Article

Oleuropein-rich leaf extract as a broad inhibitor of tumour and macrophage iNOS in Apc mutant rat model

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Abstract: Oleuropein, the major compound of olive leaves, has been reported to exert numerous pharmacological properties, including anti-inflammatory, antidiabetic and anticancer. The purpose of this study is to evaluate, for the first time, the effect of oleuropein-rich leaf extracts (ORLE) in already-developed colon tumours colon tumours arising in an *Apc* (adenomatous polyposis coli) mutated PIRC rats (F344/NTac-Ap^{cam1137}). Here, we were able to investigate in parallel the anti-cancer effect of ORLE, both *in vivo* and *in vitro*, and its anti-inflammatory effect on macrophages, which represents a critical and abundant population in most solid tumours microenvironment. We found that *in vivo* ORLE treatment promoted apoptosis and attenuated iNOS activity both in colon tumours as in peritoneal macrophages of PIRC rats. We confirmed *in vitro* using primary RAW264.7 cells: ORLE reduced iNOS activity in parallel with COX-2 and pro-inflammatory cytokines, such as IL-1β, IL-6 and TGF-β. These findings suggest that ORLE possess a strong anti-inflammatory activity, which could be crucial for dampening the pro-tumourigenic activity elicited by a chronic inflammatory state generated by either tumour cells or tumour-associated macrophages.

Keywords: Oleuropein; colon cancer; activated macrophages; chronic inflammation, inducible nitric oxide synthetase (iNOS), cyclooxygenase-2 (COX-2), nitric oxide (NO).

1. Introduction

Cancer is currently the second leading cause of death worldwide and highly efficient anticancer drugs are currently used to counteract the uncontrolled proliferative activity of neoplastic cells. The effectiveness of most chemotherapeutic agents is accompanied by systemic toxicity, since anticancer agents do not discriminate between normal and cancerous cells. In addition, the efficacy of these treatments is still limited, due to the adverse side effects and the frequent development of resistance.

Among alternative therapies for cancer treatment, there is a growing interest in the anticancer action of natural substances, that are non-toxic, affordable, readily accessible, and, some of which, present in large amounts in byproducts from agro-food chains [1]. The beneficial effects of olive leaves or different preparations (e.g., infusions, extracts) have a several-century-long tradition and have been used for the treatment or to alleviate

the symptoms of many diseases (such as diabetes mellitus, artherial hypertension, and bronchial asthma), and are currently contemplated in the Pharmacopoeia Ph. Eur. 5 [2].

In particular, *Olea Europaea L.* leaves are rich in oleuropein (Ole), a secoiridoid compound that exhibits a wide range of antioxidant, anti-inflammatory, anti-diabetic, neuro- and cardio-protective, anti-microbic and immunomodulatory activities [3–6].Recently, preclinical studies have provided convincing evidence that Ole has, also, peculiar properties as autophagic, and proapoptotic inducer and amyloid fibril growth inhibitor [7–11]. In our experience, we found that Ole might exert an anticancer activity, alone or in combination with conventional anticancer treatments, through different mechanism in different cancer cell lines [11,12].

Colorectal cancer (CRC), one of most common type of cancer in the Western world of both men and women [13], is one of the solid human tumour that may take advantage on a nutritional intervention. Indeed, in addition to a complex genetic susceptibility, key environmental factor for colon cancer includes diet. Preclinical evidences demonstrated that olive oil derived substances have a beneficial effect against colorectal cancer through the modulation of gut microbiota composition or activity [14]. In particular, Ole was able to reduce crypt dysplasia in an *in vivo* rat short-term colon carcinogenesis experiment [15] and to showed protective effects in colitis-associated CRC in mice, suggesting, together with the results obtained in cancer cells *in vitro,* that this molecule may decrease colon tumorigenesis. Whether these protective effects can be extended also to already-developed colon tumours is not known.

Based on these considerations, the aim of the present study is to explore, for the first time, whether oleuropein-rich leaf extracts (ORLE), exerts anti-tumoral and anti-inflammatory activity in colon tumours and peritoneal activated macrophages of PIRC rats carrying a heterozygous germline mutation in the *Apc* gene. APC mutation is the first event triggering colon carcinogenesis both in the majority of sporadic cases and in Familial Adenomatous Polyposis (FAP) syndrome, a hereditary form of colon cancer [16]. Accordingly, PIRC rat spontaneously develops multiple tumours in the colon and small intestine, thus standing as a robust model to study the protective effect of ORLE, derived from olive leaves, on colon cancer progression.

We found that ORLE enriched diet reduces cell proliferation and increases cell apoptosis in tumours and reduced nitric oxide synthase (iNOS) in colon tumour lesions and peritoneal macrophages of PIRC rats. We confirmed that ORLE inhibits the pro-inflammatory features of activated murine macrophages through the reduction of iNOS, cyclooxygenase-2 (COX-2), interleukin (IL)-1β, IL-6 and TGF-β expression, both in acute as in a chronic exposure.

We suggest that an ORLE-enriched diet contributes to switching-off the pro-inflammatory signal released either by tumour cells or by inflammatory cells of tumour microenvironment critical for colon cancer progression.

2. Materials and Methods

2.1 Olive Leaf Extract's Preparation and Toxicity

The organic leaves (Leccino cultivar) were harvested in Tuscany (Vinci, Florence, Italy) and immediately processed to obtain a powder extract rich in active compounds, as previously described in Romani et al., 2020 **[17]**. The characterization of the minor polar compounds and the phenolic profile of olive leaves extract was carried out with HPLC-DAD-MS (high-performance liquid chromatography coupled with diode-array detection and mass spectrometry). The total polyphenol content of dry extract is about 400mg/g, which oleuropein was about 379mg/g. For *in vitro* experiments ORLE was reconstituted to a final concentration of 14 mM in PBS.

2.2 *Cell Lines and Culture Conditions*

HCT-116, colorectal carcinoma cells were purchased from European Collection of Authenticated Cell Cultures (ECACC). The murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium high glucose (DMEM 4500, EuroClone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, EuroClone) and maintained at 37°C in humidified atmosphere containing 90% air and 10% CO_2 and they harvested from subconfluent cultures by incubation with a trypsin-EDTA solution (EuroClone), and propagated every three days. Viability of the cells was determined by trypan blue exclusion test. Cultures were periodically monitored for mycoplasma contamination using Chen's fluorochrome test. HCT116 or RAW cells were exposed for 24h or 72h to 50μM ORLE in complete medium according to different experimental procedures. This concentration was tested on colon and macrophage cell lines in preliminary experiments and chosen because resulted non-toxic (see Figure 3a).

2.3 MTT assay

HCT116 cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay (Sigma Aldrich, Milan, Italy) as described in **[11]**. Cells ($2.5x10^3$) were plated into 96-multiwell plates in complete medium without red phenol. The ORLE treatment was added to the medium colture at different dose for 72h. Then the MTT reagent was added to the medium, and plates were incubated at 37 °C. After 2 h, MTT was removed and the blue MTT–formazan product was solubilized with dimethyl sulfoxide (DMSO, Sigma Aldrich). The absorbance of the formazan solution was read at 595 nm using the microplate reader (Bio-Rad, Milan, Italy).

2.4 *Animal maintenance and ex-vivo analysis*

PIRC rats (F344/NTac-Apcam1137) and wild type (wt) (Fisher F344) rats were originally obtained by the National Institutes of Health (NIH), Rat Resource and Research Center (RRRC) (University of Missouri, Columbia, MO, USA) and bred in Ce.S.A.L. (Housing Center for Experimental Animals of the University of Florence, Italy) in accordance with the Commission for Animal Experimentation of the Italian Ministry of Health (EU Directive 2010/63/EU for animal experiments), as described **[18]**; rats were maintained in polyethylene cages and fed with a standard AIN-76 diet (Laboratorio Dottori Piccioni, s.r.l., Italy). Eight PIRC rats aged 12 months were randomly assigned to the AIN-76 diet (Control diet) or to the same diet containing 2,7 g/kg of diet (ORLE group) as reported **[11]**. Considering that rats eat about 11 g of diet/day, and a mean body-weight of 300 g, we administered a dose of ORLE of about 100 mg/kg b.w **[11]**. Rats were euthanized by CO2 asphyxia after one week of treatment, in line with the experimental protocol approved by the Commission for Animal Experimentation of the Italian Ministry of Health. The entire colon and small intestine were flushed with saline solution and opened to check for the presence of tumours which were collected and processed for histological procedure and RNA-extraction as reported **[18]**.

Expression of CD68, as a measure of macrophage infiltration, and expression of proliferating cell nuclear antigen (PCNA), as a measure of proliferative activity, were determined in the tumour lesions of PIRC rats fed with different diets. Longitudinal colon sections (4 μm) were mounted on electrostatic-treated slides (Superfrost® Plus, Medite, Italy) and processed as described **[19]** using as primary antibodies: mouse monoclonal antibodies against PCNA (PC-10, Santa Cruz, CA, USA) and rat CD68 (AbD Serotec, Oxford, UK). Both antibodies were diluted in PBS 1:200. CD68 reactivity was quantified as number of labeled cells/areas scored evaluated with the ACT-2U software program (Nikon, Instruments Europe, Badhoevedorp, NL) connected via a camera to the microscope (Optiphot-2, Nikon, NL). Evaluation was performed at 400 x magnification.

2.5 *Peritoneal macrophages isolation*

Macrophage cultures were established from peritoneal exudates collected from rats fed with different diets. Briefly, 20 ml of iced cold PBS were injected in the peritoneal cavity of the rats and collected immediately. Peritoneal exudates were washed by centrifugation and macrophage monolayers allowed to adhere to plastic dishes in DMEM4500 medium (without phenol red) containing 250µg/ml bovine serum albumin (BSA), at the density of 125×10^3 cells/cm^2 in a 24-well dishes. After adhesion, macrophages cultures were washed with PBS and then incubated in DMEM4500 (w BSA, w/o PhR), at 37 °C in a 10% CO_2 humidified atmosphere and exposed to rIFNγ (50U/ml) (Immunotools) and LPS (10 ng/ml) (SIGMA) **[20]** for 48h.

2.6 *Mucosal samples collection and RT-PCR analysis*

Mucosal samples were collected in RNAlater and stored at −80 °C until extraction of nucleic acids. DNA quality was assessed by gel electrophoresis and spectrophotometry, measuring OD 260/280.

2.7 *Determination of apoptosis*

Apoptosis was evaluated in histological sections (4 µm thick) of tumours stained with hematoxylin eosin, determining cells with the following characteristics of apoptosis: cell shrinkage, loss of normal contact with the adjacent cells of the crypt, chromatin condensation, or formation of round or oval nuclear fragments. Apoptosis was quantified as the number of apoptotic cells/area measured using the ACT-2U software program (Nikon, Instruments Europe, Badhoevedorp, NL) connected via a camera to the microscope (Optiphot-2, Nikon, NL). The evaluation was performed at 1000× magnification.

2.8 *Nitric oxide assay*

NO concentration was measured in the culture medium of rat peritoneal macrophages or RAW cells using the Griess reaction. Namely, NO production was measured in rat peritoneal macrophages cultures after 48h of an *in vitro* treatment with IFNγ and LPS, while in RAW264.7 cells (1.6×10^5 cells/well) was measured after 24h treatment with 1µg/mL LPS **[21]**. In particular, RAW264.7 cells were exposed for 24h to a co-treatment with LPS and 50µM ORLE, or for 72h to 50µM ORLE pre-treatment and a sequential 24h treatment with LPS. Briefly, 100 µl of cell culture medium from RAW264.7 cells or 250 µl from peritoneal macrophages cultures were mixed with an equal volume of Griess reagent (1 % sulfanilamide, 0.1 % N-1-naphthalenediamine dihydrochloride, and 2.5 % H3PO4) and transferred to 96-well plates. Plates were incubated at room temperature for 10 min. Then the absorbance was measured at 540 nm in a microplate reader (Biotek, Winooski, VT, USA). The amount of nitrite in the media was calculated from sodium nitrite (NaNO2) standard curve. Results were normalized to protein concentration. For RAW264.7 cells NO production was expressed referred to LPS as 100%.

2.9 *Cytofluorimetric assay of iNOS in HCT116 cells*

The expression of intracellular iNOS in HCT116 cells was assessed by flow cytometry using iNOS monoclonal antibody recommended for the detection of NOS2 of mouse, rat and human origin. HCT116 cells were exposed to a standard medium of a medium containing 50µM ORLE for 72h. At the end of the incubation, cells were harvested, fixed in ethanol 70%, permeabilized (triton-X100, 0.01%), and then stained using anti-human iNOS mouse IgG1 monoclonal antibody (Santa Cruz sc-7271) (1 µg/ 10^5 cells). At the end of the incubation (45', 4°C), cells were exposed to PE-labeled goat anti-mouse IgG as secondary antibody (Immunotools #22549814). Stained cells were analyzed on a fluorescence-activated cell sorting (FACS) flow cytometer (FACScan, Becton Dickinson).

2.10 Western blotting analysis

RAW cells were exposed to 1 μg/ml LPS alone, or 50 μM ORLE, or LPS/ORLE in complete medium as previously described. After incubation, cells and supernatants were lysed together and separated using electrophoresis **[11]**. Cells were washed with ice cold PBS containing 1 μM Na_4VO_3, and lysed in 100 μL of cell RIPA lysis buffer (Merck Millipore, Vimodrone, Milan, Italy) containing PMSF (Sigma-Aldrich), sodium orthovanadate (Sigma-Aldrich) and protease inhibitor cocktail (Calbiochem). Aliquots of supernatants containing equal amounts of protein (40 ng) in Laemmli buffer were separated on Bolt® Bis-Tris Plus gels 4-12% precast polyacrylamide gels (Life Technologies, Monza, Italy). Fractionated proteins were transferred from the gel to a PVDF (polyvinylidene difluoride) membrane using iBlot 2 system (Life Technologies, Monza, Italy). Membranes were blocked for 1 h, at room temperature, with Odyssey blocking buffer (Dasit Science, Cornaredo, Milan, Italy). Subsequently, the membrane was probed at 4 °C overnight with primary antibodies diluted in a solution of 1:1 Odyssey blocking buffer/T-PBS buffer. The primary antibodies were (1:1000): COX-2 rabbit anti h/m/r mAb (Cell Signaling #4842S, 74kDa), iNOS rabbit anti mouse mAb (Cell Signaling #13120S, 130 kDa), and IL-1βRI rabbit anti mouse mAb (Santa Cruz sc-689, 80 kDa). The membranes were washed in T-PBS buffer and incubated for 1 h at room temperature with goat anti-rabbit IgG Alexa Flour 750 antibody or with goat anti-mouse IgG Alexa Fluor 680 antibody (Invitrogen) (1:10000). Membranes were then visualized by an Odyssey Infrared Imaging System (LI-COR® Bioscience, Lincoln, NE, USA). Vinculin rabbit anti h/m/r/mk mAb (Cell Signaling #13901, 124 kDa) (1:1000) and anti h/m/r/mk mAb α-tubulin (Cell Signaling #3873, 52 kDa) were used to assess equal amount of protein loaded in each lane.

2.11 RNA extraction and quantitative PCR

Total RNA from rat tumours or RAW264.7 cells was extracted using the Trizol reagent. The first-strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Biorad #1708891) following instructions provided. Quantitative real-time polymerase chain reaction (RT-PCR) was carried out using Applied Biosystems SYBR Green PCR Master Mix (#4309155). The results were analyzed on the (Biorad CFX96 qPCR Instrument). Values were normalized to 18S, and all the results were obtained from at least three experiments independently. The sequences of primers used in this study are listed in Table 1.

Table 1. Primer sequences for qRT-PCR.

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
IL-1β	CCT GCA GCT GGA GAG TGT GGA	CCC ATC AGA GGC AAG GAG GAA
IL-6	CTT CCA TCC AGT TGC CTT CT	TGC ATC ATC GTT GTT CAT AC
TGF-β	GGC TTC TAG TGC TGA CG	GGG TGC TGT TGT ACA AAG
18S	CGC CGC TAG AGG TGA AAT TCT	CGA ACC TCC GAC TTT CGT TCT

2.12 Statistical Analysis

Densitometric data are expressed as means ± standard errors of the mean (SEM) depicted by vertical bars from representative experiment of at least three independent experiments. Statistical analysis of the data was performed by ANOVA and Tukey's multiple comparisons test, and $p \leq 0.05$ was considered statistically significant.

3. Results

3.1 *In vivo effect of ORLE on PIRC rats*

One-year old PIRC rats with consolidated colon tumours were fed for one week with ORLE diet or standard diet. At the end of treatment, no differences in body weight were found between the two groups, indicating an unchanged caloric intake. At sacrifice, colon tumours were present in both controls and ORLE treated groups, and were processed and analyzed for cell proliferation and apoptosis level. ORLE induced a significant reduction in cell proliferation and augmented the levels of apoptotic bodies in the tumour lesions of PIRC rats, when compared to those of control group fed with a standard diet (Figure 1a).

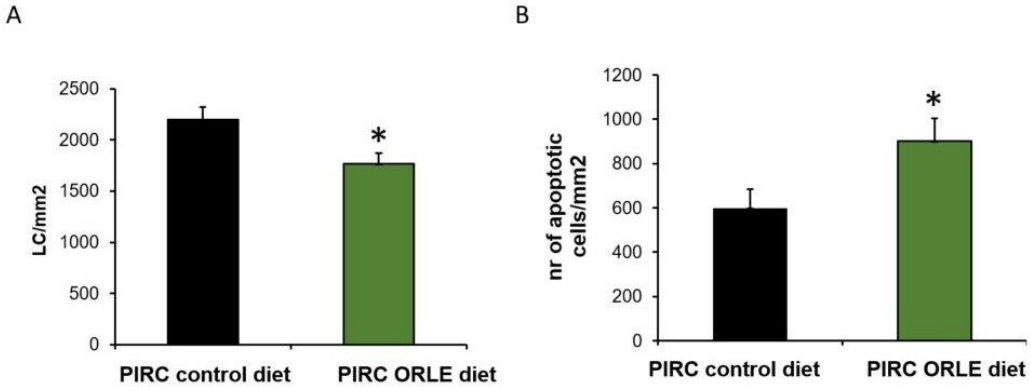

Figure. 1. A Proliferative activity ((PCNA-Labelled cells (LC))/neoplastic lesion region), and (B) apoptosis (apoptotic cells/neoplastic lesion region) in PIRC rats fed with different diets. The densities of proliferative cells and apoptotic cells were measured in histological sections of colorectal mucosa stained with hematoxylin-eosin. * P: < 0.05, with t-test, compared to control diet fed rats (black columns).

To explore the *in vivo* effect of ORLE, we also evaluated the response of ORLE enriched diet on macrophages. Macrophage recruitment to neoplastic mucosa was determined with the evaluation of CD68 antigen expression in the colon tumours. As showed in Figure 2a, ORLE intake did not alter the number of macrophages infiltrating the lesions. In addition, as NO is often associated with cancer aggressiveness, we determined mRNA level of iNOS in the colon tumours and compared to that expressed in normal mucosa of the same rats. We found that iNOS expression was significantly augmented in tumour lesions compared to their normal mucosa, while in rats fed with an ORLE diet, iNOS mRNA expression in tumour lesions reduced to a level of normal mucosa (Figure 2b). The effect of the ORLE diet was also explored in peritoneal macrophages collected at sacrifice from rats fed with the two diets. In particular, we examined the *ex-vivo* NO production by collected macrophages after *in vitro* treatment with IFNγ and LPS. Macrophages recovered from PIRC rats fed with ORLE diet were unresponsive to the exposure to IFNγ/LPS. Indeed, the release of NO was comparable to that of relative untreated macrophages. Conversely, peritoneal macrophages collected from PIRC rats fed with a control diet were extremely responsive to the exposure to IFNγ/LPS. Indeed, the NO release was remarkably increased compared to relative untreated macrophages. (Figure 2c).

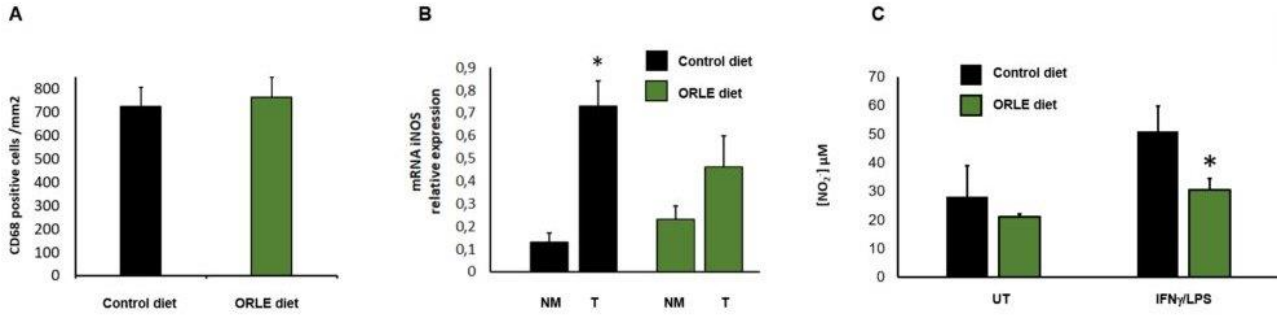

Figure 2. Effect of ORLE on rat macrophages. (A) CD-68 expression (positive cells/mm^2) in colon tumors from PIRC rats fed with a control diet (black column) or a diet enriched in ORLE (green column). (A) iNOS expression in colon tumor lesions from PIRC rats fed with a control diet (black columns) or a diet enriched in ORLE (green columns) compared to iNOS expression in normal mucosa. (C) Effect of ORLE on NO production by peritoneal macrophages recovered from tumor bearing rats fed with a control diet (black columns) or a diet enriched in ORLE (green columns) and exposed to standard media or to IFNγ/LPS. Data are expressed as means ± SEM; *P < 0.05. Statistical significance of differences vs control, t-test.

Finally, since a reduction of iNOS mRNA was observed in rat colon tumours, we aimed to determine whether the expression of iNOS protein by colon cancer cells changed after the treatment with ORLE. By using flow cytometry analysis, we found a decreased iNOS protein expression in HCT116 cells treated with 50µM ORLE for 72h. HCT116 cells exposed to 72h treatment did not show a significant reduction of cell viability at 50µM but at 100 µM ORLE, a resistance probably related to their high aggressiveness (Figure 3a and 3b).

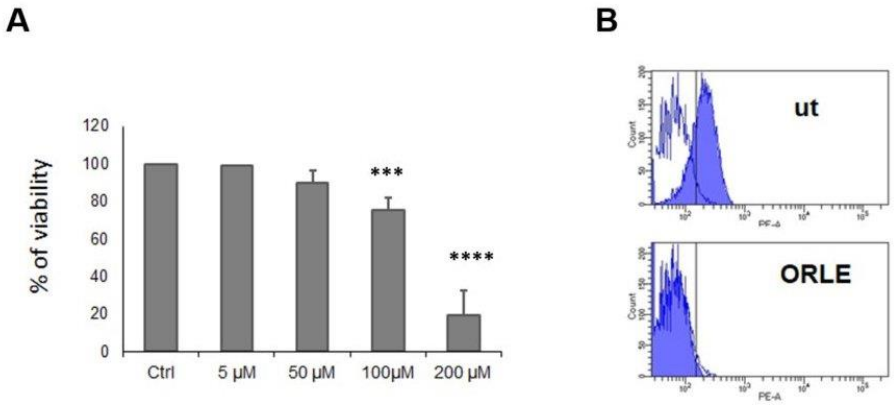

Figure 3. MTT assay of HTC116 cells exposed for 72h to different concentration of ORLE (A). Data are expressed as means±SEM of the percentage of viability and are representative of three independent experiments (n=4). ***P<0.01 ****P<0.0001 vs untreated cells. (B) Inhibition of intracellular protein expression of iNOS in HCT116 cells exposed for 72h to ORLE (50 µM). Blu istograms represents HCT116 cells exposed to primary and secondary antibody, while white istograms represents cells exposed to secondary antibody only. Representative results of at least three independent experiments.

3.2 Effect of ORLE on primed murine RAW264.7 cells

To further explore ORLE effect on macrophages pro-inflammatory activity, we evaluated the release of NO in RAW264.7 cells treated with 50 µM ORLE for 24h in the presence of LPS (acute exposure) or pretreated with 50 µM ORLE for 72h (chronic exposure) and next exposed to LPS. NO production, in quiescent RAW264.7 cells, was not affected by the treatment with ORLE, neither after an acute or chronic modality. NO production in RAW264.7 cells activated with ORLE acute exposure was significantly inhibited compared to that measured in absence of it (50% reduction). Interestingly, NO production by LPS-activated macrophages, after ORLE chronic exposure, was strongly inhibited (70%

compared to LPS-treated) (Figure 4a). In parallel, ORLE treatment strongly inhibited the expression of iNOS after ORLE acute or chronic exposure (Figure 4b).

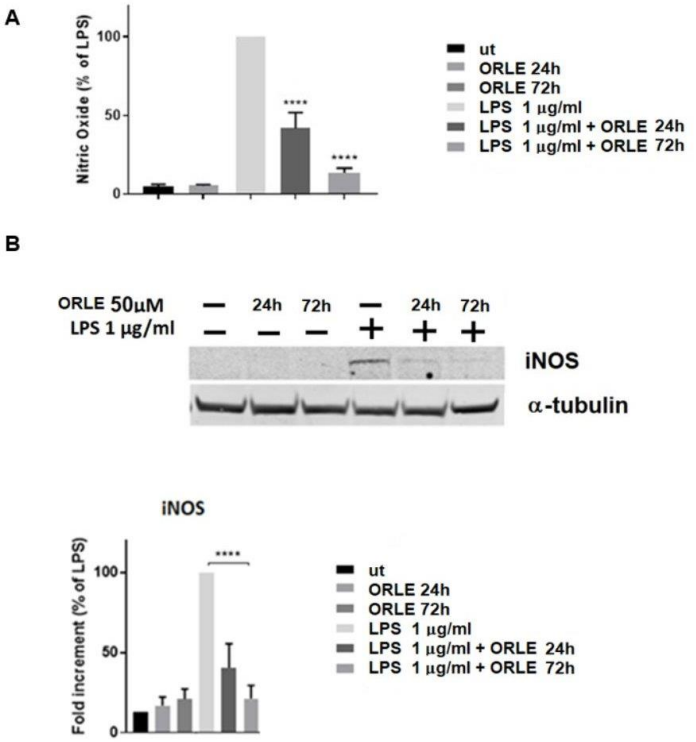

Figure 4. Inhibition of NO production in RAW264.7 macrophages: (A) exposed to 50 µM ORLE and 1 µg/ml LPS. Data are expressed as means±SEM of the percentage of inhibition compared to LPS treated cells. Data are representative of three independent experiments (n=4). ****P<0.0001 vs LPS-stimulated RAW264.7 macrophages. (B) Upper panel, representative Western blot of inducible nitric oxide synthase (iNOS) protein expression, lower panel, densitometric analysis of iNOS expression in RAW.264.7 cells. Data are expressed as means±SEM of percentage compared to LPS-stimulated cells ****P<0.0001.

Further, ORLE inhibits the expression of COX-2 by an acute treatment and completely abolished it after a chronic exposure (Figure 5). It has to be noted that the expression of iNOS and COX-2 was evaluated on the same set of samples, and, necessarily, the same tubulin reference was used. This result strengthens the close correlation between inhibition of iNOS and COX-2. We hypothesize a possible cooperation with iNOS abrogation during tumor lesion regression. Indeed, over-expression of iNOS may generate reactive mutagenic agents causing as DNA damage or impairment of DNA repair, while COX-2 stimulation leads to sustain tumor growth *[22]*.

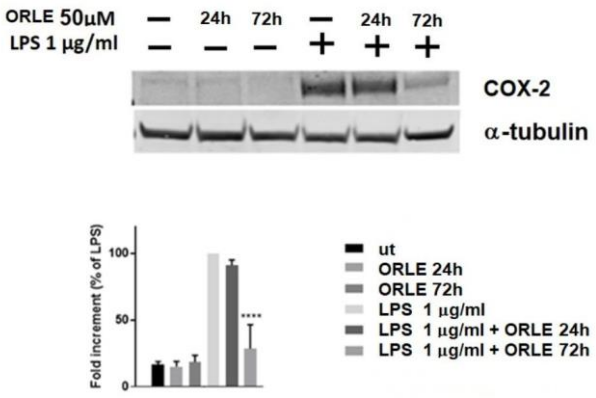

Figure 5. Inhibition of COX-2 expression. Upper panel, representative Western blot of inducible cyclooxygenase-2 (COX-2) protein expression, lower panel, densitometric analysis of COX-2 expression in RAW.264.7 cells. Data are expressed as means±SEM of percentage compared to LPS-stimulated cells ****P<0.0001.

The evaluation of anti-inflammatory effect of ORLE, either acutely or chronically, on RAW 264.7 macrophages exposed to LPS was finally explored through the analysis of the relative mRNA expression of IL-1β and IL-6 cytokines and TGF-β. The results indicated that ORLE decreased the mRNA expression of IL-1β, and IL-6, in LPS-induced RAW264.7 cells in time-dependent manner, the more prolonged was the exposure to ORLE, the more significant was the inhibitory effect (Figure 6a and 6b). In addition to this inhibitory effect, ORLE promotes, in an acute exposure, a reduction of IL-1βR protein expression and a reduction of mRNA expression for TGF-β. (Figure 6c and 6d). This finding discloses how may be extended the anti-inflammatory profile of ORLE during a complementary therapy of colon cancer.

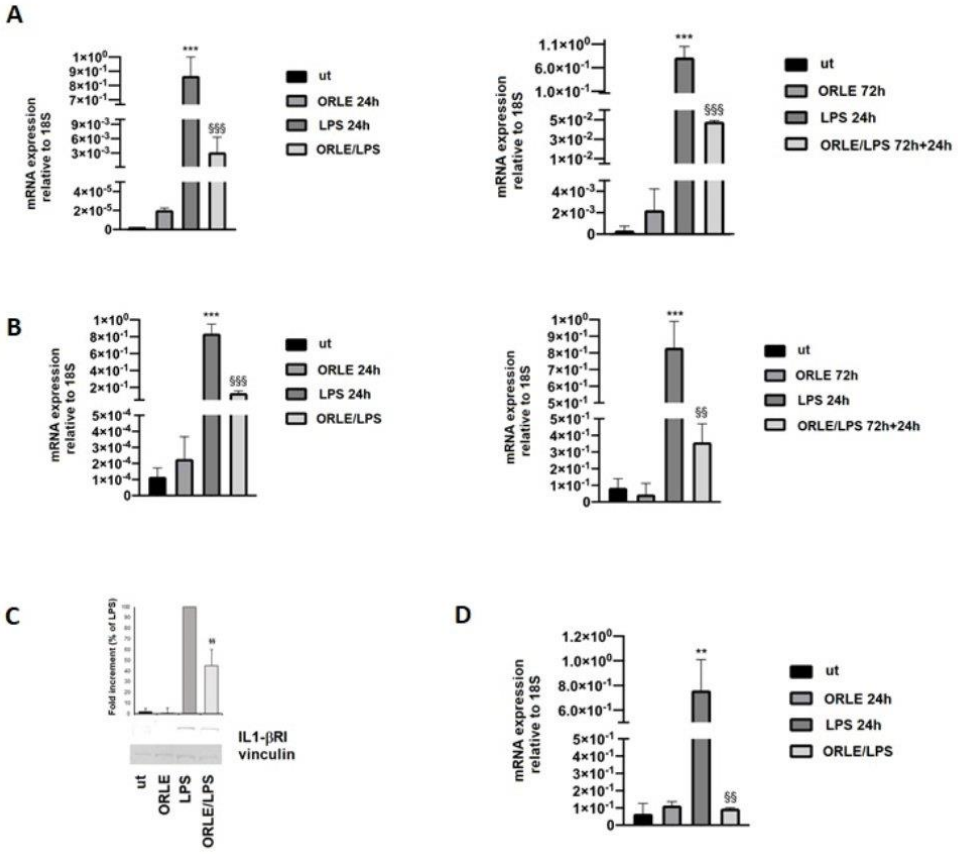

Figure 6. Evaluation by quantitative real-time PCR of IL-6 mRNA, and IL-1β mRNA (panel A and B respectively) in RAW264.7 cells exposed to ORLE and LPS after acute (left panels) or chronic (right panels) exposure. Expression of IL-1RI and relative densitometric analysis (C) and quantitative real-time PCR of of TGF-β mRNA in RAW264.7 cells exposed to ORLE and LPS after acute exposure. mRNA levels were normalized to 18S as an endogenous control, and they are expressed as reduction relative to LPS. Representative results of at least three independent experiments. ** and *** refers to P<0.001 and P<0.0001, respectively, for LPS treatment vs untreated cells (UT) and ORLE treatment; $$ and $$$ refers to refers to P<0.001 and P<0.0001, respectively, for LPS+ORLE treatment vs LPS.

4. Discussion

The anti-inflammatory properties of phenolic secoiridoids have been recognized since a long time, and the reduction of oxidative stress and inflammatory cells recruitment, have been clearly demonstrated [23]. Ole is one of the most intriguing members of secoiridoids family, and is able to dampen systemic inflammation through the modulation of pro-inflammatory cell recruitment [24,25]. Given the close correlation between the perseverance of chronic inflammation and tumour progression, Ole and the other phenolic compounds have been studied for their beneficial effect on different models of *in vitro* and *in vivo* cancer progression.

The aim of the present study is to evaluate, for the first time, the impact of an ORLE-enriched diet, in an animal model with already-developed colon tumours, exploring different aspects of colon cancer and its associated systemic inflammation [26]. We focused our interest on *in vivo* model of colon cancer, using the PIRC rats that spontaneously develop tumours in the colon [27,28]. We choose one-week treatment of ORLE-enriched diet equivalent to the consumption of a Ole dose (100 mg/kg of ORLE) according to previous studies [11,15], to better highlight whether this low-dose treatment might exert a beneficial effect against established cancer lesions and local and systemic inflammation . Secondly, we confirm anti-inflammation of ORLE in murine activated peritoneal macrophages.

The novelty of our study is that PIRC rats were fed with ORLE-enriched diet for one week at one year old and thus bearing consolidated tumours. On the contrary, previous studies explored the chemoprevention effects of Ole/ORLE during the first phases of the development of the tumors [15,29]. We focused our attention on one of the key mediators released in tumoral microenvironment, namely NO, mostly because the role of NO in colon cancer progression is controversial and not fully defined [30]. We demonstrated that ORLE-enriched diet decreased cell proliferation and increased apoptosis in colon tumours in vivo; we also documented that ORLE diet was able to counteract the iNOS tumour-associated over-expression present in control tumours.

It has to be noted that, peritoneal macrophages not only monitor and maintain local homeostasis, but also play the role of sentinel cells against threats such as infections, tissue damages, and tumours [31]. Thus, in some instances, the evaluation of peritoneal macrophages activation could be used as a measure of local and systemic responsiveness to pro-inflammatory stimuli [32]. We found that ORLE-enriched diet dampened the pro-inflammatory behavior of peritoneal macrophages from tumour-bearing rats. Indeed, one-week exposure to ORLE-enriched diet was sufficient to clearly reduce peritoneal macrophages responsiveness to the *in vitro* treatment with IFNγ/LPS. Thereafter, we demonstrate that ORLE was also effective in reducing *in vitro* iNOS expression in human adenocarcinoma cells. It has to be noted that we observed an *in vitro* anti-proliferative effect on colon carcinoma cells only at higher ORLE concentrations. Similar results have been found by others using ORLE [11] or Ole at higher concentrations [33,34]

The up-regulation of iNOS and the elevated NO release is an unavoidable aspect of tumour microenvironment. The NO unpaired electron rapidly reacts with other radical species present in the tumour microenvironment driving to an increased DNA damage and the acquisition of additional mutations on surrounding cells which contribute in the maintenance of an aggressive tumour microenvironment [35–37].

Further, in colon cancer the release of cytokines as IL-1β, IL-6 and the activation of COX-2, collectively, support neoplastic transformation and malignant progression [38,39].

Thus, we investigated whether ORLE treatment might modulate COX-2 and cytokine release of activated macrophages. There is an intense debate regarding the role of tumour-associated macrophages and cancer outcomes, mostly due to the highly plastic behavior of these immune cell population [40,41]. Despite this, clinical evidence suggests that, in colon cancer, the presence of a rich macrophages infiltrate account for a better prognosis [40]. Interestingly, we found that the number of macrophages infiltrating tumour lesions of ORLE enriched diet fed rats was similar to that of macrophages infiltrating tumour lesions in rats fed a regular diet. Conversely, we found that ORLE treatment significantly inhibited LPS activated macrophages, in term of NO release and iNOS expression, consistent with our *in vivo* results and other studies [21]. In addition to this, we found that COX-2 expression in LPS-activated macrophages was completely abolished after ORLE chronic exposure. We also found that ORLE treatment significantly downregulates LPS-macrophage activation in term of IL-1β, IL-6, and TGF-β mRNA expression. These cytokines and growth factor strongly cooperate in the maintenance of a pro-inflammatory and toxic microenvironment, that correlates with the progression of the disease and the resistance to therapy [32,42–44].

On the whole, our results give evidence for a key role of ORLE in downregulating inflammation-driven colon cancer progression through the inhibition of NO and relative proinflammatory mediators.

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