Food & Function

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c5fo00475f

Anti-inflammatory activity of horseradish (*Armoracia rusticana*) root extracts in LPS-stimulated macrophages

Stefania Marzocco,*[a] Luana Calabrone,[b] Simona Adesso,[a] Marilena Larocca,[b] Silvia Franceschelli,[a] Giuseppina Autore,[a] Giuseppe Martelli[b] and Rocco Rossano[b]

Received 30th April 2015,
Accepted 8th September 2015

DOI: 10.1039/c5fo00475f

www.rsc.org/foodfunction

Horseradish (*Armoracia rusticana*) is a perennial crop belonging to the Brassicaceae family. Horseradish root is used as a condiment due to its extremely pungent flavour, deriving from the high content of glucosinolates and their breakdown products such as isothiocyanates and other sulfur compounds. Horseradish also has a long history in ethnomedicine. In this study the anti-inflammatory potential of three accessions of *Armoracia rusticana* on lipopolysaccharide from *E. coli* treated J774A.1 murine macrophages was evaluated. Our results demonstrate that *Armoracia rusticana* reduced nitric oxide, tumor necrosis factor-α and interleukin-6 release and nitric oxide synthase and cyclooxygenase-2 expression in macrophages, acting on nuclear transcription factor NF-κB p65 activation. Moreover *Armoracia rusticana* reduced reactive oxygen species release and increased heme-oxygenase-1 expression, thus contributing to the cytoprotective cellular effect during inflammation.

Introduction

Inflammation is a biological response induced by microbial infection or tissue injury that involves an enormous expenditure of metabolic energy, damage and destruction of host tissues, even involving risk of sepsis, multiple organ failure and death. In principle, inflammation is an essential response to eliminate aggressors but can be deleterious when the initial reaction is not limited. In these cases, anti-inflammatory compounds are therapeutically useful and are administered to control the inflammation response.

During inflammation macrophages play a major role in defending the host; however, their excessive activation may contribute to extensive tissue damage. Macrophage activation by bacterial cell wall components such as lipopolysaccharide from *E. coli* (LPS), a component of the Gram-negative bacteria, promotes the synthesis and release of large amounts of mediators involved in the inflammatory onset such as cytokines (*e.g.* Tumor Necrosis Factor-α, TNF-α, and Interleukin-6, IL-6), nitric oxide (NO), pro-inflammatory enzymes (*e.g.* cyclooxygenase-2, COX-2) and reactive oxygen species[1] (ROS). Transient activation of the nuclear transcription factor NF-κB constitutes an important step in the course of inflammatory responses and plays a key role in the regulated expression of several pro-inflammatory mediators including cytokines and pro-inflammatory enzymes[2] (*e.g.* nitric oxide synthase, iNOS) and COX-2. Because of this pivotal role NF-κB is a relevant target for the pharmacological action of anti-inflammatory molecule activation in a variety of inflammatory conditions.[2] Also the oxidative response regulates many physiological responses in human health and, as inflammation, if not properly regulated it could also lead to a number of deleterious effects mediating many aspects of inflammatory-induced tissue damage and dysfunctions.[3,4] Nowadays, the study of oxygen-containing free radicals in humans and their role has been of growing interest among scientists. Synthetic antioxidants are known to have free radical inhibition properties in the human body but these compounds could also be toxic as they present hazards to the human body as well. The most important and useful source of such inhibitors, with both anti-inflammatory and anti-oxidant properties, is the area of natural products.

Horseradish (*Armoracia rusticana* Gaertn) is a perennial crop belonging to the Brassicaceae family. Due to its extremely pungent root, horseradish is used freshly grated as a condiment for meat and fish products or in sauces. The ethnomedical uses of *Armoracia rusticana* leaves and roots have a long history. Horseradish is rich in glucosinolates (GLSs) that provide the characteristic flavor[5] and aroma as a result of their breakdown into isothiocyanates (ITCs) and other sulfur compounds. Horseradish, as well as the other members of the Brassicaceae family, represents a rich source of health-promot-

[a]Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, 84084 Fisciano (Salerno), Italy. E-mail: smarzocco@unisa.it; Tel: +39 089969250
[b]Department of Sciences, University of Basilicata, Viale dell'Ateneo Lucano, 10, I-85100 Potenza, Italy

ing phytochemicals. Their beneficial effects have been principally attributed to the anticancer properties of GLSs and their ITC derivatives,[6] complex mixtures of phenolic compounds possessing antioxidant activity[7,8] and vitamins.[9] Several studies have been conducted on the allyl-isothiocyanate (AITC) effects, its main ITC resulting from the hydrolysis of the corresponding glucosinolate sinigrin by myrosinase enzyme, highlighting its anticancer properties.[6,10,11]

Several authors reported on the AITC action on inflammatory mediator production in macrophage cells.[12–15] Also, some authors[16] reported on the tumor cell proliferation inhibition by monogalactosyl diacylglycerides isolated from horseradish rhizomes.

In this study we investigated the anti-inflammatory effect of root horseradish extracts, deriving from three accessions, on LPS-induced inflammation in J774A.1 macrophages. After identifying the most active accession we investigated, in particular, its anti-inflammatory effect and the myrosinase contribution to (1) NO production; (2) iNOS and COX-2 expression; (3) TNF-α and IL-6 release; (4) p65 NF-κB nuclear translocation; (5) ROS production and (6) the cytoprotective enzyme heme-oxygenase (HO-1) expression.

Results and discussion

Myrosinase activity and sinigrin content

Plants such as the Brassicaceae, which are always able to synthesize glucosinolates, also possess a β-thioglucoside glucohydrolase commonly known as myrosinase (EC 3.2.3.1). Myrosinase represents a group of isoenzymes present in all Brassicaceae species examined and is also found in 14 other plant families and in the colon microflora.[5] Glucosinolates are hydrolyzed by myrosinase leading to the generation of several compounds such as isothiocyanates, thiocyanates, sulfate, glucose, nitriles and epithioalkanes.[17] Among the three horseradish samples analyzed (Fig. 1A), the extract obtained from the accession GUA showed the highest myrosinase activity (1.02 ± 0.14 U per mg protein). Regarding sinigrin content, the predominant glucosinolate in the horseradish plant, in our study the sinigrin concentration ranged from 52.71 to 66.58 μmol per g DW, with the sample TRI showing the highest value (Table 1).

GUA, MIN, and TRI do not affect J774A.1 macrophage cell proliferation

Cell viability of J774A.1 macrophages treated with GUA, MIN, and TRI, also in the presence of myrosinase, was not significantly different from untreated cells. Cell viability, expressed as a percentage of viability *vs.* the control, for GUA, MIN, TRI and TRI plus myrosinase was 94.9 ± 0.9, 95.1 ± 1.2, 94.8 ± 1.9, 94.1 ± 1.5 respectively (200 μg mL^{-1}); 96.1 ± 1.3, 97.1 ± 0.8, 94.2 ± 0.9, 96.3 ± 1.3 respectively (150 μg mL^{-1}); 95.2 ± 1.5, 96.4 ± 1.4, 96.5 ± 1.7, 96.8 ± 1.6 respectively (100 μg mL^{-1}); 97.2 ± 1.3, 97.6 ± 1.8, 95.7 ± 0.8, 95.1 ± 1.9 respectively (50 μg mL^{-1}); and 98.1 ± 1.5, 96.9 ± 1.9, 98.4 ± 1.0, 98.3 ± 1.3 respectively (25 μg mL^{-1}; $P > 0.05$ *vs.* control). Thus the IC_{50} for all the tested extracts on J774A.1 macrophages was >200 μg mL^{-1}. The absence of antiproliferative activity under our experimental conditions indicated that all the evaluated parameters to assess the anti-inflammatory potential of the extracts were uninfluenced by the cytotoxic activity on J774A.1 macrophages.

GUA, MIN, TRI reduce NO release and iNOS and COX-2 expression in LPS-treated J774A.1 macrophages

LPS induces an inflammatory response that culminates in the release of pro-inflammatory mediators such as NO, iNOS, COX-2 and ROS associated with mechanisms aimed at protecting the cell, such as HO-1 enzyme expression. NO is a pleiotropic mediator that acts on a variety of physiological and pathophysiological processes. This molecule is produced from the oxidation of L-arginine by the NOS enzyme, which occurs in the form of two major classes: one is constitutive (including endothelial and neuronal isoforms) and the other is inducible (including macrophagic isoform). iNOS may be expressed in different cell types (*e.g.* macrophages, smooth muscle cells, epithelia) by various proinflammatory agents such as LPS. When macrophages are activated by LPS or IFN-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, is essential for the up-regulation of the inflammatory response. Furthermore, NO contributes to tissue damage both directly *via* the formation of peroxynitrite, with its associated toxicity, and indirectly through the amplification of the inflammatory response. In our experiments, LPS induced in J774A.1 macrophages a marked increase in NO release associated with an increased iNOS expression: GUA, MIN, TRI (200–50 μg mL^{-1}) significantly reduced NO release (200–150 μg mL^{-1}; $P < 0.001$ *vs.* LPS alone; Fig. 1B). iNOS is the main NOS isoform involved in NO during inflammation and, under the same experimental conditions, GUA, MIN, and TRI (200–50 μg mL^{-1}) significantly reduced iNOS expression too ($P < 0.01$ *vs.* LPS alone; Fig. 1C). An interaction between iNOS and COX pathways represents an important mechanism for the modulation of the inflammatory response. COX-2 is a well known pro-inflammatory enzyme triggered by agents such as LPS, it is involved in macrophage response and its expression was also influenced by NO.[18] Thus, we evaluated the effect of GUA, MIN, TRI (200–50 μg mL^{-1}) on COX-2 expression. Our data show that, similarly to NO and iNOS, also COX-2 protein expression was significantly inhibited by GUA, MIN, TRI, further contributing to the reduction of LPS-induced inflammation in J774A.1 macrophages (250–50 μg mL^{-1}; $P < 0.01$ *vs.* LPS alone; Fig. 1D). Among the tested samples TRI showed the highest anti-inflammatory potential (Fig. 1).

TRI, in the presence of myrosinase, further reduces NO and ROS release and iNOS and COX-2 expression in LPS-stimulated macrophages

Because myrosinase is a key enzyme in ITC production, we evaluated the effect of TRI on LPS-stimulated J774A.1 macrophages

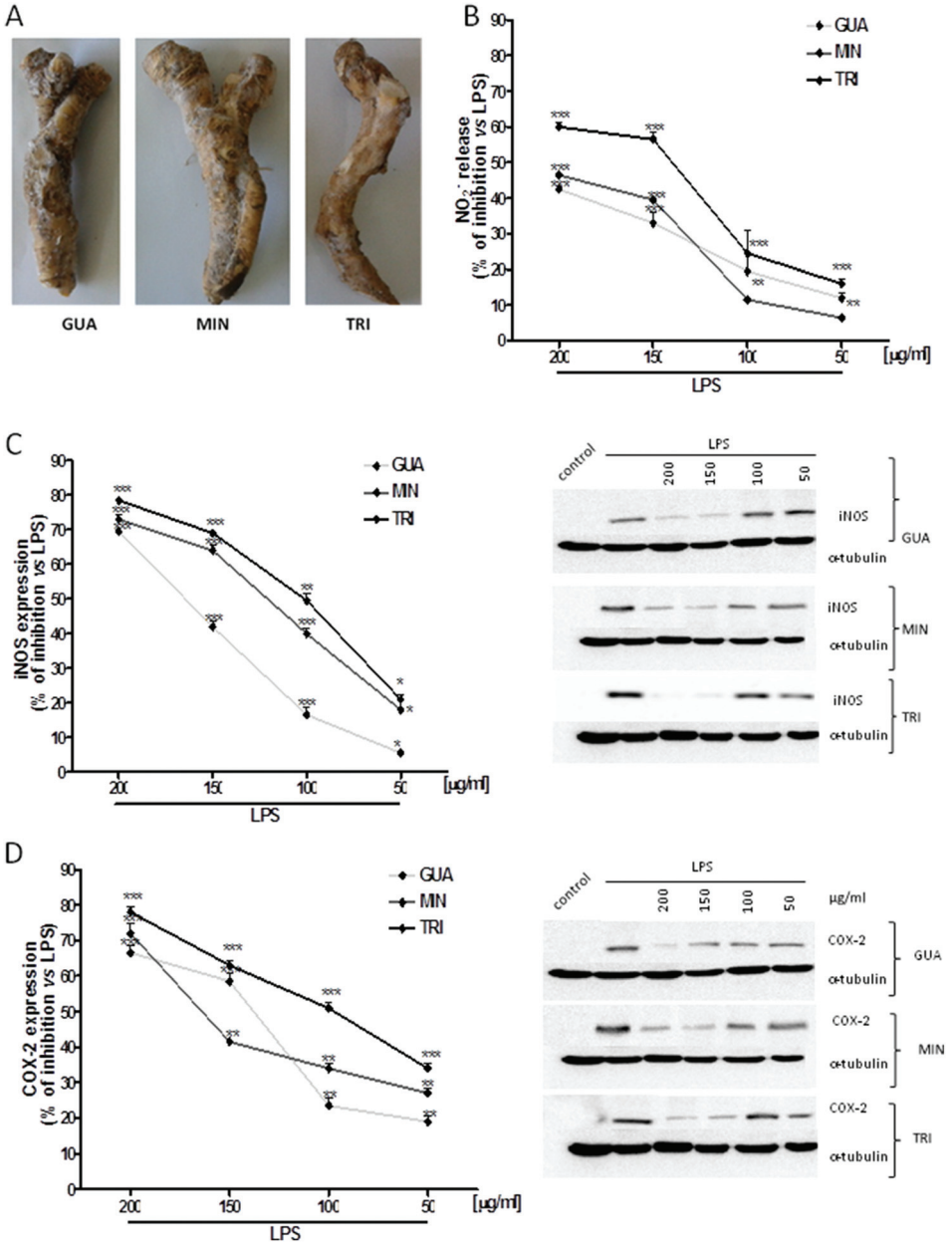

Fig. 1 Effect of GUA, MIN, and TRI on NO release and iNOS and COX-2 expression. Representative root samples of the three horseradish accessions. (Panel A) NO release, evaluated as NO_2^- (μM), by macrophages J774A.1 stimulated with LPS. (Panel B) Representative western blot of iNOS; (Panel C) COX-2; (Panel D) densitometric analysis of the concentration dependent effect of GUA, MIN, TRI (200–50 μg ml^{-1}) on LPS-induced iNOS and COX-2 in J774A.1 macrophages. Values, means ± S.E.M., are expressed as NO_2^- (μM) release (% of inhibition *vs.* LPS), iNOS expression (% of inhibition *vs.* LPS), and COX-2 expression (% of inhibition *vs.* LPS). Comparisons were made using one-way analysis of variance, and multiple comparisons were made by Bonferroni's test. ***, ** denote $P < 0.001$ and $P < 0.01$ *vs.* LPS.

in the presence of myrosinase, using sinigrin as the standard compound. To investigate if TRI influences NO production and iNOS and COX-2 expression, even in the presence of myrosinase, in another set of experiments J774A.1 macrophage were treated with TRI (200–25 μg ml^{-1}) or sinigrin (30 μM) for 1 h and in combination with LPS (1 μg ml^{-1}) for 24 h. In some experiments myrosinase (0.1 U ml^{-1}) was added 30 minutes before TRI or sinigrin treatments. The presence of myrosinase significantly increased the inhibitory effect of TRI on NO release (Fig. 2B; $P < 0.001$ *vs.* TRI alone), on iNOS (Fig. 2D; $P < 0.001$ *vs.* TRI alone) and COX-2 expression (Fig. 3B; $P < 0.001$ *vs.* TRI alone) compared to TRI alone.

These results clearly showed that the major anti-inflammatory activity of sample TRI when myrosinase was added could be due to the increased AITC availability. In this regard, AITC has been shown to exert an anti-inflammatory action,[19] research has shown that hydrolyzed form of sinigrin suppresses the formation of NO in macrophages.[20] Also, regarding

Table 1 Sinigrin content and myrosinase activity in horseradish roots

Samples	Myrosinase activity[a] (U per mg protein)	Sinigrin content[b] (μmol per g DW)
MIN	0.71 ± 0.11	50.77 ± 3.18
TRI	0.73 ± 0.07	66.58 ± 4.42*
GUA	1.02 ± 0.14*	52.71 ± 3.57

[a] Myrosinase activity was determined by the decrease in absorbance of sinigrin at 227 nm. One unit of activity (U) is defined as the amount of myrosinase that catalyzes the hydrolysis of 1 μmol of sinigrin per minute (pH = 6.0; T = 37 °C). [b] Sinigrin was desulfated and determined using HPLC; results were reported as micromoles of sinigrin per gram of dry weight. Values are reported as means ± SD of three independent experiments. *One-way ANOVA was carried out to test for significant differences and results were considered to be statistically significant at $p < 0.05$ (Tukey's test).

the anti-inflammatory activity, in a previous study[8] we have reported on the presence of flavonoids in the extract obtained from the horseradish roots. It is assumed that the biological activity of the TRI extract could be due to synergistic effects among flavonoids and AITC.

ROS, as well as NO, generation in the inflammatory site is typically induced as part of a defensive reaction intended to clear infectious and environmental threats, including microbial agents and particulate material. Alternatively, ROS activation could act as a significant and adverse participant in abnormal inflammatory disease. LPS (1 μg ml^{-1}) induced significant ROS production in macrophages after 24 h. Treatment with TRI, at all tested concentrations (200–25 μg mL^{-1}), reduced ROS production in macrophages ($P < 0.001$ *vs.* LPS alone; Fig. 3C), thus indicating its antioxidant effects. The simultaneous presence of TRI and myrosinase further reduced

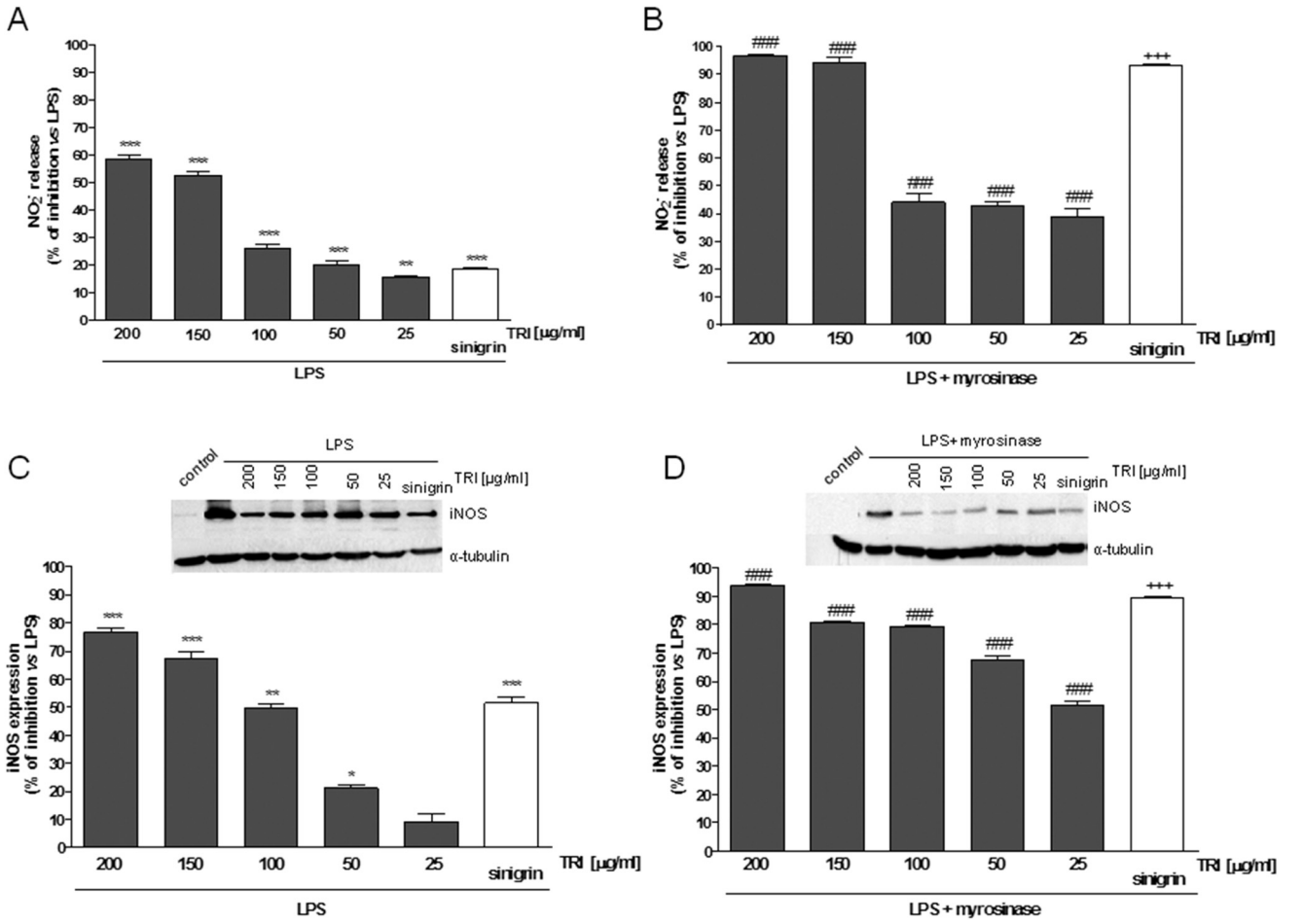

Fig. 2 Effect of TRI and sinigrin, alone and in combination with myrosinase, on NO release and iNOS expression. NO, evaluated as NO_2^- (μM), by macrophages J774A.1 stimulated with LPS (Panel A) and stimulated with LPS and myrosinase (0.1 U ml^{-1}; Panel B). Representative western blot of iNOS and densitometric analysis of the concentration dependent effect of TRI (200–25 μg ml^{-1}) on J774A.1 macrophages stimulated with LPS (Panel C) and stimulated with LPS and myrosinase (0.1 U ml^{-1}; Panel D). Values, means ± S.E.M., are expressed as NO_2^- (μM) release (% of inhibition *vs.* LPS), and iNOS expression (% of inhibition *vs.* LPS). Comparisons were performed using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. ***, ** and * denote $P < 0.001$, $P < 0.01$ and $P < 0.05$ *vs.* LPS. ### denotes $P < 0.001$ *vs.* TRI alone. +++ denotes $P < 0.001$ *vs.* sinigrin.

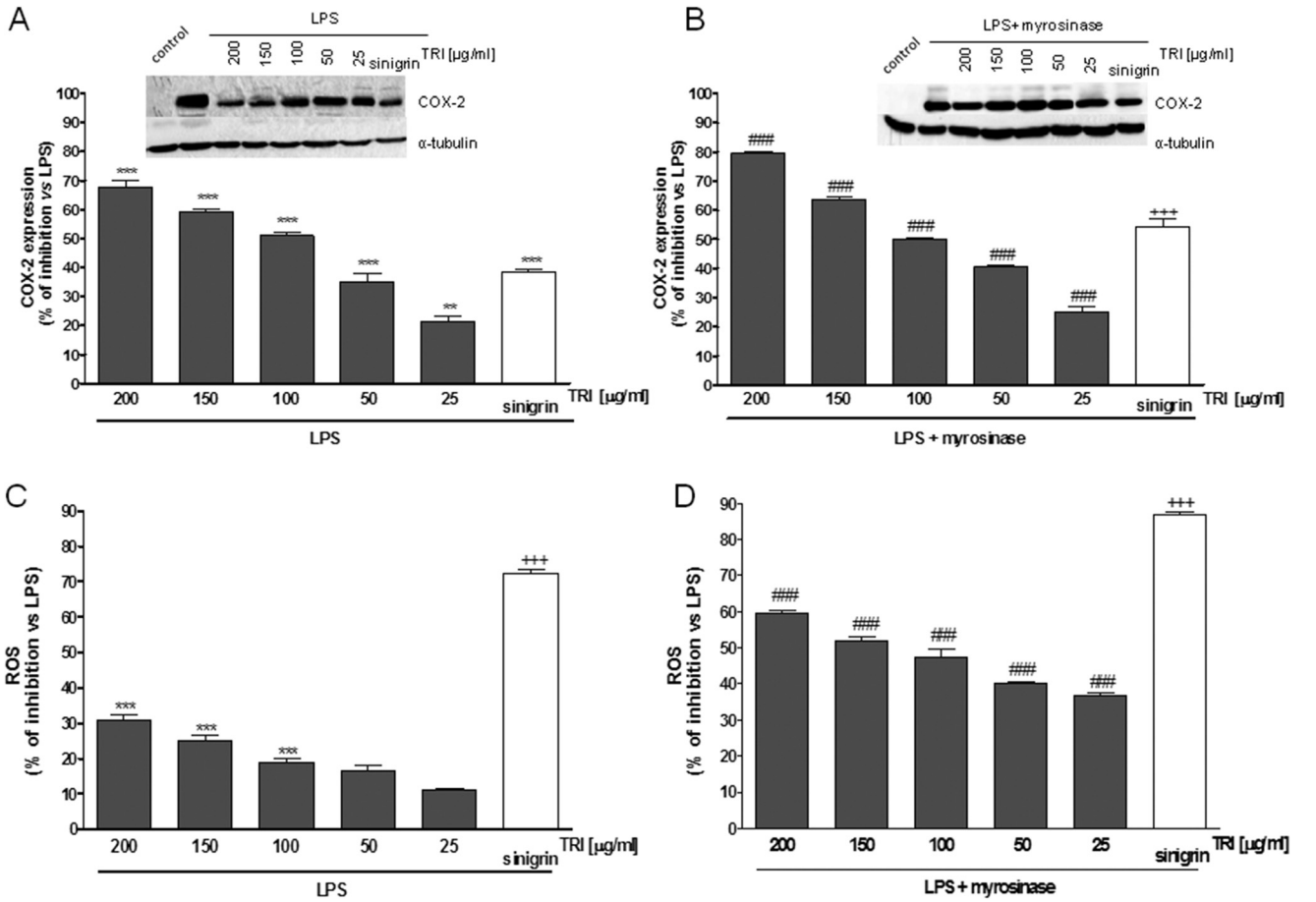

Fig. 3 Effect of TRI and sinigrin, alone and in combination with myrosinase, on COX-2 expression and ROS release. Representative western blot of COX-2 and densitometric analysis of the concentration dependent effect of TRI (200–25 μg ml^{-1}) on LPS-induced COX-2 on J774A.1 macrophages stimulated with LPS (Panel A) and stimulated with LPS and myrosinase (0.1 U ml^{-1}; Panel B). Effect of TRI (200–25 μg ml^{-1}) on ROS formation, evaluated by means of the probe 2′,7′-dichlorofluorescein-diacetate (H2DCF-DA), on J774A.1 macrophages stimulated with LPS (Panel C) and treated with LPS and myrosinase (0.1 U ml^{-1}; Panel D). Values, means ± S.E.M., are expressed as COX-2 expression (% of inhibition *vs.* LPS) and as ROS (% of inhibition *vs.* LPS). Comparisons were performed using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. ***, ** denote $P < 0.001$, $P < 0.01$ *vs.* LPS. ### denotes $P < 0.001$ *vs.* TRI alone. +++ denotes $P < 0.001$ *vs.* sinigrin.

ROS production in LPS-treated J774A.1 macrophages ($P < 0.001$ *vs.* TRI alone; Fig. 3D).

TRI reduces LPS-induced TNF-α and IL-6 in J774A.1 macrophages

TNF-α and IL-6 are pro-inflammatory cytokines elevated in sepsis. Moreover TNF-α itself induces iNOS expression and large amounts of NO production.[21] 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 TRI (200–25 μg ml^{-1}) added 1 h before and simultaneously to LPS significantly reduced TNF-α release ($P < 0.001$ *vs.* LPS alone; Fig. 4A) and IL-6 ($P < 0.01$ *vs.* LPS alone; Fig. 4C) at all tested concentrations in macrophages. The presence of myrosinase further reduced TNF-α release ($P < 0.001$ *vs.* TRI alone; Fig. 4B) and IL-6 ($P < 0.05$ *vs.* TRI alone; Fig. 4D) with respect to TRI alone.

TRI inhibits p65 NF-κB nuclear translocation in LPS-treated macrophages

LPS is known to activate the pro-inflammatory transcription factor NF-κB, also regulated *via* a number of second messengers, including ROS.[22] One of the primary physiological functions of NF-κB is the regulation of immune responses, including pro-inflammatory enzyme production (*e.g.* iNOS, COX-2), antigen presentation, pattern recognition and phagocytosis. Following p65 phosphorylation, the free NF-κB dimers translocate into the nucleus and bind to specific sequences to regulate the downstream gene expression.[23] So we labelled p65 with a green fluorescence to track the influence of TRI tested at two medium concentrations of TRI (150–100 μg mL^{-1}) and added 1 h before LPS (1 μg mL^{-1}) on NF-κB translocation. As shown in Fig. 5, nuclear NF-κB p65 was increased after 15 minutes by LPS. NF-κB translocation is reduced by TRI in J774A.1 treated macrophages compared to LPS alone. The presence of myrosinase increased the inhibitory effect of TRI on

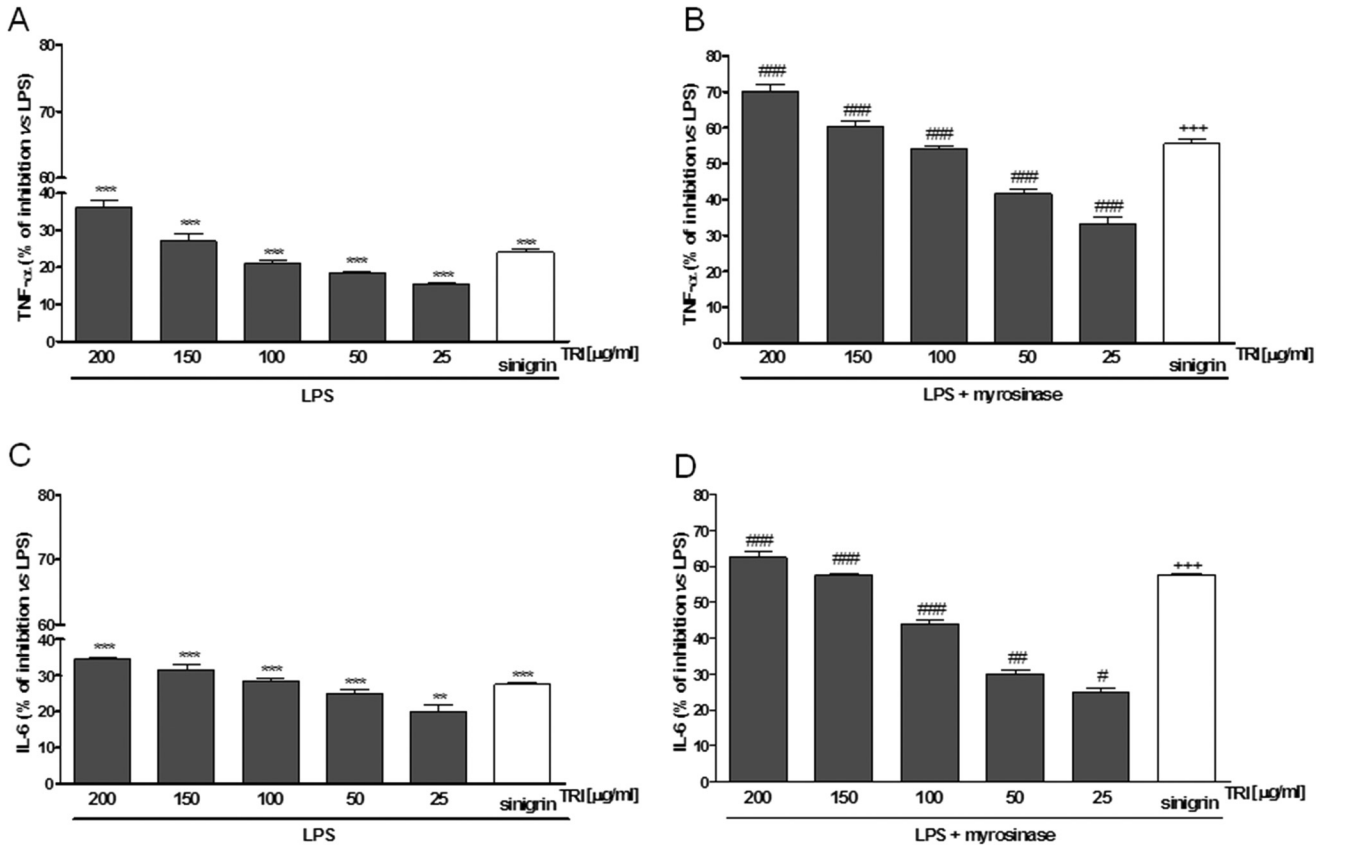

Fig. 4 Effect of TRI and sinigrin, alone and in combination with myrosinase, on TNF-α and IL-6 production. TNF-α production was measured in the supernatants of J774A.1 cells treated with TRI (200–25 μg ml^{-1}) and LPS (Panel A) and treated with LPS and myrosinase (0.1 U ml^{-1}; Panel B) by the ELISA assay. Effect of TRI (200–25 μg ml^{-1}) on LPS-induced IL-6 production in J774A.1 macrophages. IL-6 production was measured in the supernatants of J774A.1 cells treated with TRI (200–25 μg ml^{-1}) and LPS (Panel C) and treated with LPS and myrosinase (0.1 U ml^{-1}; Panel D) by the ELISA assay. Values, means ± S.E.M., are expressed as TNF-α (% of inhibition *vs.* LPS) and IL-6 (% of inhibition *vs.* LPS). Data were analyzed by ANOVA test, and multiple comparisons were made by Bonferroni's test. *** and ** denote $P < 0.001$ and $P < 0.01$ *vs.* LPS. ###, ## and # denote $P < 0.001$, $P < 0.01$ and $P < 0.05$ *vs.* TRI alone. +++ denotes $P < 0.001$ *vs.* sinigrin.

p65 translocation. Our evidence indicates that in the presence of LPS, TRI inhibits the nuclear translocation of the p65 subunits, thus reducing NF-κB activity in J774A.1 macrophages (Fig. 5). These results indicate an activity of TRI in the early steps of the inflammatory response.

TRI increases HO-1 expression in LPS-treated macrophages

In order to protect themselves against inflammatory and oxidative injury, cells, such as macrophages, up-regulate some defence mechanisms such as HO-1 expression. HO-1, the rate-limiting enzyme in heme degradation, catalyzes the oxidation of heme to generate several biologically active molecules such as carbon monoxide (CO), biliverdin, and ferrous ions.[24] HO-1 can increase the cellular anti-oxidant status by generating anti-oxidants such as bilirubin, which can inhibit iNOS protein expression and suppress NO production.[25] Moreover, CO, a major product of HO-1 activity, was shown to inhibit COX-2 expression. CO was also shown to inhibit iNOS enzymatic activity, thus decreasing NO production.[26] HO-1 is normally expressed at low levels in most tissues/organs except for the spleen; however, it is highly inducible in response to a variety of stimuli, such as LPS, to protect cells against oxidative and inflammatory injury.[24] Considering the beneficial role of HO-1 in controlling various inflammatory mediators, we evaluated whether its expression was influenced by TRI. HO-1 expression in J774A.1 macrophages was increased by LPS, and TRI treatment further increased HO-1 ($P < 0.05$ *vs.* LPS alone; Fig. 6A) mostly in the presence of myrosinase ($P < 0.05$ *vs.* TRI alone; Fig. 6B).

Thus during LPS-induced inflammation in macrophages, TRI on the one hand inhibits pro-inflammatory mediators and on the other stimulates a cytoprotective response, with respect to TRI alone.

Experimental

Reagents

Unless stated otherwise, all reagents and compounds were purchased from Sigma Chemical Company (Sigma, Milan, Italy).

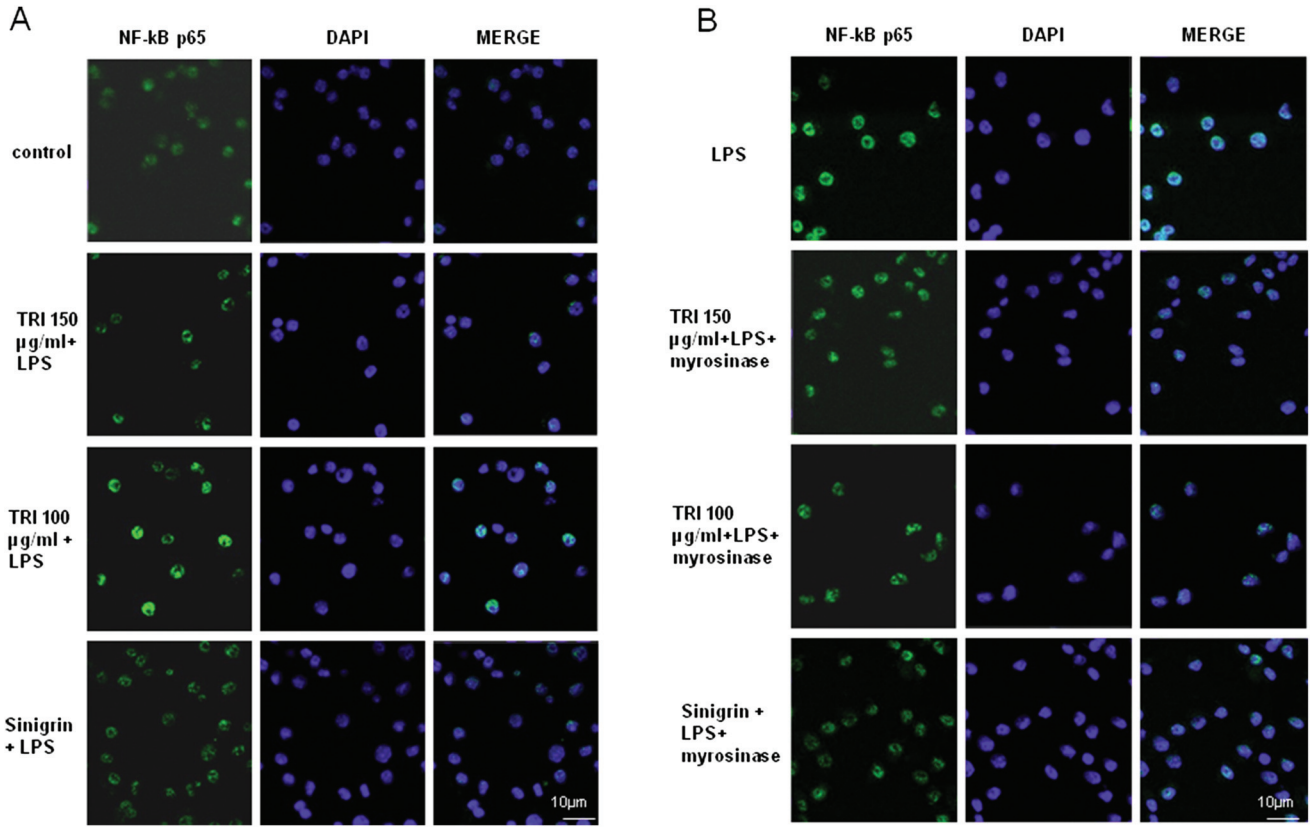

Fig. 5 Effect of TRI and sinigrin, alone and in combination with myrosinase, on NF-κB activation. p65 NF-κB nuclear translocation in J774A.1 macrophages was induced by LPS (Panel A). TRI alone (A) and in the presence of myrosinase (0.1 U ml^{-1}; B) reduced p65 NF-κB nuclear translocation. Nuclear translocation of NF-κB p65 subunit was detected using the immunofluorescence assay by confocal microscopy. Scale bar, 10 µm. Blue and green fluorescence indicates localization of the nucleus (DAPI) and p65 NF-κB respectively. Analysis was performed by confocal laser scanning microscopy.

Plant material

Three different accessions of horseradish (*Armoracia rusticana*, Gaertn) samples named GUA (Acc1), MIN (Acc2), and TRI (Acc3) were harvested in December 2012 from a private crop grown in Southern Italy (Accettura – latitude 40° 29′ N, longitude 16° 9′ E – Basilicata, Italy).

For each sample, roots (35–40 cm long and 2.5–3.0 cm in diameter) were collected from the middle of the plants, cleaned with distilled water, dried with paper towels and quickly frozen in liquid nitrogen. Samples were then lyophilized, ground into a fine powder and stored at −70 °C.

Myrosinase activity

Aliquots of root powder were dissolved (1 : 5, w/v) in a cold 2 mM DTT, 5 mM EDTA, phosphate buffer (pH 6.0) for 1 h (vortexing 30 s every 5 min), the resuspended samples were centrifuged at 12 000*g* for 15 min at 4 °C and the supernatants were assayed for myrosinase activity. Enzyme activity was determined spectrophotometrically by the decrease in absorbance at 227 nm deriving from the hydrolysis of sinigrin.[27] Briefly, 100 µL of the enzymatic extract was added to the reaction mixture (pre-equilibrate at 37 °C) consisting of 2.3 mL deionised water, 400 µL of 100 mM phosphate buffer pH 6.0, 20 µL of 100 mM ascorbic acid and 10 µL of 40 mM sinigrin. The decrease in absorbance as a result of sinigrin breakdown was plotted at 227 nm over 4 min. Activity was determined from the linear slope representing the disappearance of sinigrin in time. One unit of activity (U) is defined as the amount of myrosinase that catalyzes the hydrolysis of 1 µmol of sinigrin per minute at 37 °C and pH 6.0. Specific activity was expressed as units per milligram of protein. Protein content of the extracts was determined according to the method of Bradford (1976),[28] using the Bio-Rad reagent and bovine serum albumin as the standard protein.

Sinigrin determination

Sinigrin was extracted with hot methanol from freeze-dried samples followed by an enzymatic desulfation. Briefly, for each sample, 500 mg of freeze-dried samples was added to 0.3 mL of 2 µM benzylglucosinolate (internal standard), extracted in 4 mL of 100% methanol at 70 °C and heated for 20 min on a heating block. The extracting procedure was repeated twice and the supernatants were combined. After centrifugation (10 000*g* for 8 min) 1 mL of the supernatant was added to a

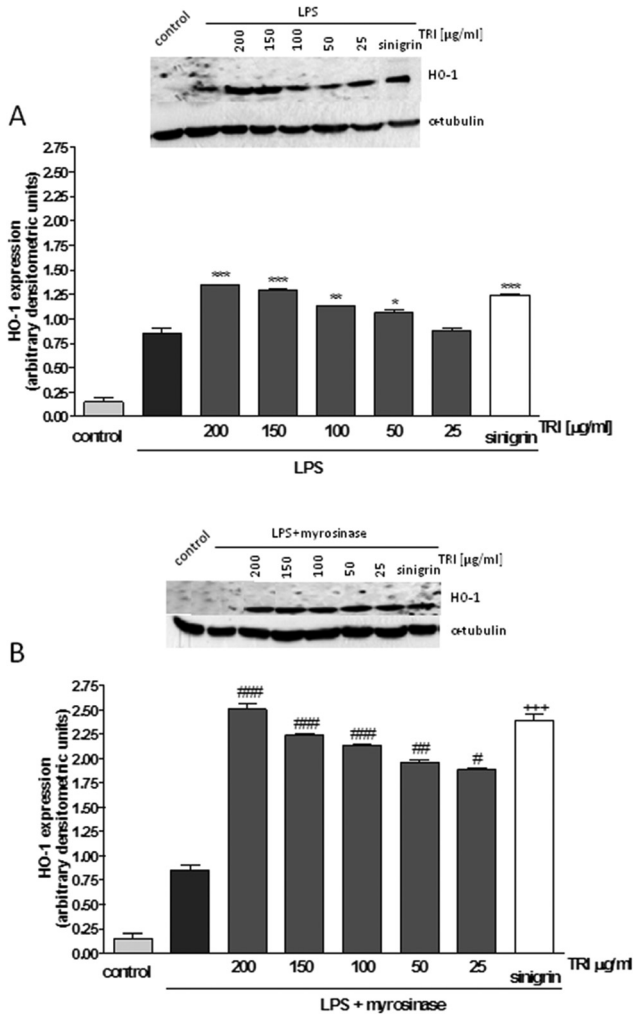

Fig. 6 Effect of TRI and sinigrin, alone and in combination with myrosinase, on HO-1 expression. Representative western blot of HO-1 enzyme expression and densitometric analysis of the concentration dependent effect of TRI (200–25 μg ml^{-1}) on J774A.1 macrophages stimulated with LPS (Panel A) and stimulated with LPS and myrosinase (0.1 U ml^{-1}; Panel B). Values, means ± S.E.M., are expressed as HO-1 expression (% of inhibition *vs.* LPS). Comparisons were made using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. ***, ** and * denote $P < 0.001$, $P < 0.01$ and $P < 0.05$ *vs.* LPS. ###, ## and # denote $P < 0.001$, $P < 0.01$ and $P < 0.05$ *vs.* TRI alone. $^{+++}$ denotes $P < 0.001$ *vs.* sinigrin.

Sephadex A25 column and desulfated (for 16 h) using 0.5 mL of aryl sulfatase (20 U mL^{-1}; Sigma, St. Louis, MO). Desulfosinigrin was eluted with 2 mL of deionised water, filtered through a 0.2 μm filter and separated with an Alliance Waters 2695 HPLC (Waters, Milford, MA) equipped with a UV detector set at 229 nm and a Spherisorb ODS2 column (150 × 4.6 mm, 3 μm, Waters). Elution was performed by a linear gradient mobile phase from 100% A (0.5% trifluoroacetic acid in water) to 30% B (acetonitrile) in 30 min, at 1 mL min^{-1}. Desulfosinigrin was identified and quantified by comparison of the HPLC retention time with that of a sinigrin standard (Sigma) after on-column sulfatase treatment as described above. Samples were independently analyzed in triplicate, and the results were reported as micromoles of sinigrin per gram of dry weight (μmol per g DW).

Preparation of extracts

For each sample ten grams of root powders were extracted for 12 h at 30 °C in a conical flask at 150 rpm with 100 mL of methanol. The extracts were recovered after centrifugation (18 000*g* for 4 min at 20 °C), filtered through filter paper and freed of solvent in a rotary vacuum evaporator. The dried crude extracts were weighed to calculate the yield and stored at −70 °C.

Cell culture

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

Antiproliferative activity

J774A.1 macrophages (5×10^4 per well) were plated on 96-well plates and allowed to adhere for 4 h. The medium was then replaced with fresh medium alone or containing serial dilutions of GUA, MIN, TRI (200–50 μg ml^{-1}), also in the presence of myrosinase, and cells were incubated for 24 h. Cell viability was assessed using the MTT assay as previously reported.[29,30] Briefly, 25 μL of MTT (5 mg mL^{-1}) 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) 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) equipped with a 620 nm filter. Macrophage viability in response to treatment with the extracts was calculated as % dead cells = 100 × (OD treated/OD control) × 100], as previously reported.[31]

Cell treatment for the anti-inflammatory activity evaluation

Macrophages J774A.1 were plated in P60 (1.8×10^6) and allowed to adhere for 4 h. Thereafter, the medium was replaced with fresh medium alone or containing serial dilutions of GUA, MIN, TRI (200–50 μg ml^{-1}) for 1 h and then co-exposed to LPS (1 μg ml^{-1}) for a further 18–24 h for NO detection and iNOS and COX-2 expression.

In another set of experiments TRI (200–25 μg ml^{-1}), activity was compared to sinigrin (30 μM) also in the presence of myrosinase (0.1 U ml^{-1}) added 30 minutes before TRI or sinigrin treatment.

Nitrite determination and western blot analysis for iNOS, COX-2 and HO-1 expression

NO generation was measured as nitrite (NO_2^-), the index of NO released by cells, in the culture medium 24 h after LPS

stimulation by Griess reaction, as previously reported.[32] Briefly, 100 mL of cell culture medium were mixed with 100 mL of Griess reagent – equal volumes of 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-hydrogen chloride and incubated at room temperature for 10 min, and then the absorbance was measured at 550 nm on a microplate reader Titertek (Dasit, Cornaredo, Milan, Italy). The amount of NO_2^-, as μM concentration, in the samples was calculated by using a sodium NO_2^- standard curve. iNOS and COX-2 protein expression in J774A.1 macrophages was assessed by western blot analysis. As previously reported,[33] after NO_2^- determination, cells were scraped off, washed with ice-cold phosphate-buffered saline (PBS), and centrifuged at 5000*g* 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 per ml leupeptin, 10 mM sodium fluoride, 150 mM sodium chloride, 10 mg per ml trypsin inhibitor, and 1% Tween-20. Protein concentration was estimated by using the Bio-Rad protein assay using bovine serum albumin as the 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 to a hybond polyvinylidene difluoride membrane for 40 min at 5 mA cm^2. Membranes were blocked for 40 min in PBS and 5% (w/v) nonfat milk and subsequently probed overnight at 4 °C with mouse monoclonal anti-iNOS, anti-COX-2 antibody (BD Laboratories) and anti-HO-1 antibody (Santa Cruz Biotechnology, Inc.) in PBS, 5% w/v nonfat milk, and 0.1% Tween-20. Blots were then incubated with horseradish-peroxidase conjugated goat anti-mouse immunoglobulin (I_g)G (1 : 5000) for 1 h at room temperature. Immunoreactive bands were visualized using the electro-chemiluminescence assay (ECL) detection system according to the manufacturer's instructions and exposed to Kodak X-Omat films. The protein bands of iNOS, COX-2, HO-1 and tubulin on X Omat films were quantified by scanning densitometry (Imaging Densitometer GS-700, Bio-Rad, U.S.A.). Data are normalized with tubulin expression, used as the reference protein, and are expressed as arbitrary densitometric units as previously reported.[34]

TNF-α and IL-6 determination

TNF-α and IL-6 concentrations were assessed by an Enzyme-Linked Immuno Sorbent Assay (ELISA) using a commercial kit for murine TNF-α or IL-6 according to the manufacturer's instructions (e-Biosciences, CA, USA) in J774A.1 culture medium stimulated for 18 h with TRI (200–25 μg ml^{-1}) and/or sinigrin (30 μM) for 1 h and in combination with LPS (1 μg ml^{-1}) for 24 h and, in other experiments, myrosinase (0.1 U ml^{-1}) was added 30 minutes before treatment.

Immunofluorescence analysis with confocal microscopy

For the immunofluorescence assay, J774A.1 cells (3×10^5 per well) were seeded on coverslips in 12 well plates and treated with TRI using two medium concentrations (150–100 μg ml^{-1}) for 1 h and then simultaneously with LPS (1 μg ml^{-1}) for 20 min for NF-κB. Then the cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% saponin in PBS for 15 min. After blocking with BSA and PBS for 1 h, cells were incubated with rabbit anti-phospho p65 antibody (Santa Cruz Biotechnologies) for 1 h at room temperature. The slides were then washed three times with PBS and fluorescein conjugated secondary antibody (FITC) was added for 1 h, and DAPI was used for counterstaining of nuclei. Coverslips were finally mounted in a mounting medium and fluorescent images were taken under a Laser Confocal Microscope (Leica TCS SP5) as previously reported.[35]

Measurement of intracellular ROS

ROS formation was evaluated by means of the probe 2′,7′-dichlorofluorescein-diacetate (H_2DCF-DA). H_2DCF-DA is a non-fluorescent permeant molecule that passively diffuses into cells, where the acetates are cleaved by intracellular esterases to form H_2DCF and thereby trap it within the cell. In the presence of intracellular ROS, H_2DCF is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Briefly, J774A.1 cells were plated at a density of 3×10^5 cells per well into 24-well plates. Cells were allowed to grow for 24 h; thereafter, the medium was replaced with fresh medium and cells were stimulated with TRI (200–25 μg ml^{-1}) and/or sinigrin (30 μM) for 1 h and in combination with LPS (1 μg ml^{-1}) for 24 h. In other experiments myrosinase (0.1 U ml^{-1}) was added 30 minutes before treatment. Cells were then collected, washed twice with phosphate buffer saline (PBS) and then incubated in PBS containing H_2DCF-DA (10 μM) at 37 °C. After 15 minutes, cell fluorescence was evaluated using fluorescence-activated cell sorting (FACScan; Becton Dickinson) and elaborated with Cell Quest software as previously reported.[36]

Data analysis

Data are reported as the mean ± standard error of the mean (S.E.M.) values of at least three independent experiments, each performed in triplicate. Statistical analysis was performed by the analysis of variance test, and multiple comparisons were made by Bonferroni's test. A *P*-value less than 0.05 was considered significant.

Conclusions

In the last few years, the search for natural products from folk medicine has been widely investigated for their potential anti-inflammatory activity. The ethno-medical uses of *Armoracia rusticana* leaves and roots have a long history; juice extracted from horseradish has been used to treat sinusitis, urinary and respiratory tract infections. In this study we have demonstrated the anti-inflammatory potential of three accessions of *Armoracia rusticana* on LPS-treated J774A.1 murine macrophages. Our results indicate that horseradish reduced NO, iNOS and COX-2 expressions, in particular for the TRI extract. This result was

probably due to the highest content of sinigrin than the other accessions that was converted to AITC.

TRI extract was also able to reduce ROS and cytokine release acting on NF-κB p65 activation, a pivotal transcription factor in chronic inflammatory diseases.[37] Moreover *Armoracia rusticana* TRI extract increased HO-1 expression, thus contributing to the cytoprotective cellular effect during inflammation. TRI anti-inflammatory effects were further enhanced by the presence of myrosinase presumably due to the increase of allyl-isothiocyanate (AITC) concentration, derived from the action of myrosinase on sinigrin, the predominant glucosinolate in horseradish.[5]

These results indicate that horseradish has inhibitory effects on the production of pro-inflammatory mediators, such as many anti-inflammatory drugs, and are consistent with several studies on the biological effects of herbal medication extracts on LPS-induced inflammation in macrophages.[38–41]

Conflict of interest

The authors declare no competing financial interest.

Acknowledgements

This study was supported by a grant from University of Basilicata (Ricerca Interesse Locale, 2011) and by a grant from Basilicata Region (Mis 214 Azione 5 "Agrobiodiversità: Biodiversità di specie orticole ed areali lucani: da patrimonio a strumento di sviluppo"). The authors are grateful to Mr Paolo Giannelli for editing the manuscript.

References

1 J. MacMicking, Q. W. Xie and C. Nathan, *Annu. Rev. Immunol.*, 1997, **15**, 323–350.
2 P. A. Baeuerle, *Cell*, 1998, **95**, 729–731.
3 J. Pike and R. K. Chandra, *Int. J. Vitam. Nutr. Res.*, 1995, **65**, 117–121.
4 A. Popolo, G. Autore, A. Pinto and S. Marzocco, *Free Radical Res.*, 2013, **47**, 346–356.
5 X. Li and M. M. Kushad, *J. Agric. Food Chem.*, 2004, **52**, 6950–6955.
6 Y. Zhang, *Mol. Nutr. Food Res.*, 2010, **54**, 127–135.
7 A. Majewska, B. Bałasińska and B. Dąbrowska, *Folia Hort.*, 2004, **16**, 15–22.
8 L. Calabrone, M. Larocca, S. Marzocco, G. Martelli and R. Rossano, *Food Nutr. Sci.*, 2015, **6**, 64–74.
9 G. Mazza, *Can. Inst. Food Sci. Technol. J.*, 1984, **17**, 18–23.
10 L. Tang, G. Li, L. Song and Y. Zhang, *Anti-Cancer Drugs*, 2006, **17**, 297–305.
11 A. Bhattacharya, Y. Li, K. L. Wade, J. D. Paonessa, J. W. Fahey and Y. Zhang, *Carcinogenesis*, 2010, **31**, 2105–2110.
12 K. Ippoushi, H. Itou, K. Azuma and H. Higashio, *Life Sci.*, 2002, **71**, 411–419.
13 P. Thejass and G. Kuttan, *Nitric Oxide*, 2007, **16**, 247–257.
14 A. E. Wagner, C. Boesch-Saadatmandi, J. Dose, G. Schultheiss and G. Rimbach, *J. Cell Mol. Med.*, 2012, **16**, 836–843.
15 S. J. Kim, H. J. Park, H. J. Shin, D. H. Shon, H. Kim do and H. S. Youn, *Int. Immunopharmacol.*, 2012, **13**, 403–407.
16 M. J. Weil, Y. Zhang and M. G. Nair, *J. Agric. Food Chem.*, 2005, **53**, 1440–1444.
17 A. M. Bones and J. T. Rossiter, *Physiol. Plant.*, 1996, **97**, 194–208.
18 N. Ahmad, L. C. Chen, M. A. Gordon, J. D. Laskin and D. L. Laskin, *J. Leukocyte Biol.*, 2002, **71**, 1005–1011.
19 H. M. Woo, J. H. Kang, T. Kawada, H. Yoo, M. K. Sung and R. Yu, *Life Sci.*, 2007, **80**, 926–931.
20 D. K. Patel, K. Patel, M. Gadewar and V. A. Tahilyani, *AsianPac. J. Trop. Biomed.*, 2012, S446–S448.
21 C. Thiemermann, C. C. Wu, C. Szabó, M. Perretti and J. R. Vane, *Br. J. Pharmacol.*, 1993, **110**, 177–182.
22 D. M. Brown, K. Donaldson, P. J. Borm, R. P. Schins, M. Dehnhardt, P. Gilmour, L. A. Jimenez and V. Stone, *Am. J. Physiol.: Lung Cell. Mol. Physiol.*, 2004, **286**, 344–353.
23 P. P. Tak and G. S. Firestein, *J. Clin. Invest.*, 2001, **107**, 7–11.
24 M. L. Wu, Y. C. Ho, C. Y. Lin and S. F. Yet, *Am. J. Cardiovasc. Dis.*, 2011, **1**, 150–158.
25 W. W. Wang, D. L. Smith and S. D. Zucker, *Hepatology*, 2004, **40**, 424–433.
26 C. Thorup, C. L. Jones, S. S. Gross, L. C. Moore and M. S. Goligorsky, *Am. J. Physiol.*, 1999, **277**, F882–F889.
27 S. Palmieri, R. Leoni and R. Iori, *Anal. Biochem.*, 1982, **123**, 320–324.
28 M. Bradford, *Anal. Biochem.*, 1976, **72**, 248–254.
29 G. Autore, S. Marzocco, C. Formisano, M. Bruno, S. Rosselli, M. B. Jemia and F. Senatore, *Molecules*, 2015, **20** (1), 571–1578.
30 G. Cioffi, R. Sanogo, A. Vassallo, F. Dal Piaz, G. Autore, S. Marzocco and N. De Tommasi, *J. Nat. Prod.*, 2006, **69**(4), 625–635.
31 M. Ben Jemia, M. E. Kchouk, F. Senatore, G. Autore, S. Marzocco, V. De Feo and M. Bruno, *Chem. Cent. J.*, 2013, **7**(1), 47.
32 G. Bianco, R. Russo, S. Marzocco, S. Velotto, G. Autore and L. Severino, *Toxicon*, 2012, **59**, 644–650.
33 E. Sommella, G. Pepe, F. Pagano, G. C. Tenore, S. Marzocco, M. Manfra, G. Calabrese, R. P. Aquino and P. Campiglia, *J. Funct. Foods*, 2014, **7**, 641–649.
34 F. Cianchi, S. Cuzzocrea, M. C. Vinci, L. Messerini, C. E. Comin, G. Navarra, G. Perigli, T. Centorrino, S. Marzocco, E. Lenzi, N. Battisti, G. Trallori and E. Masini, *Dig Liver Dis.*, 2010, **42**(1), 20–27.
35 M. Del Regno, S. Adesso, A. Popolo, A. Quaroni, G. Autore, L. Severino and S. Marzocco, *Toxicol. Appl. Pharmacol.*, 2015, **285**(2), 118–127.
36 S. Adesso, A. Popolo, G. Bianco, R. Sorrentino, A. Pinto, G. Autore and S. Marzocco, *PLoS One*, 2013, **8**, e76778.
37 P. J. Barnes and M. Karin, *N. Engl. J. Med.*, 1997, **336**, 1066–1071.

38 Y. C. Oh, Y. H. Jeong, W. K. Cho, J. H. Ha, M. J. Gu and J. Y. Ma, *Int. J. Mol. Sci.*, 2015, **16**(1), 1232–1251.
39 Y. C. Oh, Y. H. Jeong, J. H. Ha, W. K. Cho and J. Y. Ma, *BMC Complementary Altern. Med.*, 2014, **14**, 242.
40 D. J. Kwon, S. M. Ju, G. S. Youn, S. Y. Choi and J. Park, *Food Chem. Toxicol.*, 2013, **58**, 479–486.
41 A. Braca, F. Dal Piaz, S. Marzocco, G. Autore, A. Vassallo and N. De Tommasi, *Curr. Drug Targets*, 2011, **12**(3), 302–321.