatory enzymes, as inducible nitric oxide synthase (iNOS) and cycloxygenase-2 (COX-2), and highly reactive species, as nitric oxide (NO) and reactive oxygen species (ROS). Despite oxidative response regulates many physiological responses, if not properly regulated it can also leads to a number of deleterious effects, thus its regulation results of great importance for human health (Popolo, Autore, Pinto, & Marzocco, 2013). In our study, we evaluated the anti-inflammatory and anti-oxidant potential of the polyphenolic extract of Maravilla de Verano green lettuce on J774A.1 macrophages stimulated with Escherichia coli lipopolysaccharide (LPS), showing an important reduction in the release of pro-inflammatory mediators. Furthermore, the quali/quantitative polyphenolic content of lettuce extracts was determined through a ultra high pressure-diode array detector-ion trap-time of flight (UHPLC–DAD–IT-TOF) platform, obtaining a fast and accurate profiling of its polyphenols.

2. Experimental

2.1. Chemicals

Ultra pure water (H_2O) was obtained by a Milli-Q system (Millipore, Milan, Italy). The following chemicals have been all purchased from Sigma Aldrich (Milan, Italy) acetonitrile (ACN), and formic acid LC–MS grade (HCOOH), methanol and hydrochloric acid (HCl), standards for calibration curves caffeic acid, quercetin 3β-glucoside and esculetin. Unless stated otherwise all other reagents employed in the sections below have been purchased from Sigma Aldrich. Two different columns were employed in this work: a Kinetex C18 150 × 2.1 mm, 2.6 μm (Phenomenex, Bologna, Italy) for UHPLC–IT-TOF qualitative analyses, and a Kinetex C18 150 × 4.6 mm, 2.6 μm for UHPLC–DAD quantitative analysis.

2.2. Sample preparation

The experiment was conducted in 2011 in a conventional farm (Gerstaberg) located in central Sweden. Green lettuce (*L. sativa* L.), var. Maravilla de Verano was used. At harvest, five plant were randomly picked in the field, in order to minimise soil differences among the treatments and avoid the borderline effect. The extraction were carried out as reported in literature (Llorach et al., 2008) with little differences. For each plant, outer leaves (second stage of leaves) were detached and slightly washed with distilled water to remove eventual residues. Five grams of outer fresh leaf taken from the central part were collected with a scalpel and placed in an extracting solution of 49 mL methanol + 1 mL HCl 37%. The solution was covered to prevent evaporation of extraction solvent, shaken at 100 rpm at 20 °C in the dark for 45 min, then the extracts were evaporated to dryness in vacuo. The resulting extracts were filtered through 0.20 μm Minisart SFCA sterile filters (Sartorius Stedim Biotech GmbG, Goettingen, Germany), and immediately stored at −20 °C.

2.3. Cell culture

J774A.1 murine monocyte/macrophage cell line (American Type Culture Collection, Rockville, MD), was grown in adhesion on Petri dishes and maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere.

2.3.1. Antiproliferative activity

Cells (5×10^4/well) were plated on 96-well plates and allowed to adhere for 4 h. Thereafter, the medium was replaced with of fresh medium and of serial dilutions of lettuce extracts (250–10 μg/mL) were added and cells were incubated for 24, 48 and 72 h. Cell viability was assessed through MTT assay. Briefly, 25 mL of 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) were added and cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilised with 100 mL of a solution containing 50% (v:v) N,N dimethylformamide, 20% (w:v) sodium dodecyl sulphate (SDS) with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. Macrophage viability in response to treatment with lettuce extracts, was calculated as: % dead cells = 100 × (OD treated/OD control).

2.3.2. Measurement of intracellular ROS

ROS formation was evaluated through the probe 2′,7′-dichlorofluorescin-diacetate (H2DCF-DA) as previously reported (Adesso et al., 2013). H2DCF-DA is a non-fluorescent permeant molecule that passively diffuses into cells, where the acetates are cleaved by intracellular esterases to form H2DCF and thereby traps it within the cell. In the presence of intracellular ROS, H2DCF is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Briefly, J774A.1 cells were plated at a density of 3.0×10^5 cells/well into 24-well plates. Cells were allowed to grow for 4 h; thereafter, the medium was replaced with fresh medium and cells were incubated with lettuce extracts (250–10 μg/mL) for 1 h and then co-exposed to LPS (1 μg/mL) for 24 h. Cells were then collected, washed twice with phosphate buffer saline (PBS) buffer and incubated in PBS containing H2DCF-DA (10 μM) at 37 °C. After 45 min, cells fluorescence was evaluated using a fluorescence-activated cell sorting (FACSscan; Becton Dickinson) and elaborated with Cell Quest software. Data are then expressed as mean fluorescence intensity.

2.3.3. Nitrite determination and Western blot analysis for iNOS, COX-2 and HO-1 expression

Macrophages J774A.1 were seeded in P60 plates (1.8×10^6/P60) and allowed to adhere for 4 h. Thereafter, the medium was replaced with fresh medium and cells were pretreated with lettuce extracts (250–10 μg/mL) for 1 h and then co-exposed to LPS (1 μg/mL) for further 24 h.

NO generation was measured as nitrite (NO_2^-, μM), index of NO released by cells, in the culture medium, as previously reported. NO_2^- amounts were measured by Griess reaction. Briefly, 100 μL of cell culture medium were mixed with 100 μL of Griess reagent – equal volumes of 1% (w:v) sulphanilamide in 5% (v:v) phosphoric acid and 0.1% (w:v) naphtylethylenediamine–HCl and incubated at room temperature for 10 min, then the absorbance was measured at 550 nm in a microplate reader Titertek (Dasit, Cornaredo, Milan, Italy). The amount of NO_2^-, as μM concentration, in the samples was calculated by a sodium nitrite standard curve iNOS, COX-2 and HO-1 expression was assessed by Western blot as previously reported (Bianco et al., 2012). Briefly after NO_2^- determination in cellular medium, cells were scraped off, washed with ice-cold PBS, and centrifugated at 5000 rpm for 10 min at 4 °C. The cell pellet was lysed in a buffer containing 20 mM Tris HCl (pH 7.5), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride,

10 μg/mL leupeptin, 10 mM sodium fluoride, 150 mM sodium chloride, 10 mg/mL trypsin inhibitor, and 1% Tween-20. Protein concentration was estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (50 μg) were dissolved in Laemmli's sample buffer, boiled, and run on a SDS polyacrylamide gel electrophoresis (SDS–PAGE) minigel (8% polyacrylamide) and then transferred for 40 min at 5 mA cm^2 into 0.45 μm hybond polyvinylidene difluoride membrane. Membranes were blocked for 40 min in PBS and 5% (w/v) non fat milk and subsequently probed overnight at 4 °C with mouse monoclonal anti-iNOS, anti-COX-2 (BD Laboratories), anti HO-1 or anti-tubulin antibody (Santa Cruz Biotechnologies) in PBS, 5% w/v non fat milk, and 0.1% Tween-20. Blots were then incubated with horseradish peroxidase conjugated goat anti-mouse immunoglobulin (Ig)G (1:5.000) for 1 h at room temperature. Immunoreactive bands were visualised using electrochemiluminescence assay (ECL) detection system according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of iNOS, COX-2 and HO-1 on XOmat films were quantified by scanning densitometry (Imaging Densitometer GS-700 BIO-RAD USA). Data are normalised with tubulin expression, used as reference protein, and expressed as arbitrary densitometric units as previously reported (Bianco et al., 2012).

2.3.4. Data analysis

Anti-inflammatory activity data are reported as mean ± standard error mean (s.e.m.) values of independent experiments, done at least three times, with three or more independent observations in each. Statistical analysis was performed by analysis of variance test, and multiple comparisons were made by Bonferroni's test. A *P*-value less than 0.05 was considered significant.

2.4. Instrumentation

UHPLC analyses were performed on a Shimadzu Nexera UHPLC system, consisting of a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20 A5 degasser, an SPD-M20A photo diode array detector (equipped with a semi-micro flow cell of 2.5 μL), a CTO-20A column oven, a SIL-30AC autosampler. The UHPLC system was coupled online to an LCMS–IT-TOF mass spectrometer through an ESI source (Shimadzu, Kyoto, Japan). LC–MS data elaboration was performed by the LCMSsolution® software (Version 3.50.346, Shimadzu).

2.4.1. UHPLC–PDA–MS–IT-TOF conditions

Mobile phase were (A): 0.1% HCOOH in water, (B) 0.1% HCOOH in ACN, analysis was performed in gradient elution as follows: 0–2.90 min, 15–20% B; 2.90–3.10 min, 20% B; 3.10–3.80 min, 20–27% B; 3.80–5.30 min, 27–35% B, 5.30–6.60 min, 35–55% B, 6.60–10 min 55–100% B. Flow rate was 2.2 mL/min. Column oven temperature was set to 48 °C. Injection volume was 2 μL of methanolic extract. The following DAD parameters were applied: sampling rate, 40 Hz; detector time constant, 0.160 s; cell temperature, 40 °C. Data acquisition was set in the range 190–400 nm and chromatograms were monitored at 330 and 350 nm at the maximum absorbance of the compounds of interest. UHPLC system was coupled on-line to a hybrid IT-TOF instrument, flow rate from LC was prior of the electrospray (ESI) source by means of a stainless steel Tee union (1/16 in., 0.15 mm bore, Valco HX, Texas US). Resolution, sensitivity, and mass number calibration of the ion trap and the TOF analyzer were tuned using a standard sample solution of sodium trifluoroacetate. MS detection was operated in negative ionisation mode with the following parameters: detector voltage, 1.53 kV; interface voltage, −3.5 kV, CDL (curve desolvation line) temperature, 200 °C; block heater temperature, 200 °C; nebulizing gas flow (N_2), 1.5 L/min, drying gas pressure, 100 kPa. Full scan MS data were acquired in the range of 200–900 *m*/*z* (ion accumulation time, 40 ms; IT, (repeat = 2). MS/MS experiments were conducted in data dependent acquisition, precursor ions were acquired in the range 150–800 *m*/*z*; peak width, 3 Da; ion accumulation time, 60 ms; Collision induced dissociation (CID) energy, 60%, collision gas 50%, repeat = 1; execution trigger (BPC) intensity, at 95% stop level.

2.5. Antioxidant activity

For each antioxidant assay, a Trolox aliquot was used to develop a 50–500 μmol/L standard curve. All data were expressed as Trolox Equivalents (μmol TE per gram of fresh weight). Spectrophotometric analyses were performed using a Jasco V-530 UV–vis spectrophotometer (Tokyo, Japan) set at appropriate wavelengths to each assay.

2.5.1. DPPH radical-scavenging assay

The ability of the sample extracts to scavenge the 2,2-diphenyl-1-picrylhydrazyl. (DPPH) radical was measured as described previously (Brand-Williams & Cuvelier, 1995). Aliquots (100 μL) of extracts were added to 3 mL of DPPH solution (6×10^{-5} mol/L) and the absorbance was determined at 515 nm every 5 min until the steady state using a Jasco V-530 UV–vis spectrophotometer (Tokyo, Japan).

2.5.2. Reducing potential assay

The antioxidant potential of the samples was determined using the ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996). A solution of 10 mmol/L TPTZ in 40 mmol/L HCl and 12 mmol/L ferric chloride was diluted in 300 mmol/L sodium acetate buffer (pH 3.6) at a ratio of 1:1:10. Aliquots (100 μL) of extracts were added to 3 mL of the FRAP solution and the absorbance was determined at 593 nm every 5 min until the steady state using a Jasco V-530 UV–vis spectrophotometer (Tokyo, Japan).

2.5.3. Total phenolic content

The concentration of total phenolics was measured by the method described by Singleton (Singleton, Orthofer, & Lamuela-Raventos, 1998), with some modifications. Briefly, an aliquot (100 μL) of the extract and calibration solutions of gallic acid (20, 40, 60, 80 and 100 mg/L) was added to a 25 volumetric flask containing 9 mL of double distilled water (ddH_2O). A reagent blank using ddH_2O was prepared. One millilitre of Folin & Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 mL of Na_2CO_3 aqueous solution (7 g/100 mL) was added with mixing. The solution was then immediately diluted to volume with ddH_2O and mixed thoroughly. After incubation for 90 min at 23 °C, the absorbance versus prepared blank was read at 765 nm. Total phenolic content was expressed as mg gallic acid equivalents (GAE)/100 mL sample.

3. Results and discussion

3.1. Analytical characterisation

The advantages of UHPLC for the separation of polyphenols have been demonstrated for numerous matrices in terms of speed and resolution, and only very recently for the separation of lettuce polyphenolic compounds (Abu-Reidah, Contreras, Arráez-Roman, Segura-Carretero, & Fernandez-Gutierrez, 2013). Conventional HPLC techniques based on the employment of fully porous particle are characterised by longer analysis time and often not satisfactory resolution. With respect to the other methods reported (Heimler, Isolani, Vignolini, Tombelli, & Romani, 2007), as can be observed

from chromatogram depicted in Fig. 1, a considerable reduction of analysis time (10 min) and a good resolution were obtained, which is important for analyse and compare numerous different batches of samples, together with less solvent consumption and thus a lower environmental impact.

3.1.1. Quantification of phenolic compounds

Quantification of individual polyphenolic compounds was performed by DAD at the maximum absorbance of the compounds of interest. Stock solution (1 mg/mL) were prepared in methanol, the calibration curves were obtained in a concentration range of 1–100 μg/mL with six concentration levels (1, 5, 10, 25, 50, 100 μg mL^{-1}) and triplicate injection of each level were run. Hydroxycinnamic acids derivatives, were quantified by comparison with an external standard of caffeic acid, flavonols were quantified as quercetin 3-*O*-glucoside, while coumarins were quantified as esculetin, the quantification of the compounds for which there was not a standard available was made using the calibration curve of the compound that was included in their structure. Peak areas of each standard were plotted against corresponding concentrations (μg/mL). The amount of the compounds in the sample was expressed as milligram per gram of extract, linear regression was used to generate calibration curve, R^2 values were $\geqslant$0.999, retention times and areas repeatability was also evaluated showing RSD% values below 0.80 and 8.68 respectively, proving good linearity and reproducibility (see Table S1) and could be a valuable tool for the analysis of polyphenols in different species of lettuce. Quali-quantitative results are shown in Table S2, as can be observed from chromatogram depicted in Fig. 1 most abundant compounds were peaks **3**, **5**, **10**, **11**, **14**, and **16**. As previously reported (Romani et al., 2002) for this matrix, hydroxycinnamic acid derivatives, represent nearly 65% of the total phenolic compounds, among these feruloylquinic acid (**3**), and caffeoyl feruloyl quinic derivatives (**12**, **14**) are the compounds in the highest concentration (see Table S2). Flavon-3-ol compounds were represented mainly from quercetin-3-*O*-glucoside and kaempferol derivatives, in accordance with previous studies (DuPont, Mondin, Williamson, & Price, 2000).

3.1.2. ESI–IT-TOF elucidation of polyphenolic profile

Identification of polyphenols was carried out using both DAD spectra and MS/MS data, comparing the fragmentation pattern with data, when present, in literature. Molecular formula was calculated by the Formula Predictor software (Shimadzu), setting a low tolerance so that most of the identified compounds were in position 1 in the list of the possible candidates. Results are shown in Table S2 in order of peak elution. Peak 1 and 2 showed two main fragment ions at *m*/*z* 191 and m/z 163, corresponding to the loss of the sugar moiety: $[M-H-C_7H_{12}O_6]^-$ and the tartaric residue $[M-H-C_4H_6O_6-H_2O]^-$ respectively, and were identified as chlorogenic acid and feruloyl tartaric acid. The most abundant compound, peak 3, showed two intense fragment ions at *m*/*z* 191 $[M-H^-]^-$ and at *m*/*z* 179 $[M-H-C_7H_{12}O_6-CH_3]^-$ and was tentatively identified as feruloylquinic acid, whose presence in lettuce has been previously reported (Tomas-Barberan, Velarde, Bonfanti, & Saltveit, 1997). Peak 4 and 5, were identified as chicoric acid and quercetin 3-*O*-glucoside respectively, by comparison with retention time of the commercial standards, and MS/MS fragments reported in literature. Peak 6 showed two fragment ions, the most abundant with *m*/*z* 315 $[M-H-C_5H_8O_6]^-$ and *m*/*z* 301 $[M-H-C_5H_8O_7]^-$ and was tentatively identified as isorhamnetin 3-*O*-glucuronide. A glucoronidate form (Carazzone et al., 2013) was also observed for peak 10, with a main fragment ion with *m*/*z* 299 deriving from the loss of the sugar, and thus was tentatively identified as kaempferide3-*O*-glucuronide. A loss of 162 amu $[M-H-180-H_2O]^-$ was observed for peak 7, this compound was identified as esculin. Compounds 11 was characterised from an intense fragment with *m*/*z* 367 relative to the loss of a caffeoyl residue $[M-H-C_9H_8O_4]^-$ and was identified as caffeoylferuloylquinic acid. The peak 13 showed a MS parent ion at *m*/*z* 301 and MS^2 fragments at *m*/*z* 179 and 151 and was recognised as quercetin. The fragmentation pattern of peaks 12 and 14, showed again a loss of a caffeoyl residue $[M-H-C_9H_8O_4]^-$, and they were identified as methyl-caffeoyl-feruloyl-tartaric acid isomers. The difference in retention times is probably due to a different interaction with stationary phase. Last eluting compounds, peaks 15 and 16, were both characterised by same mass and fragmentation ions

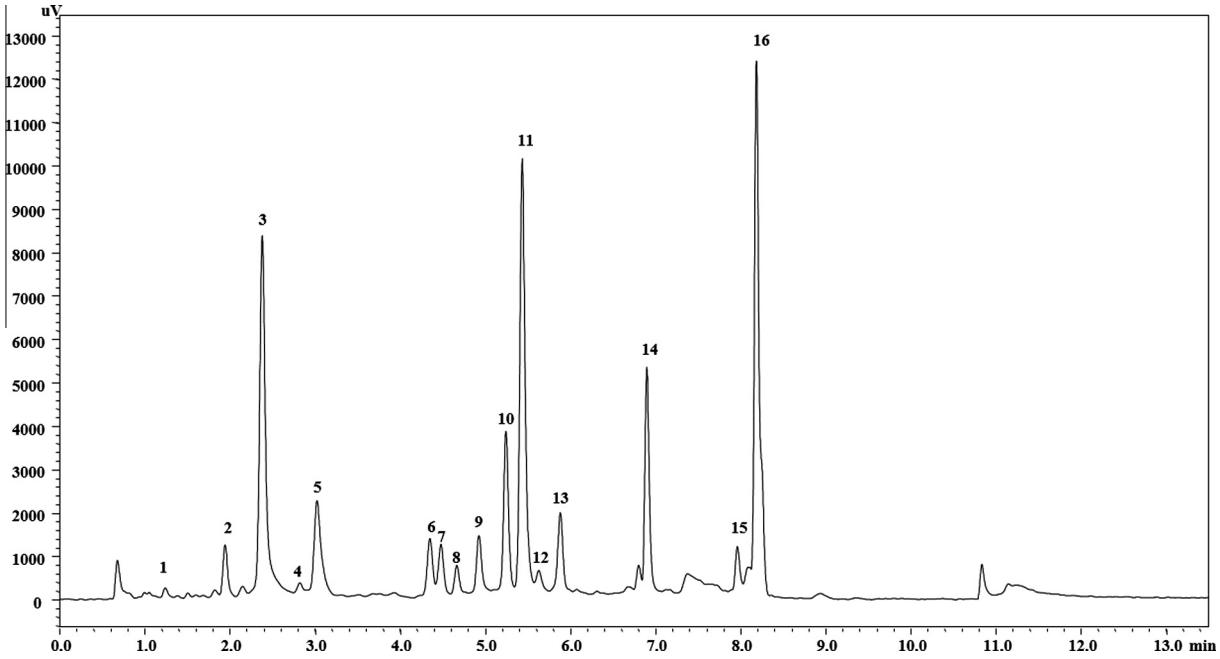

Fig. 1. UHPLC–PDA chromatogram of green lettuce polyphenolic extract, column: Kinetex C18 150 × 4.6 mm, 2.6 μm, flow: 2.2 mL/min, inj.vol 2 μL, column oven 48 °C.

$[M-H-162-H_2O]^-$, but a little difference in retention times, the fragmentation pattern and the prediction of molecular formula let us hypothesize a potential caffeoyl derivative. Unambiguous identification of almost all compounds was attained, through the employment of the hybrid mass spectrometer, for peaks 8, 9, 15–16 identification is currently under confirmation through HR-MS^n and NMR experiments.

3.2. Antioxidant activity

Due to the complex reactivity of phytochemicals, the antioxidant activities of natural matrices and food extracts cannot be evaluated by a single method, but at least two test systems have been recommended for the determination of antioxidant activity (Schlesier, Harwat, Bohm, & Bitsch, 2002). For this reason, the antioxidant activity of sample was determined by two spectrophotometric methods, DPPH and FRAP tests, and expressed as Trolox equivalents (TEs). As can be seen from Fig. S1 results (expressed as μmol TE/100 ml as mean ± standard deviation of four extracts) show that lettuce extract possess a discrete antioxidant activity when compared with that of authentic standard solutions of widely employed food preservatives and strong hydrophilic or lipophilic antioxidants, at the same concentration. The reduction of DPPH absorption is indicative of the capacity of the samples to scavenge free radicals, whereas the FRAP method is used to determine the capacity of reductants in a sample. DPPH test showed higher antiradical activity of lettuce extract than that of FRAP test. Results are in accordance with previous studies reported in literature, and this is attributed not only to the presence in high amounts of hydroxicinnamic acids, represented mainly from feruloylquinic acid methylcaffeoylferuloyltartaric acid, but also to the presence of 3-*O*-glucosidic flavonols, such as quercetin 3-*O* glucoside and kaempferide 3-*O* glucuronide, which contribute to the antioxidant activity, since they have a demonstrated scavenging activity (Heimler et al., 2007). The total phenolic content was in good accordance (1.21 mg(GAE)/100 ml) with the quantification proposed by HPLC–DAD, with subtle differences due to the more accurate quantification of the chromatographic method, proving however that the widely used Folin & Ciocalteu method provides a rapid and useful overall estimate of phenolic contents.

3.3. Effect of lettuce extracts on LPS-stimulated macrophages

LPS is an important structural component of the outer membrane of Gram-negative bacteria and it is known to modulate macrophage response during sepsis. LPS induces an inflammatory response that culminates in the release of inflammatory mediators, as NO, iNOS, COX-2 and ROS. NO is produced from the oxidation of L-arginine by NOS that occurs in two major classes: constitutive (including endothelial and neuronal isoforms), and inducible (including macrophagic isoform) (Moncada & Higgs, 1993). The iNOS may be expressed in different cell types (e.g. macrophages) by various proinflammatory agents, such as LPS. NO can be considered an immune modulator owing to its complex activity during host cellular defence (Schmidt & Walter, 1994). When macrophages are activated by the endotoxin from the bacterial wall components LPS or by interferon-γ, iNOS is significantly expressed, and massive amounts of NO are produced to exert a nonspecific immune response. Induced NO, in addition to being a 'final common mediator' of inflammation, also contributes to tissue damage, both directly via the formation of peroxynitrite, and indirectly through the amplification of the inflammatory response (Bhattacharyya, Biswas, & Datta, 2004). In our experiments, LPS induced in J774A.1 macrophages a marked increase in NO release associated to an increase in iNOS expression. Lettuce extracts at concentrations of 250–25 μg/mL significantly reduced NO release ($P < 0.01$ vs. LPS alone; Fig. 2) and iNOS expression ($P < 0.01$ vs. LPS alone; Fig. 3). An interaction between NOS and COX pathway represents an important mechanism for the modulation of the inflammatory response. COX-2 is a well known pro-inflammatory enzyme triggered by agents as LPS, it is involved in macrophage response and its expression is also influenced by NO. Thus, we evaluated the effect of lettuce extracts on COX-2 expression. Our data showed that, similarly to NO and iNOS, also COX-2 protein expression resulted significantly inhibited by lettuce extracts (250–25 μg/mL, $P < 0.05$ vs. LPS alone; Fig. 4), further contributing to the reduction of LPS-induced inflammation in J774A.1 macrophages. ROS generation in the inflammatory site, as well as NO, is typically induced as part of defensive reaction intended to clear infectious and environmental threats, including microbial agents and particulate material. Alternatively, ROS activation could act

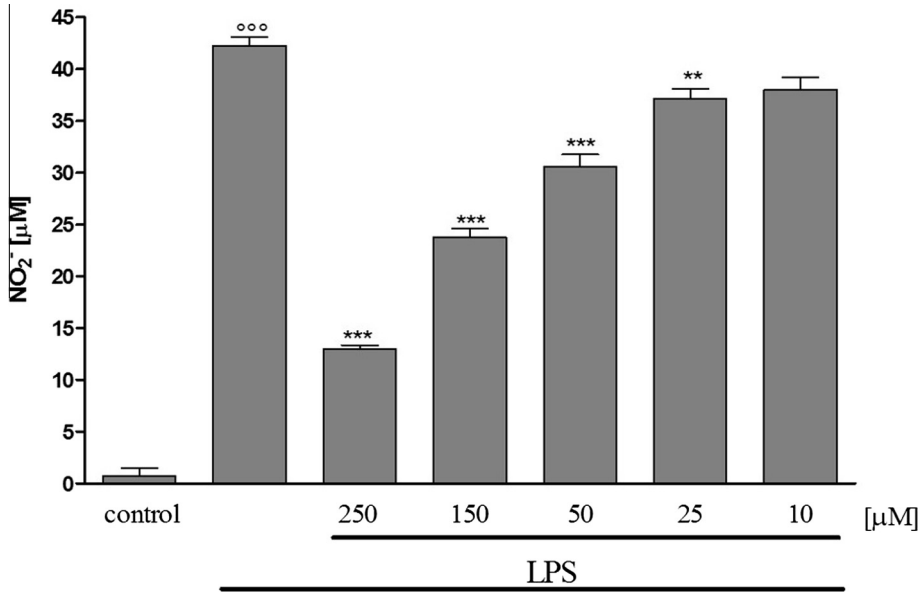

Fig. 2. Effect of green lettuce extracts (250–10 μg/mL) on NO release, evaluated as NO_2^- (μM), by macrophages J774A.1 stimulated with LPS. Values are expressed as mean ± s.e.m of NO_2^- (μM), of at least three independent experiments with three replicates each. Comparisons were performed using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. °°° Denotes $P < 0.001$ vs. control; *** and ** denotes $P < 0.001$ and $P < 0.01$ respectively vs. LPS alone.

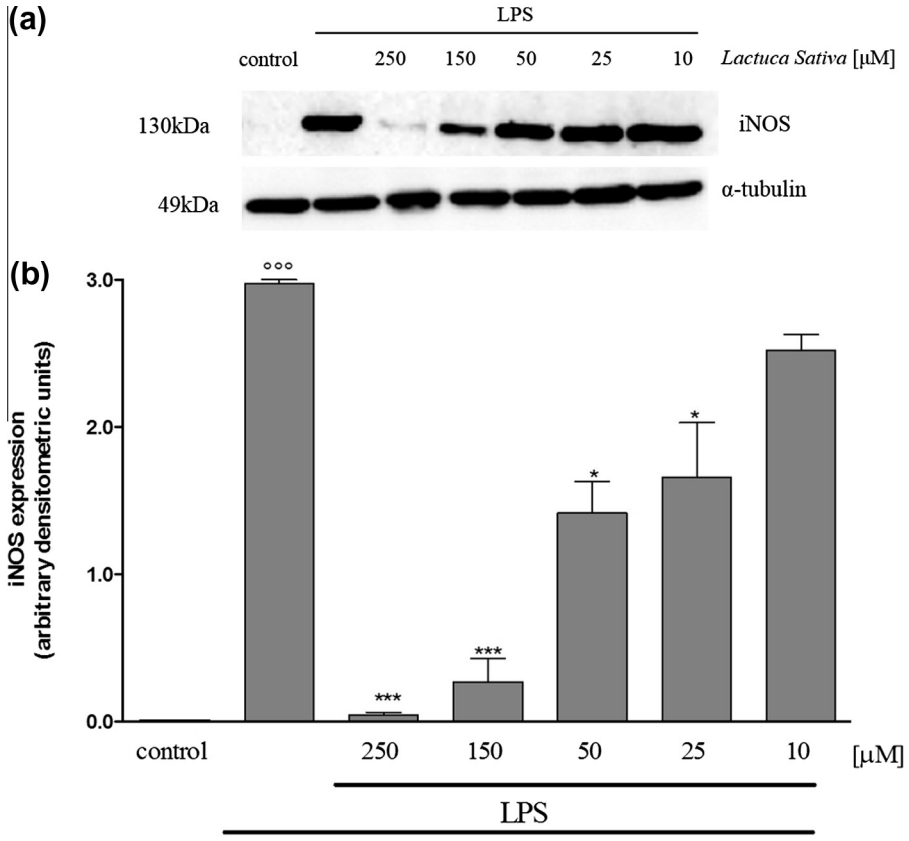

Fig. 3. Representative Western blot of inducible nitric oxide synthase (iNOS) expression (a). Densitometric analysis of the concentration dependent effect of green lettuce extracts (250–10 μg/mL) on LPS-induced iNOS expression in J774A.1 macrophages (b). Values, mean ± s.e.m., are expressed as arbitrary densitometric units at least 3 independent experiments with three replicates each. Comparisons were performed using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. °°° Denotes $P < 0.001$ vs. control; *** and ** denotes $P < 0.001$ and $P < 0.01$ respectively vs. LPS alone.

as a significant and adverse participant in abnormal inflammatory disease. Because of the involvement of ROS not only in inflammation but also in the ethiology of human diseases, such as atherosclerosis, neurodegeneration, diabetes, and ageing, there is a growing interest in the use of antioxidants. Thus, a large number of exogenous chemicals (naturally occurring as well as synthetic) have been accredited as agents aimed to reduce ROS release and inflammation (Geronikaki & Gavalas, 2006). In J774A.1 macrophages, LPS induce ROS generation and lettuce extracts (250–25 μg/mL, $P < 0.05$ vs. LPS alone; Fig. 5) significantly inhibits ROS release, index of its antioxidant effect on macrophages. Cells, as macrophages, in order to protect themselves against inflammatory and oxidative injury up-regulate some defence mechanisms as HO-1 expression. HO-1, the rate-limiting enzyme in heme degradation, catalyzes the oxidation of heme to generate several biologically active molecules (Wu, Ho, Lin, & Yet, 2011). This enzyme is normally expressed at low levels in most tissues/organs except for spleen. However, it is highly inducible in response to a variety of stimuli, as LPS, to protect cells against oxidative and inflammatory injury (Ryter, Alam, & Choi, 2006). Present in J774A.1 macrophages at low levels in basal condition, OH-1 resulted significantly increased by LPS ($P < 0.001$ vs. control; Fig. 6). Lettuce extracts (250–150 μg/mL) significantly, and in a concentration-related manner, further increased HO-1 enzyme expression in J774A.1 macrophages ($P < 0.01$ vs. LPS alone; Fig. 5), resulting in a protective effect for macrophages in the presence of LPS.

HO-1 can increase cellular anti-oxidant status by generating antioxidants such as bilirubin that can inhibit iNOS protein expression and suppress NO production (Wang, Smith, & Zucker, 2004). Moreover, carbon monoxide, a major product of HO-1 activity, was shown to inhibit COX-2 protein expression (Suh, Jin, Yi, Wang, & Choi, 2006). Thus, considering these studies in field, we can also hypothesize the important contribution of OH-1 expression in reducing inflammatory response associated to lettuce in LPS-stimulated J774A.1 macrophages. Moreover, MTT assay revealed that at all concentrations (250–10 μg/mL) and incubation times (24, 48 and 72 h) did not affect macrophage proliferation (data not shown), indicating its absence of toxic effects on macrophages, and that the observed effects were not due to disruption of normal cellular function.

The seeds of lettuce were traditionally used in Iran for relieving of inflammation, gastrodynia and osteodynia (Aqili Khorasani, 1991). Anticonvulsant and sedative-hypnotic effects have been mentioned for lettuce leaves (Zargari, 1989) and the analgesic and antinflammatory activity of lettuce seed extracts in rats it has been also reported (Sayyah, Hadidi, & Kamalinejad, 2004). More recently, the antioxidant activity of lettuce edible portion has been reported in CaCo-2 intestinal cells (Durazzo et al., 2014). Our results are in accordance with these previous study reporting the antinflammatory and antioxidant effect of lettuce also in macrophage during inflammatory response. This study, at our knowledge, firstly reports the antinflammatory and antioxidant effect of lettuce leaves on LPS-stimulated macrophages showing the involvement of important regulators of inflammatory response. Quantification of phenolic compounds in lettuce extracts performed in this study revealed the main presence of hydroxycinnamic acids derivatives, flavonols and coumarins, as highlighted recently in literature (Baslam, Morales, Garmendia, & Goicoechea, 2013). Hydroxycinnamic acid derivatives have been described to exert anti-oxidant and anti-inflammatory actions (Nagasaka et al., 2007). The beneficial effects of flavonoids has been

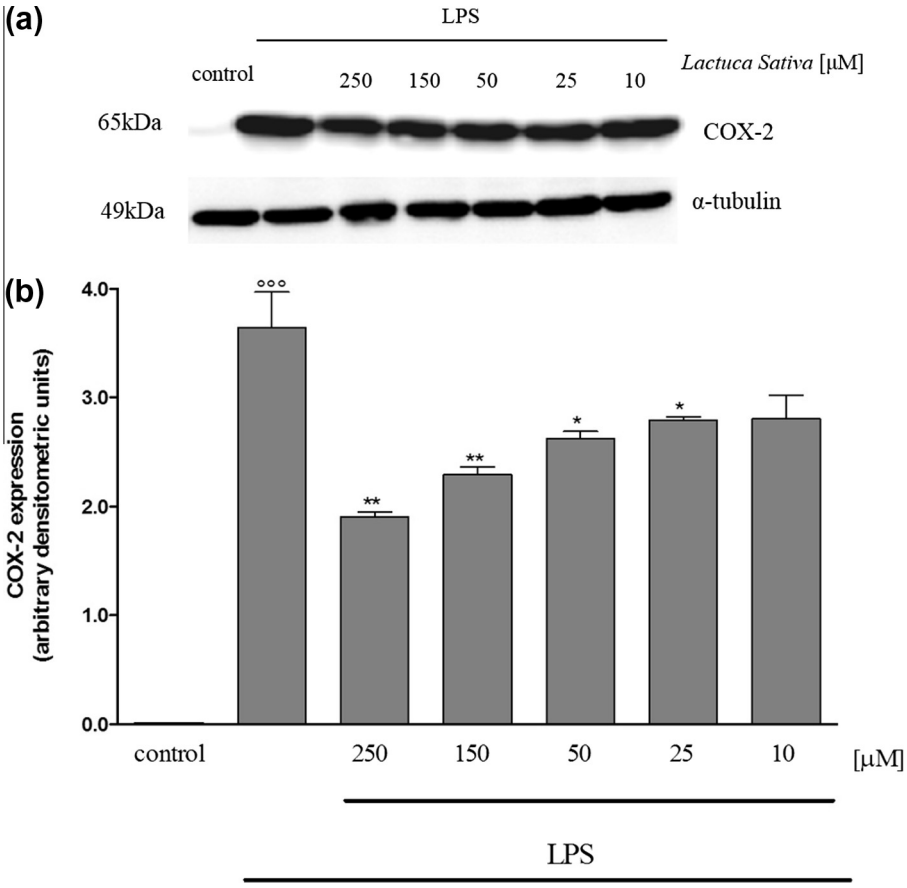

Fig. 4. Representative Western blot of cicloxygenase-2 (COX-2) expression (a). Densitometric analysis of the concentration dependent effect of green lettuce extracts (250–10 μg/mL) on LPS-induced COX-2 expression in J774A.1 macrophages (b). Values, mean ± s.e.m., are expressed as arbitrary densitometric units at least 3 independent experiments with three replicates each. Comparisons were performed using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. °°° Denotes $P < 0.001$ vs. control; ** and * denotes $P < 0.01$ and $P < 0.05$ vs. respectively LPS.

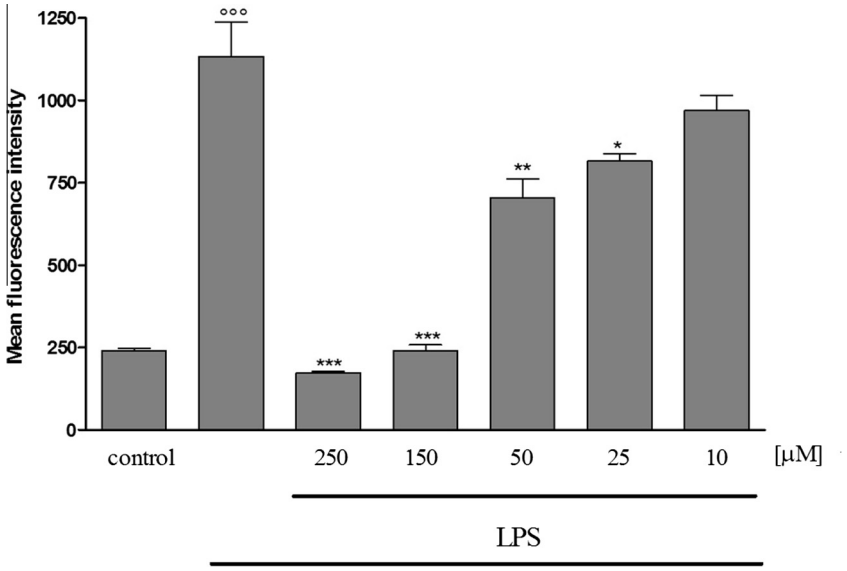

Fig. 5. Effect of green lettuce extracts (250–10 μg/mL) on ROS formation, evaluated by means of the probe 2′,7′ dichlorofluorescein-diacetate (H2DCF-DA), in LPS-stimulated J774A.1 macrophages. Values, mean ± s.e.m., are expressed as arbitrary densitometric units at least 3 independent experiments with three replicates each. Comparisons were performed using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. °°° Denotes $P < 0.001$ vs. control; ***, ** and * denotes $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively vs. LPS-treated macrophages.

extensively reported and in particular several mechanisms explaining the anti-inflammatory activity of flavonoids have been described, including (1) antioxidative and radical scavenging activities, (2) regulation of cellular activities of inflammation-related cells, (3) modulation of the activities of arachidonic acid metabolism enzymes (phospholipase A2, COX, lipoxygenase) and

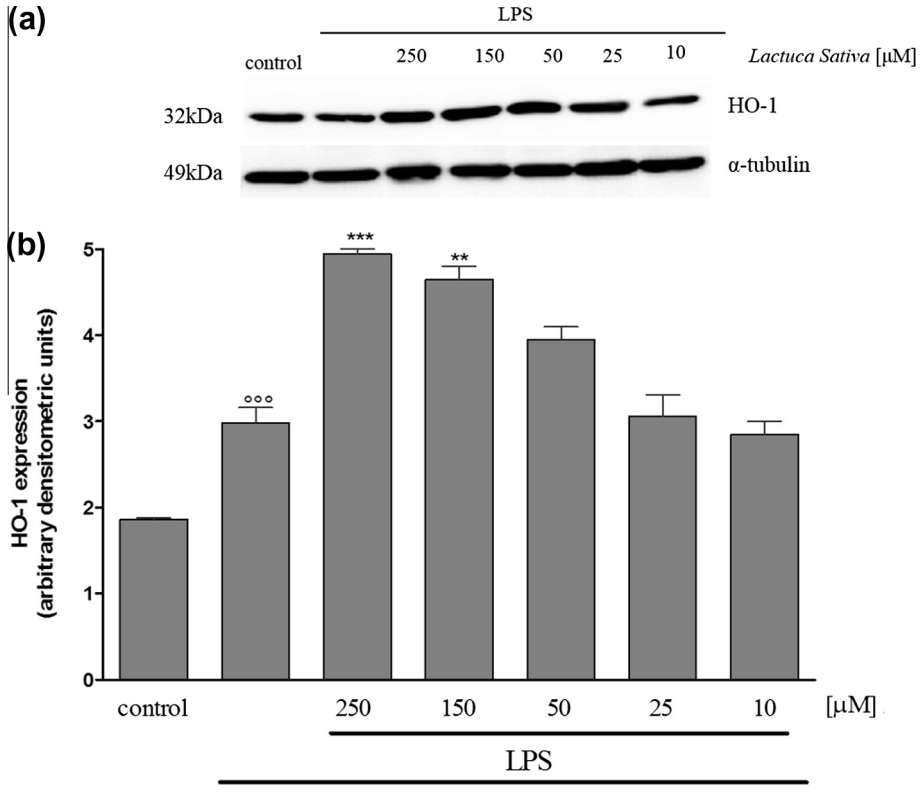

Fig. 6. Representative Western blot of heme-oxygenase (HO-1) enzyme expression (a). Densitometric analysis of the concentration dependent effect of green lettuce extracts (250–10 μg/mL) on LPS-induced OH-1 expression in J774A.1 macrophages (b). Values, mean ± s.e.m., are expressed as arbitrary densitometric units at least 3 independent experiments with three replicates each. Comparisons were performed using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. °°° Denotes $P < 0.001$ vs. control; *** and ** denotes $P < 0.001$, $P < 0.01$ and vs. respectively J774A.1 treated with LPS alone.

NOS, (4) modulation of the production of other proinflammatory molecules, (5) modulation of proinflammatory gene expression (García-Lafuente, Guillamón, Villares, Rostagno, & Martínez, 2009). More recently also cumarin has been proposed as nucleus for antinflammatory molecules. Coumarins and their derivatives have acquired much attention from the pharmacological and pharmaceutical field due to their broad range of therapeutic potential. Several coumarins analogs have been reported as potential antiinflammatory agents by inhibiting also mediators of inflammation in macrophages, such as iNOS and COX-2 (Hemshekhar, Sunitha, Thushara, Sebastin Santhosh, et al., 2013; Li et al., 2012). On this basis, we can hypothesize that the antinflammatory and antioxidant effect of lettuce extracts on LPS-stimulated macrophages could be in partly addressed to the presence of these biological active compounds.

3.4. Conclusion

Green lettuce is an important dietary leafy vegetable and our results have shown that it possess strong antioxidant and anti-inflammatory activities. The analytical method, based on a fast UHPLC–DAD–IT-TOF platform, was able to characterise the polyphenolic compounds in a very short time, with respect to other methods reported. After the analytical analysis we showed how lettuce extract significantly decreased reactive oxygen species, nitric oxide release, inducible nitric oxide synthase and cycloxygenase-2 expression, leading to the global reduction of the inflammation process. The polyphenolic profile described above let us to hypothesize that biological activity of green lettuce could be addressed to the presence of high amounts of hydroxycinnamic acids derivatives, as well as of flavon-3-ols and coumarins, suggesting that its value is not only nutritional and this nutraceutical potential could provide important health-promoting benefits.

Conflict of interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.06.105.

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