Research Article

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Anti-inflammatory and antioxidant activity of polyphenolic extracts from *Lactuca sativa* (var. *Maravilla de Verano*) under different farming methods

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Abstract

BACKGROUND: Besides their nutritional value, vegetables are a source of health-promoting compounds, such as polyphenols, and their content can be influenced by the particular farming method. In this study polyphenolic extracts from *Lactuca sativa* (var. *Maravilla de verano*) plants cultivated with different farming methods were chemically characterised and tested *in vitro* and *ex vivo* inflammation models.

RESULTS: The tested extacts (250 – 2.5 μg mL^{-1}) were able to reduce both the inflammatory and oxidative stress in LPS-stimulated J774A.1 murine monocyte macrophage cells, by lowering the release of nitric oxide (NO) and reactive oxygen species (ROS) and promoting nuclear translocation of nuclear factor (erythroid-derived 2)-like 2; (Nrf2) and nuclear factor-κB (NF-κB). In this regard, quantitative profiles revealed different amounts of polyphenols, in particular quercetin levels were higher in plants under mineral fertilised treatment. Those extract showed an enhanced anti-inflammatory and antioxidant activity.

CONCLUSION: Our data showed the anti-inflammatory and antioxidant potential of *Maravilla de Verano* polyphenolic extracts. The effect of farming methods on polyphenolic levels was highlighted. The higher reduction of inflammatory mediators release in extracts from plants cultivated under mineral fertilisation treatment was correlated to the higher amount of quercetin. These results can be useful for both nutraceutical or agronomic purposes.

Supporting information may be found in the online version of this article.

Keywords: polyphenols; lettuce; anti-inflammatory; antioxidant; mineral fertilisation

INTRODUCTION

Several pieces of scientific evidence promote a balanced, health-promoting type of diet, based on vegetables and fruit, as the basis to prevent the onset of chronic and age-related diseases. Edible plants are an important source of mineral components and natural antioxidants with healthy activity, such as polyphenols.[1,2] Among vegetables, green lettuce (*Lactuca sativa* L.) is widely consumed worldwide as a fresh product.[3] The absence of any cooking process emphasises the importance of this product, since the degradation of the thermolabile phytochemical species having potential nutraceutical interest is preserved. The most abundant classes of polyphenols in lettuce are phenolic acids (caffeic acid derivatives) and flavonols.[4–6] As they act as free radical scavengers, both these classes of secondary plant metabolites are used to counter oxidative stress caused by the environmental adversities and agronomic conditions experienced by plants.[3,7–9] The polyphenolic content of lettuce can be

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influenced by environmental factors such as excessive light and UV, extreme temperature, water deficit, soil mineral composition, diseases, and climatic conditions, as well as agronomical practices, such as fertilisation plans, soil management, and cultivation techniques can cause quantitative and qualitative changes in phenolic compounds.[7,10–14] Various studies have investigated the effects of the polyphenolic compounds present in green lettuce, in reducing oxidative and anti-inflammatory stresses.[15–20] Modulation of chronic inflammation and oxidative stress represent today one of the main research area. While acute inflammation and controlled release of reactive oxygen species (ROS) are therapeutic, chronic inflammation and oxidative stress cause numerous chronic diseases.[21] Macrophages play a major role in host defence during inflammatory and immune response and it is widely known 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 ROS and nitric oxide (NO). Moreover, during inflammatory and oxidative stress conditions, in order to protect themselves from damage, cells also activate protective response [e.g. nuclear factor (erythroid-derived 2)-like 2; Nrf2]. In this work we focused on the anti-inflammatory and antioxidant activity of polyphenolic extracts of green lettuce (var. *Maravilla de Verano)* under different farming treatments. Extracts were firstly chemically characterised and then tested on macrophages, both *in vitro* and *ex vivo*. Inflammatory and oxidative stress parameters, such as iNOS and COX-2 expression, NF-κB activation, cytokines and ROS release, nitrotyrosine formation and cytoprotective factors, Nrf-2 and haem oxygenase-1, were evaluated and the extracts showed different activities in relation to their polyphenolic content.

MATERIALS AND METHODS

Reagents

Unless stated otherwise, all reagents and compounds were purchased from Sigma–Aldrich Chemicals Company (Milan, Italy). Ultra-pure water (H_2O) was obtained by a Milli-Q Direct 8 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. Two different columns were employed in this work: a Kinetex C18, 150 × 2.1 mm, (100 Å), length × internal diameter (L. × I.D.), packed with 2.6 µm particles for LC-MS/MS analyses, and a Kinetex C18, 150 × 4.6 mm, L. × I.D., 2.6 µm (Phenomenex, Bologna, Italy) for LC-DAD quantitative analysis. Both columns were protected with C18 precolumns (Phenomenex).

Experimental site and plant material

The experiment was conducted in 2011 in an open-field farm located in Järna (central Sweden), a conventional farm which was well representative of a conventional farm that has not had animals and forage cultivation for over 50 years. Meteorological variables were monitored by weather stations of the national weather measuring system of Swedish Meteorological and Hydrological Institute, placed at 50 m from the experimental fields. Climatic variables in the period June to July 2011, when lettuce was cultivated, the rainfall in the experimental area was lower (72 mm in) than normal (123 mm; average of the last 50 years). The average temperature from June to July 2011 (18.3 °C) was warmer than normal (16.0 °C). The number of sunshine hours [photosynthetically active radiation (PAR) under clear sky, >3600 mmol m^{-2} h^{-1}] in the same period was 610, compared to 571 of a normal year. The green lettuce (*Lactuca sativa* L.) variety used was *Maravilla de Verano*. The experiment were performed as a randomised block trial with four blocks (replicates), including one unfertilised control (treatment A) and three fertilisation treatments with different types of fertilisation: [1] 120 t manure ha^{-1} (treatment C); [2] 28.8 t rock dust ha^{-1} (treatment E); [3] 120 t manure ha^{-1} + 28.8 t rock dust ha^{-1} (treatment G). An additional mineral fertilised treatment (K) with 150 kg N ha^{-1} was included. Cattle manure that had been composted for 6 months has been used in the treatments C and G. Rock dust (treatments E and G) was obtained by finely grinding (approximately 0.5 mm of diameter) local rocks using a jaw crusher (Model Nordberg C100; Metso Minerals Ltd., Tampere, Finland). Compost stone meal (treatment G) was prepared by mixing the stone powder with cattle manure (<0.2 mm) from diabase, after which the mixture was composted for 6 months. Fertilisers amounts of the treatment K were calculated on the basis of soil texture and nitrogen content in the site. Lettuce was planted on 21 June. The distance between the plants was 0.25 × 0.25 m, with a final plant density of 160 000 plants ha^{-1}. Plants were covered with a nylon cloth to reduce evaporation and protect them against pests. The harvest was carried out on 28 July.

Lettuce extracts and sample preparation

For each plant, outer leaves (second stage of leaves) were detached and lightly washed with distilled water to remove eventual residues. Five grams of 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%, according to Sofo *et al.*[22] The solution was covered with Parafilm to avoid evaporation, shaken at 100 rpm at 20 °C in the dark for 45 min. 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. The extracts were lyophilised and solubilised in methanol prior to analysis by high-performance liquid chromatography (UHPLC) for the determination of the phenolic compounds. It should be pointed out that the extraction conditions, time, temperature and acid concetration, did not affect the chemical nature of the investigated compounds.[23,24]

Instrumentation

LC-DAD-MS/MS analyses were performed on a Shimadzu Nexera UHPLC system, consisting of a CBM-20A controller, two LC-30 AD dual-plunger parallel-flow pumps, a DGU-20 A_{R5} vacuum degasser, an SPD-M20A photo diode array detector, a CTO-20 AC column oven, a SIL-30 AC 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). Quali-quantitative analyses on polyphenolic extracts were carried out by using an optimised method based as reported elsewhere.[20] Detailed UHPLC-MS/MS parameters are reported in the supporting information.

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Cell culture

J774A.1 murine monocyte macrophage cell line (American Type Culture Collection, Rockville, MD, USA), was grown adherent to Petri dishes with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 25 mmol L^{-1} HEPES, 2 mmol L^{-1} glutamine, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin at 37 °C in a 5% CO_2 atmosphere.

Antiproliferative activity

J774A.1 macrophages (5×10^4 $well^{-1}$) were plated on 96-well plates and allowed to adhere for 4 h. Serial dilutions of lettuce extracts (250–10 μg mL^{-1}) were then added for 24 h, 48 or 72 h. Cell viability was assessed using the MTT assay as previously reported.[25,26] Briefly, 25 μL of MTT (5 mg mL^{-1}) were added to cells for 3 h, cells were then lysed and the dark blue crystals solubilised with 100 mL of a solution containing 50% (v/v) *N*,*N*-dimethylformamide, 20% (w/v) 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-DASIT; Milan, Italy) equipped with a 620 nm filter. Macrophage viability in presence of the extracts was calculated as: % dead cells = 100 × (OD treated/OD control).

NO determination and western blot analysis for iNOS and COX-2 expression

To detect NO, evaluated as (NO_2^-), iNOS and COX-2 expression J774A.1 macrophages (5×10^4 $well^{-1}$) were plated on 96-well plates and serial dilutions of C, K, A, G, E lettuce extracts (250–2.5 μg mL^{-1}) were added for 1 h and then for further 24 h in the presence of LPS (1 μg mL^{-1}). For the experiments performed only with C and K extracts, cells were plated in P60 dishes (1.8×10^6) and C and K lettuce extracts (250–10 μg mL^{-1}) were added alone for 1 h and then for further 24 h in presence of LPS (1 μg mL^{-1}). NO generation, evaluated as nitrite (NO_2^-), was evaluated in the culture medium 24 h after LPS stimulation by Griess reaction, as previously reported.[27] The amount of NO_2^-, evaluated as μmol L^{-1} concentration, in the samples was calculated by a sodium nitrite standard curve. In J774A.1 cellular lysates iNOS and COX-2 protein expression was assessed by western blot analysis as previously reported.[28]

TNF-α and IL-6 determination

TNF-α and IL-6 concentrations in J774A.1 culture medium stimulated for 18 h with LPS (1 μg mL^{-1}) in the presence of C and K lettuce extracts (250–10 μg mL^{-1}), as previously described, were assessed by an enzyme-linked immunosorbent assay (ELISA) assay by using a commercial kit, for murine TNF-α or IL-6, according to manufacturer's instruction (e-Biosciences, San Diego, CA, USA). Results are expressed as pg mL^{-1} for TNF-α and IL-6.

Intracellular ROS evaluation

ROS formation was evaluated by means of the probe 2′,7′-dichlorofluorescin-diacetate (H_2DCF-DA).[29] Briefly, J774A.1 macrophages were plated at a density of 3×10^5 cells $well^{-1}$ into 24-well plates and C and K lettuce extracts (250–10 μg mL^{-1}) were added alone for 1 h and then for further 24 h in presence of LPS (1 μg mL^{-1}) or H_2O_2 (100 μmol L^{-1}). In some experiments macrophages were treated at first with LPS for 24 h and then in presence of C and K lettuce extracts for further 24 h. Cells were then collected, washed twice with phosphate buffer saline (PBS) buffer and then incubated in PBS containing H_2DCF-DA (10 μmol L^{-1}) at 37 °C. After 15 min, cell fluorescence was evaluated using fluorescence-activated cell sorting (FACSscan; Becton Dickinson, NJ, USA) and elaborated with Cell Quest software (San Diego, CA, USA).

HO-1 detection by cytofluorimetry

J774A.1 macrophages were plated into 96-well plates (5×10^4 cells $well^{-1}$) and C and K lettuce extracts (250–10 μg mL^{-1}) were added alone for 1 h and then for further 24 h in the presence of LPS (1 μg mL^{-1}). Macrophages were collected, washed twice in PBS, incubated in Fixing Solution for 20 min at 4 °C and then in Fix Perm Solution for 30 min at 4 °C. Anti-HO-1 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and then the secondary antibody in Fix solution were added. Cells fluorescence was evaluated by a fluorescence-activated cell sorting (FACSscan; Becton Dickinson) and elaborated with Cell Quest software as previously reported.[29]

Immunofluorescence analysis with confocal microscopy

Immunofluorescence analysis were performed as previously reported.[29,30] J774A.1 cells (3×10^5 per well) were seeded on coverslips in 12-well plates and treated with C and K lettuce extracts at two medium concentrations (150–50 μg mL^{-1}) for 1 h and then simultaneously with LPS (1 μg mL^{-1}) for 20 min, for nuclear factor-kappaB (NF-κB) and Nrf2, or 24 h, for nitrotyrosine. Rabbit anti-phospho p65 antibody (Santa Cruz Biotechnologies), anti-nitrotyrosine (Millipore) and rabbit anti-phospho Nrf2 antibody (Santa Cruz Biotechnologies) were then added for 1 h at room temperature and fluorescein-conjugated secondary antibody (FITC) was added for 1 h. DAPI was used for counter-staining of nuclei. Coverslips were finally mounted in mounting medium and fluorescent images were taken under a laser confocal microscope (Leica TCS SP5; Leica, Wetzlar, Germany).[30]

Primary murine peritoneal macrophages

Female C57BL/6 mice (6–8 weeks; Harlan Laboratories, Udine, Italy) were fed a standard chow diet and housed under specific pathogen-free conditions at the University of Salerno, Department of Pharmacy. All animal experiments were performed under protocols that followed the Italian and European Community Council for Animal Care (DL. no 116/92). Peritoneal cells were harvested by means of lavage of the peritoneum with 5 mL of EDTA 0.5 mmol L^{-1} plated and allowed to adhere for 2 h at 37 °C in a 5% CO_2 atmosphere. Subsequently, non-adherent cells were removed and RPMI 1640 medium with 10% FBS was added, as previously reported,[30] and cells were treated as reported above.

Evaluation of anti-inflammatory and antioxidant potential of C and K lettuce extracts in mouse peritoneal macrophages

Mouse peritoneal macrophages were plated into 96-well plates (3.5×10^4 cells $well^{-1}$). Peritoneal macrophages were allowed to adhere for 24 h at 37 °C in a 5% CO_2 atmosphere before experiments. Thereafter NO and ROS production, iNOS, COX-2 and HO-1 expression and nitrotyrosine formation were evaluated as performed in J774A.1 macrophages.

Data analysis

Data are reported as mean ± standard error mean (SEM) values of at least three independent experiments, with three replicates

each. Statistical analysis was performed by analysis of variance test, using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. A P value < 0.05 was considered as significant.

RESULTS AND DISCUSSION

Quali-quantitative profiles of lettuce extracts

Polyphenols identification was carried out on the basis of standard retention time and comparing MS/MS data with those present in literature. Results are shown in Table S1 in order of peak elution. Peaks 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. 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 identified as feruloylquinic acid, whose presence in lettuce has been previously reported.[10] Peaks 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 loss of 162 amu $[M-H-180-H_2O]^-$ was observed for peak 7; this compound was identified as esculin. A glucoronidate form[31] was also observed for peak 8, with a main fragment ion with *m/z* 299 deriving from the loss of the sugar, and thus was tentatively proposed as kaempferide-3-*O*-glucuronide. Compound 9 was characterised by 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. Peak 11 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 10 and 12, showed again a loss of a caffeoyl residue $[M-H-C_9H_8O_4]^-$, and they were tentatively identified as methylcaffeoylferuloyltartaric acid isomers.

Quantification of individual polyphenolic compounds was performed by DAD at the maximum absorbance of the compounds of interest. 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. 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.

The phenolic acids (peaks 1–4, 9, 10 and 12) and flavonols (peaks 5, 6, 8 and 12) revealed in the lettuce extracts, sorted in the order of the peaks found in the UHPLC chromatograms, are shown in Table 1. The total phenolic acids in the unfertilisated treatment A were significantly higher than in the fertilisation treatments including manure and/or rock dust (C, E, G). Compared to the other treatments, the total concentration of phenolic acids under mineral fertilisation (treatment K) was significantly lower compared to all the treatments.

The levels of phenolic acids and flavonols were significantly higher in the unfertilised treatment (A) compared to the three fertilised treatments (C, E, G), with significant differences respect to the lowest value found in plants subjected to mineral fetilisation (treatment K). The high content of phenolics in the minerally fertilised treatment (K) could be associated to the presence of more stressful conditions, in terms of plant and/or soil

Table 1. Quantitative profiles of polyphenols extracted from green lettuce cultivated under different fertilisation treatments

Treatment	Chlorogenic acid 1	Feruloyl tartaric acid 2	Feruloylquinic acid 3	Chicoric acid 4	Quercetin 3-*O*-glucoside 5	Isorhamnetin 3-*O*-glucuronide 6	Esculin 7	Kaempferide 3-*O*-glucuronide 8	Caffeoylferuloylquinic acid 9	Methylcaffeoyl feruloyltartaric acid 10	Quercetin 11	Methylcaffeoylferuloyl tartaric acid 12
A	28.01 ± 0.113	38.41 ± 0.021	564.90 ± 0.031	31.74 ± 0.016	257.88 ± 0.065	136.48 ± 0.055	34.78 ± 0.040	181.65 ± 0.032	314.89 ± 0.036	42.34 ± 0.102	39.05 ± 0.055	176.08 ± 0.064
C	20.39 ± 0.038	24.50 ± 0.039	432.16 ± 0.023	31.95 ± 0.021	111.26 ± 0.020	66.35 ± 0.033	27.83 ± 0.111	183.01 ± 0.149	230.04 ± 0.110	31.37 ± 0.050	44.05 ± 0.059	130.22 ± 0.086
E	22.38 ± 0.016	31.46 ± 0.136	507.76 ± 0.002	31.29 ± 0.003	148.64 ± 0.082	90.28 ± 0.094	30.88 ± 0.115	180.53 ± 0.117	262.97 ± 0.134	33.42 ± 0.025	39.78 ± 0.054	156.30 ± 0.029
G	28.43 ± 0.007	32.02 ± 0.011	519.36 ± 0.009	32.83 ± 0.008	183.21 ± 0.016	107.85 ± 0.030	30.44 ± 0.033	156.91 ± 0.021	255.50 ± 0.019	43.15 ± 0.091	26.23 ± 0.060	140.33 ± 0.090
K	19.40 ± 0.034	40.62 ± 0.015	257.31 ± 0.035	22.45 ± 0.214	97.87 ± 0.072	46.79 ± 0.101	33.53 ± 0.069	162.28 ± 0.046	281.63 ± 0.022	37.68 ± 0.114	74.39 ± 0.084	151.11 ± 0.117

The amount of each compound was expressed as microgram per gram of extract.
Data are reported as mean ± relative standard deviation (RSD %) values of at least three independent experiments.
Fertilisation treatment: A, unfertilised control; C, manure; E, rock dust; G, manure + rock dust; K, mineral fertilisation.

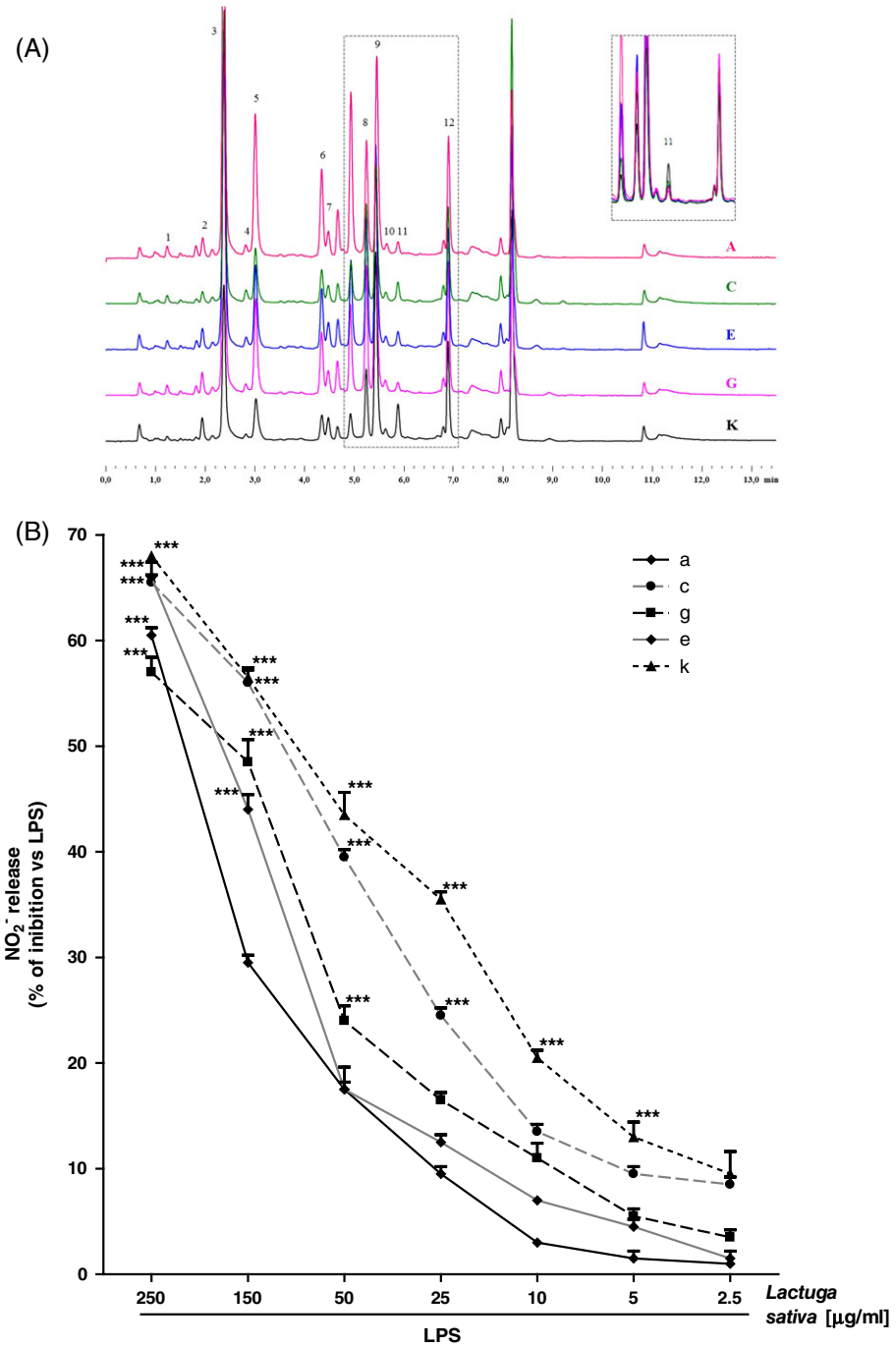

Figure 1. Overlapped chromatograms of different fertilisation treatments, the expansion show the higher abundance of quercetin in K treatment (panel A). Effect of C, K, A, G, E lettuce extracts (250–2.5 µg mL^{-1}) on NO release, evaluated as NO_2^- (µmol L^{-1}) release (% of inhibition vs. LPS), by J774A.1 macrophages stimulated with LPS. Values are expressed as mean ± SEM of NO_2^- (µmol L^{-1}) (panel B).

mineral deficits.[13,14] Despite the lower levels of phenolic acids and flavonoids, plants under mineral fertilisation (K) showed levels of quercetin significantly higher than all other treatments (Fig. 1A).

Lettuce extracts did not affect macrophage viability

To elucidate the influence all lettuce extracts in our experimental conditions on macrophage viability, cells were treated with all the tested lettuce extracts (250–2.5 µg mL^{-1}) for 24 h. Our data indicated that the viability of macrophages was not affected by lettuce extracts treatment (data not shown).

C, K, A, G, E lettuce extracts reduce LPS-induced NO: effect of the different fertilisation systems

LPS induces an inflammatory response that culminates in the release of pro-inflammatory mediators as NO. iNOS is expressed in different cell types, as macrophages, generally in response to various pro-inflammatory stimuli and is the main NOS isoform involved in NO release during inflammation. To assess the effect of different fertilisation systems of lettuce (C, K, A, G, E) in influencing NO production, we measured nitrite release. When C, K, A, G, E lettuce extracts (250–2.5 µg mL^{-1}) were added to J774A.1

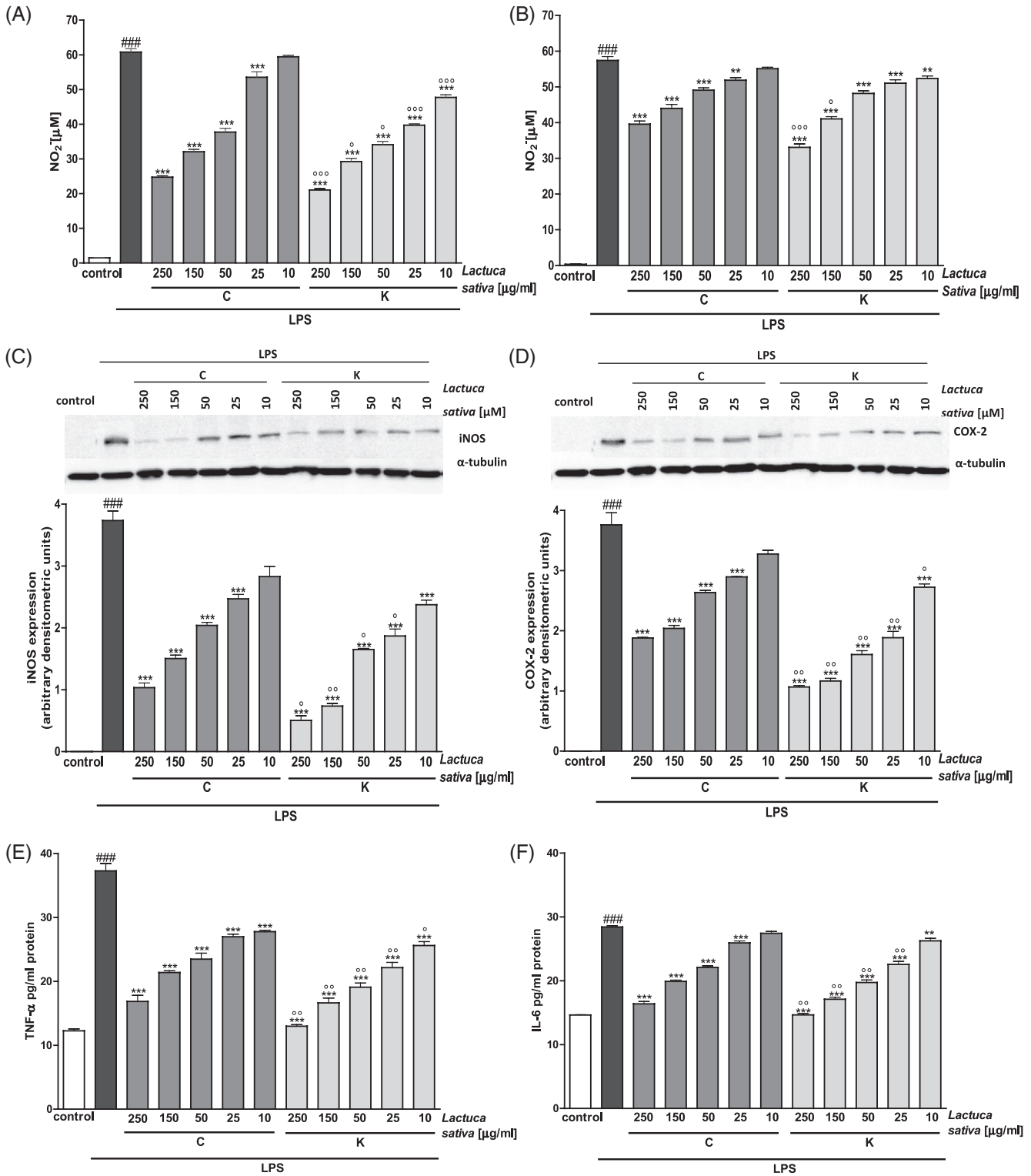

Figure 2. Effect of C and K lettuce extracts (250–10 µg mL^{-1}) on NO release, evaluated as NO_2^- (µmol L^{-1}), by macrophages J774A.1 stimulated with LPS (panels A and B). Representative western blot of inducible nitric oxide synthase (iNOS) expression and densitometric analysis of the effect of *Lactuca sativa* C and K (250–10 µg mL^{-1}) on LPS-induced iNOS expression in J774A.1 macrophages (panel C) and of COX-2 (panel D). Effect of C and K lettuce extracts on LPS-induced TNF-α (panel E) and IL-6 (panel F) production in J774A.1 macrophages. White column: J774A.1 macrophages alone (Control); black column: LPS-treated J774A.1 macrophages (LPS). Values are expressed as mean ± SEM of NO_2^- (µmol L^{-1}) and as arbitrary densitometric units and as pg mL^{-1} proteins. ### denotes $P < 0.001$ vs. control; ***, and ** denote $P < 0.001$, and $P < 0.01$ vs. LPS alone. °°°, °° and ° denote $P < 0.001$, $P < 0.01$ and $P < 0.05$ vs. C lettuce extract.

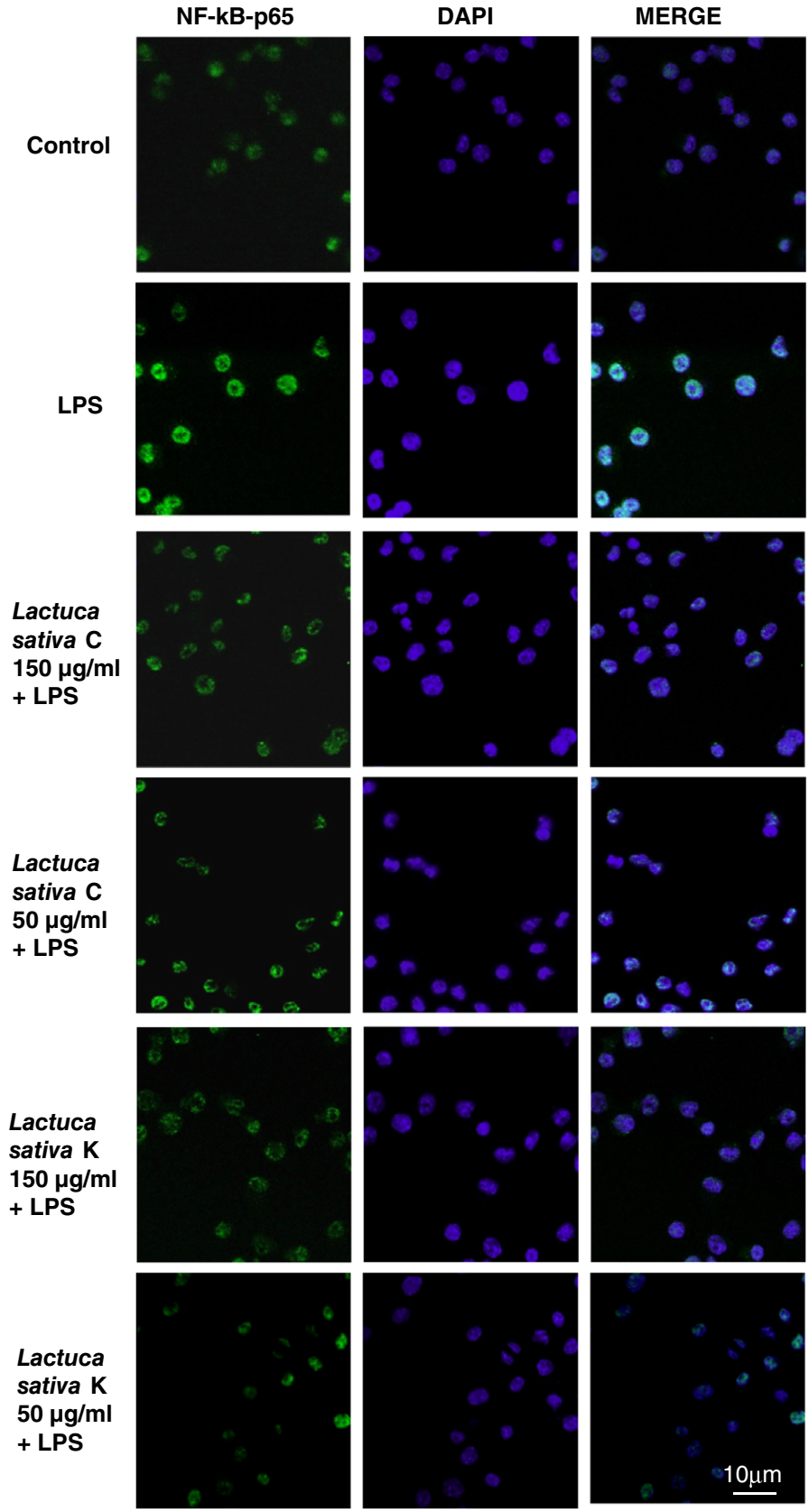

Figure 3. Effect of C and K lettuce extracts (250–10 µg mL^{-1}) on LPS-induced NF-κB p65 nuclear translocation in J774A.1 macrophages. Nuclear translocation of NF-κB p65 subunit was detected using immunofluorescence assay at confocal microscopy. Scale bar, 10 µm. Blue (second column) and green (first column) fluorescences indicate localisation of nucleus (DAPI) and p65 respectively. Analysis was performed by confocal laser scanning microscopy.

macrophages, 1 h before and simultaneously to LPS stimulation, a significantly NO reduction in J774A.1 was observed. Among all different fertilisation systems, C and K exerted the highest inhibitory effect on NO release (Fig. 1B). Based on this initial screening, a comparison between the anti-inflammatory and antioxidant potential of C and K lettuce extracts was conducted.

K lettuce extract significantly inhibit LPS-induced NO, iNOS, COX-2 and cytokine release in J774A.1 macrophages respect to C extract

C and K lettuce extracts (250–10 μg mL^{-1}), significantly inhibited NO release by J774A.1 macrophages (Fig. 2A). In particular, C extract significantly inhibited NO in at concentrations of 250–25 μg mL^{-1} while K extract significantly inhibited NO release at all tested concentrations ($P < 0.01$ vs. LPS and vs. C extract; Fig. 2A). Interestingly, also when added after LPS treatment, both C and K extracts inhibited NO release by J774.A1 indicating their inhibitory effect both on the iNOS enzyme expression and on its activity. Also, in these conditions, K extract exerted a stronger effect both versus LPS and versus C extract ($P < 0.05$; Fig. 2B). LPS macrophage response to LPS was also characterised by an increase in iNOS expression. C and K lettuce extracts (250–10 μg mL^{-1}), significantly and in a concentration-related manner inhibited iNOS expression in J774A.1 macrophages (Fig. 2C). C lettuce extract exerted its effect in a concentration range between 250 and 25 μg mL^{-1} while K extract significantly inhibited iNOS expression at all tested concentrations ($P < 0.05$ vs. LPS and vs. C lettuce extract; Fig. 2C). An interaction between iNOS and COX pathway represents an important mechanism for inflammatory response modulation. COX-2 is a well known pro-inflammatory enzyme triggered by agents as LPS, it is involved in macrophage response and its expression resulted also influenced by NO.[32] Our data showed that also COX-2 protein expression was strongly inhibited, at all concentration tested, by C and K extracts, respect to LPS-treated macrophages. In particular, also COX-2 resulted stronger inhibited by K lettuce extract respect to C extract ($P < 0.05$; Fig. 2D). In response to foreign invaders and/or chemical signals generated by the affected tissue, macrophages release various inflammatory cytokines, as TNF-α and IL-6, which contribute to the inflammatory response. A substantial body of work indicates that these mediators generated by macrophages, and dependent by NF-κB activation, can act in an autocrine or paracrine manner to regulate each other in their synthesis, production and function.[33] Although cytokine production is necessary to protect against pathogens and promote tissue repair, excessive release or decreased clearance, or both, can lead to organ failure and premature death. In our experimental model C and K lettuce extracts (250–10 μg mL^{-1}) significantly reduced TNF-α and IL-6 release ($P < 0.001$ vs. control; Fig. 2E and F, respectively). While TNF-α resulted equally inhibited by both extracts IL-6 resulted strongly inhibited by K extract ($P < 0.01$ vs. LPS and vs. extract C; Fig. 2F).

K lettuce extract inhibits p65 NF-κB nuclear translocation stronger than C extract in LPS- treated J774A.1 macrophages

LPS activates the pro-inflammatory transcription factor NF-κB which role is essential in inflammation and oxidative stress.[34] It has been reported to activate more than 500 genes, most of which implicated in inflammation and to regulate pro-inflammatory enzyme production (e.g. iNOS, COX-2), antigen presentation, pattern recognition and phagocytosis and cytokine production. Following p65 phosphorylation, the free NF-κB dimers translocate into the nucleus and bind to specific sequences to regulate the downstream genes expression.[35] Thus, we labelled p65 with a green fluorescence to track the influence of C and K lettuce extracts (150–50 μg mL^{-1}) and added 1 h before LPS (1 μg mL^{-1}) on p65 NF-κB translocation. NF-κB activation was reduced by C lettuce extract but mostly by K lettuce extract in J774A.1 treated macrophages, compared to LPS alone (Fig. 3). This result indicates the stronger activity of K lettuce extract also on the early steps of inflammatory response.

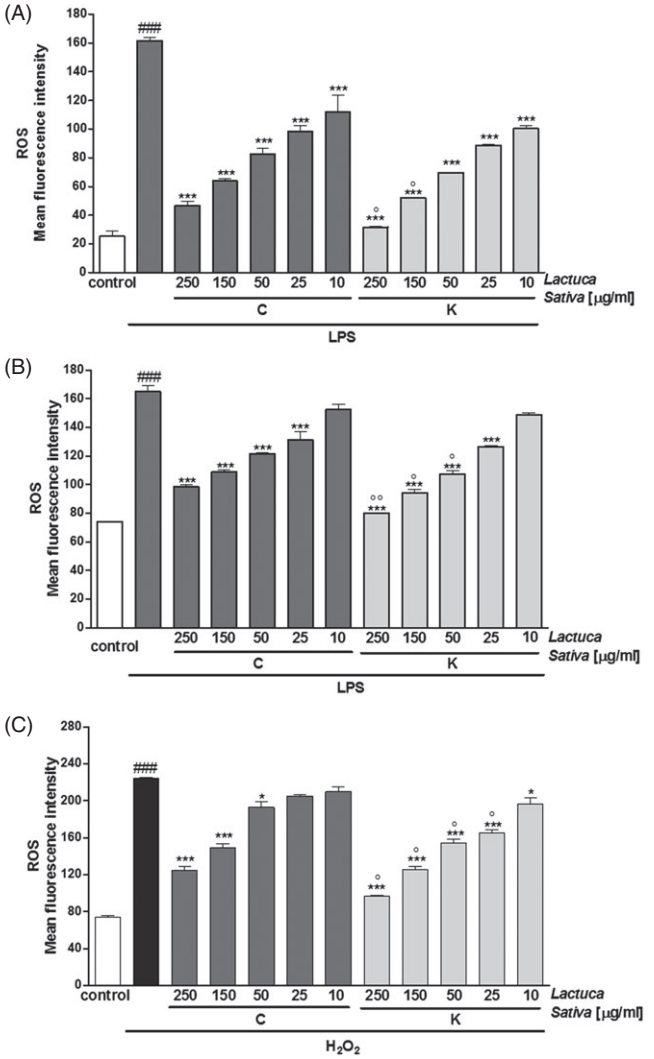

Figure 4. Effect of C and K lettuce extracts (250–10 μg mL^{-1}) on ROS formation, evaluated by means of the probe 2′,7′-dichlorofluorescein-diacetate (H_2DCF-DA). J774A.1 macrophages were treated with the extracts before (panel A) or after (panel B) LPS and before H_2O_2 (panel C). White column bar: J774A.1 macrophages alone (Control); black column: LPS-treated (panels A and B) or H_2O_2-treated (panel C) J774A.1 macrophages. ### denotes $P < 0.001$ vs. control; *** and * denote $P < 0.001$, and $P < 0.05$ vs. J774A.1 treated with LPS or H_2O_2-treated macrophages. °° and ° denote $P < 0.01$ and $P < 0.05$ vs. C lettuce extract.

K lettuce extract significantly inhibits ROS in J774A.1 macrophages respect to C extract

ROS are essential mediators both of inflammatory response and in oxidative stress conditions. C and K lettuce extracts (250–10 μg mL^{-1}) significantly inhibited ROS production in LPS-treated J774A.1 macrophages at all concentrations tested

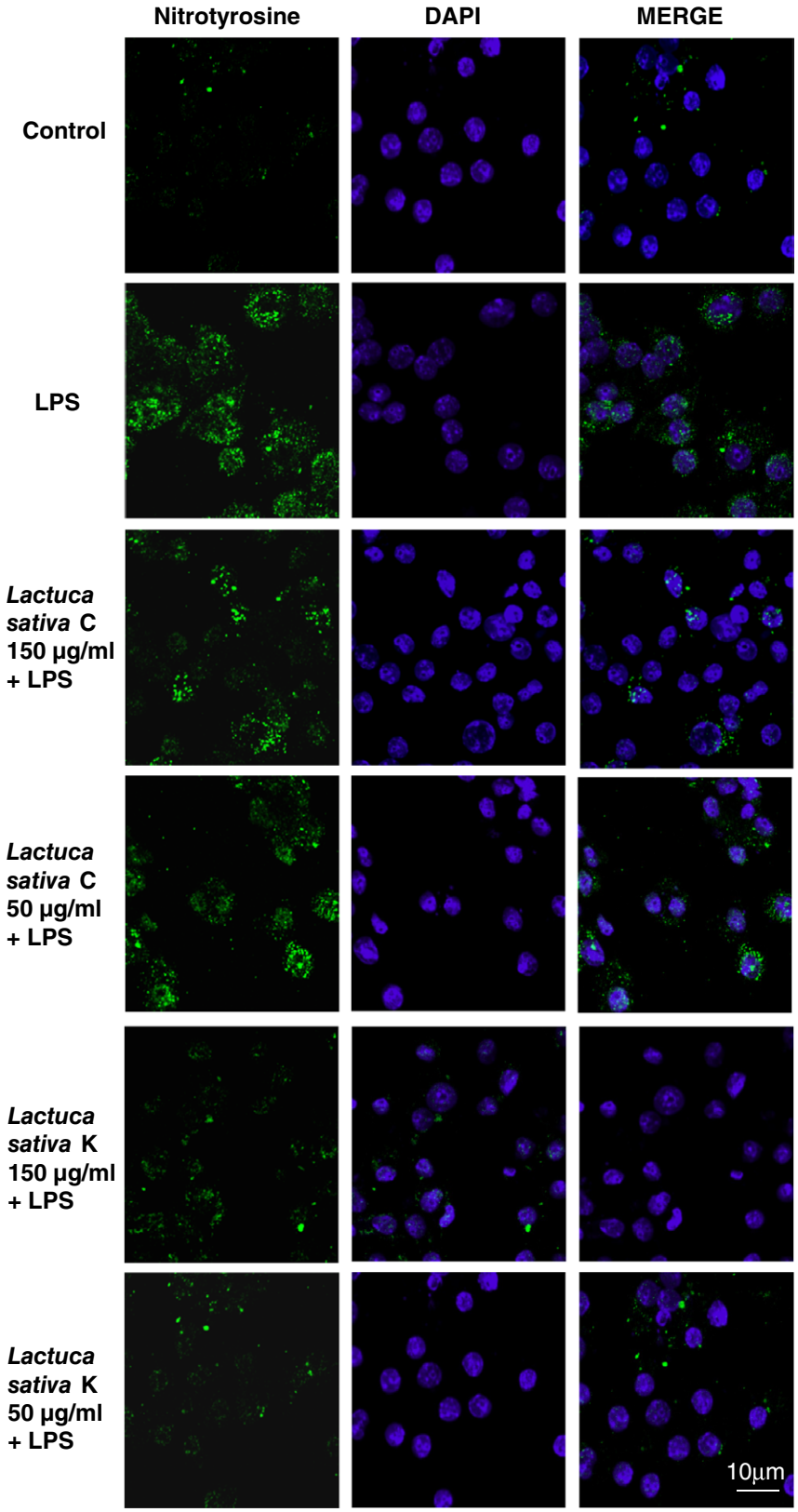

Figure 5. Effect of C and K lettuce extracts (250 – 10 µg mL^{-1}) on LPS-induced nitrotyrosine in J774A.1 macrophages. Scale bar, 10 µm. Blue (second column) and green (first column) fluorescences indicate localisation of nucleus (DAPI) and nitrotyrosine respectively. Analysis was performed by confocal laser scanning microscopy.

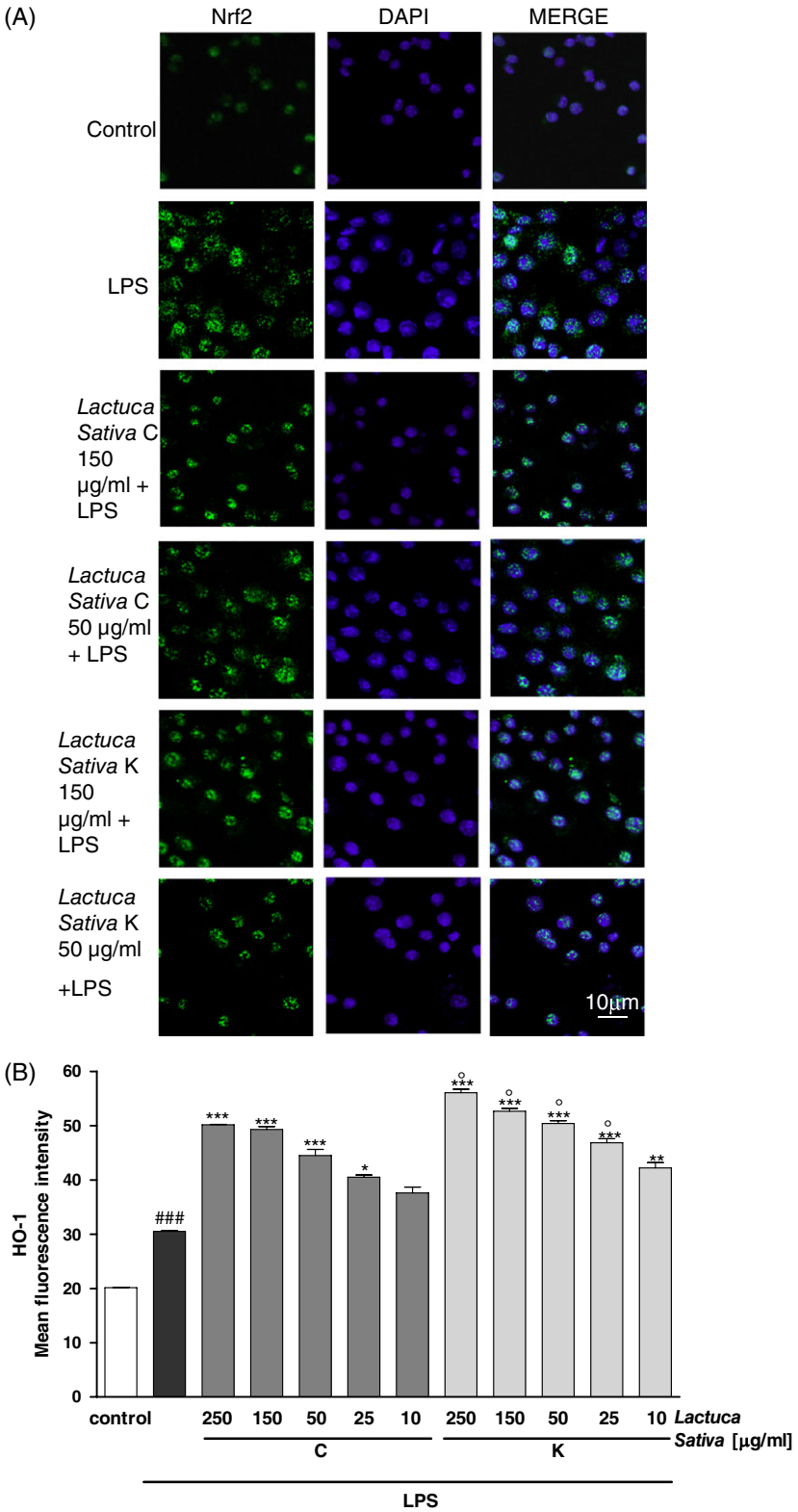

Figure 6. Effect of C and K lettuce extracts (250–10 µg mL^{-1}) on LPS-induced Nrf2 nuclear translocation in J774A.1 macrophages (panel A). Nuclear translocation of Nrf2 was detected using immunofluorescence assay at confocal microscopy. Scale bar, 10 µm. Blue (second column) and green (first column) fluorescences indicate localisation of nucleus (DAPI) and Nrf2 respectively. Analysis was performed by confocal laser scanning microscopy. Effect of C and K lettuce extract (250–10 µg mL^{-1}) on HO-1 expression in LPS-treated J774A.1 macrophages (panel B). White column: J774A.1 macrophages alone (Control); black column: LPS-treated J774A.1 macrophages (LPS). ### denotes $P < 0.001$ vs. control; ***, ** and * denote $P < 0.001$, $P < 0.01$ and $P < 0.05$ vs. J774A.1 treated with LPS or H_2O_2-treated macrophages. ^{oo} and ^{o} denote $P < 0.01$ and $P < 0.05$ vs. C lettuce extract.

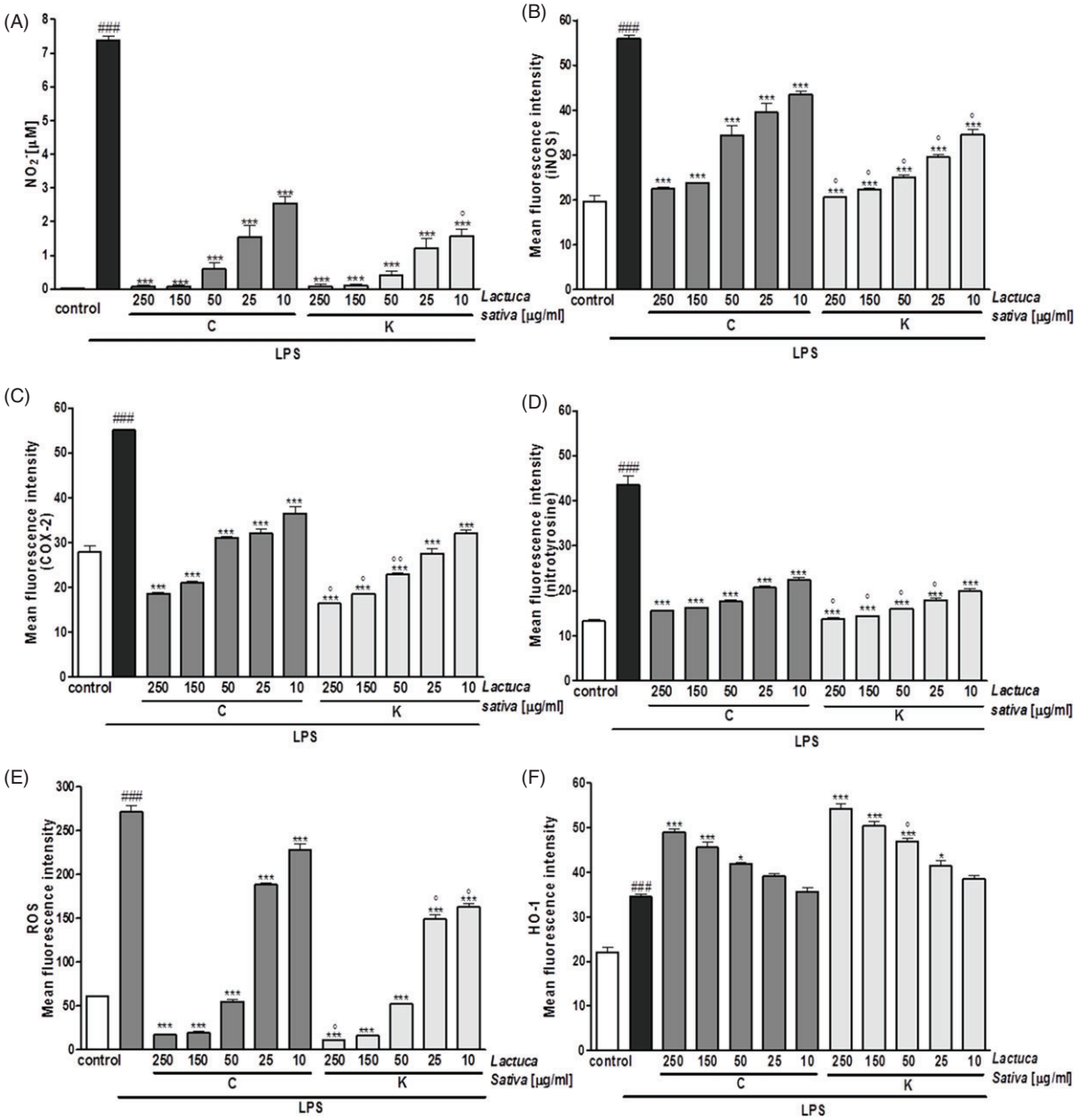

Figure 7. Effect of C and K lettuce extracts (250–10 µg mL^{-1}) on NO release, evaluated as NO_2^- (µmol L^{-1}), by primary peritoneal macrophages stimulated with LPS (panel A). Effect of *Lactuca sativa* C and K (250–10 µg mL^{-1}) on LPS-induced iNOS (panel B), COX-2 (panel C) and nitrotyrosine (panel D) in peritoneal macrophages. Effect of C and K lettuce extract (250–10 µg mL^{-1}) on ROS formation (panel E) and on HO-1 expression (panel F) in LPS-stimulated peritoneal macrophages. White column: primary peritoneal macrophages alone (Control); black column: LPS-treated primary peritoneal macrophages (LPS). ### denotes $P < 0.001$ vs. control; *** and * denote $P < 0.001$ and $P < 0.05$ vs. LPS alone. °° and ° denote $P < 0.01$ and $P < 0.05$ vs. C lettuce extracts.

($P < 0.001$). In particular, K extract (250–10 µg mL^{-1}) significantly inhibited ROS production versus C lettuce extract (250–150 µg mL^{-1}; $P < 0.05$; Fig. 4A). Interestingly, when added after LPS treatment, both C and K extracts also inhibited ROS production in J774.A1 macrophages in a concentration range of 250–25 µg mL^{-1} ($P < 0.001$; Fig. 4B) thus indicating an antioxidant potential also when added to macrophages after the inflammatory stimulus. Also in this experimental condition, K extract exerted a stronger effect respect to C extract (250–50 µg mL^{-1}; $P < 0.05$; Fig. 4B).

When macrophages were treated with H_2O_2, a pro-oxidant agent, both C and K lettuce extracts inhibited ROS production in J774.A1 macrophages but K extract exerted the stronger effect ($P < 0.05$; Fig. 4C).

K lettuce extract inhibits nitrotyrosine formation stronger than C extract in LPS-treated J774A.1 macrophages

During inflammation, NO rapidly reacts with superoxide anion generating the toxic metabolite peroxynitrite. Peroxynitrite can nitrate tyrosine residues in proteins, resulting in nitrotyrosine formation. Because tyrosine nitration is an alternative to phosphorylation at key residues, this process can affect protein enzymatic activity and intracellular signalling processes.[36] Both C and K lettuce extracts (150–50 µg mL^{-1}) reduced nitrotyrosine formation respect to LPS (Fig. 5). Also, in this case, K extract showed a stronger ability to inhibit nitrotyrosine formation, giving a further insight into its protective effect in inflammatory and oxidative stress conditions.

K lettuce extract increases Nrf2 nuclear translocation stronger than C extract in LPS-treated J774A.1 macrophages

A recurrent theme in oxidant signalling and antioxidant defence is reactive cysteine thiol-based redox signalling. Nrf2 is an emerging regulator of cellular resistance to oxidants. Nrf2 controls the basal and induced expression of an array of antioxidant response element-dependent genes that regulate the physiological and physiopathological outcomes of oxidant exposure (e.g. HO-1).

Following its activation, Nrf2 translocates into the nucleus and bind to specific sequences to regulate the downstream genes expression. As shown in Fig. 6A, nuclear Nrf2 was increased after 1 h by LPS. Nrf2 translocation was further increased by C lettuce extracts but mostly by K lettuce extract in J774A.1 treated macrophages indicating the stronger activity of K lettuce extract on the early steps of antioxidant response.

In order to protect themselves against inflammatory and oxidative injury cells, as macrophages, up-regulates some defence mechanisms as HO-1 expression. HO-1, the rate-limiting enzyme in haem degradation, catalyzes the oxidation of haem to generate several biologically active molecules carbon monoxide (CO), biliverdin, and ferrous ion. HO-1 can increase cellular antioxidant status by generating antioxidants such as bilirubin, which can inhibit iNOS protein expression and suppress NO production. HO-1 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.[37–41] Present in J774A.1 macrophages at low levels in basal condition HO-1 resulted increased by LPS ($P < 0.001$; Fig. 6B). Moreover C lettuce extracts further increased HO-1 enzyme expression in J774A.1 macrophages in a concentration range of 250–25 μg mL^{-1} ($P < 0.05$) whereas K lettuce extracts significantly increase HO-1 at all tested concentrations ($P < 0.01$ vs. LPS) and in a concentrations range of 250–25 μg mL^{-1} versus C lettuce extracts ($P < 0.05$; Fig. 6B).

K lettuce extract inhibits inflammation and oxidative stress in mouse primary macrophages stronger than C extract

To further confirm the observed stronger effect of K lettuce extract versus C extract we evaluate NO and ROS release, iNOS and COX-2 expression and nitrotyrosine formation in LPS-treated mouse peritoneal primary macrophages.

In mouse primary macrophage, both K and C lettuce extracts (250–10 μg mL^{-1}) significantly inhibited NO release at all tested concentrations ($P < 0.001$ vs. LPS alone; Fig. 7A) and the effect of K extract at the lowest concentration resulted significantly higher compared to C extracts ($P < 0.05$; Fig. 7A). In addition also iNOS (Fig. 7B) and COX-2 (Fig. 7C) expression and nitrotyrosine formation resulted significantly inhibited by K and C lettuce extract and the effect of K extract resulted significantly higher respect to C ($P < 0.05$; Fig. 7D).

In mouse primary macrophage, both the lettuce extracts (250–10 μg mL^{-1}) significantly inhibited ROS production at all tested concentrations ($P < 0.001$; Fig. 7E) with a stronger effect for K extract ($P < 0.05$; Fig. 7E). In addition also HO-1 resulted significantly increased by both lettuce with a stronger effect for K lettuce extract ($P < 0.05$; Fig. 7F). These results confirm the anti-inflammatory and antioxidant potential of lettuce extracts also in mouse primary macrophage, highlighting the stronger action of K extract, respect to C, in correlation with the major amount of quercetin, whose anti-inflammatory properties have been reported before,[42] and thus the importance of different fertilisation systems in determining the observed biological effects.

CONCLUSIONS

Green lettuce was found to be a rich source of anti-inflammatory compounds, as was shown in our experimental system. Depending upon the type of lettuce fertilisation, significant quantitative and qualitative variations in lettuce leaf phenolic composition were observed. From the overall analysis of the results, the higher content of quercetin in the lettuce treated with mineral fertilisation (K), compared to the other treatments and particularly to the organically fertilised C treatment. The high values of quercetin corresponded to an higher anti-inflammatory and antioxidant potential of these extracts. In mammals, free quercetin is the most studied flavonols for its powerful cytoprotective effect against inflammation, likely due also to an induced antioxidative stress–response.[43] The mineral fertilisation extract K revealed also an high abundance of caffeoyl derivatives which could contribute to its capability to inhibit iNOS and COX-2 expression and to activate Nrf2 antioxidant response.[44] Thus the contemporary presence in K extract of higher concentrations of quercitin and caffeoyl derivatives could explain its higher activity respect to C extract. This study could help further research concerning the bioavailability of flavonols in green lettuce, where soil parameters together with the fluctuations of nutrients in plants and crop yield should be taken into account before scheduling optimal farming systems.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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