DEPARTAMENTO DE FARMACOLOGÍA
FACULTAD DE FARMACIA
UNIVERSIDAD DE SEVILLA

"ESTUDIO PRECLINICO DE OLEUROPEINA, UN SECOIRIDOIDE DEL OLIVO Y DE SUS DERIVADOS ACETILADOS EN MODELOS EXPERIMENTALES DE INMUNOINFLAMACIÓN"

Tesis Doctoral presentada por

MARIA LUISA CASTEJÓN MARTÍNEZ

Para optar al Grado de Doctor en Farmacia con Mención Internacional

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DEPARTAMENTO DE FARMACOLOGÍA
FACULTAD DE FARMACIA
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La Dra. **Dña. María Concepción Pérez Guerrero**, Profesora Titular de Universidad y Directora del Departamento de Farmacología de la Facultad de Farmacia de la Universidad de Sevilla,

CERTIFICA:

Que la presente Tesis Doctoral titulada "**ESTUDIO PRECLINICO DE OLEUROPEINA, UN SECOIRIDOIDE DEL OLIVO Y DE SUS DERIVADOS ACETILADOS EN MODELOS EXPERIMENTALES DE INMUNOINFLAMACIÓN**" realizada por María Luisa Castejón Martínez, ha sido dirigida por la **Dra. Catalina Alarcón de la Lastra Romero** y la **Dra. Marina Sánchez Hidalgo**, para aspirar al grado de Doctora en Farmacia con Mención Internacional, cumpliendo los requisitos para este tipo de trabajo.

Y para que así conste, firmo la presente.

En Sevilla, a 31 de Octubre de 2019

Fdo.: Dra. **María Concepción Pérez Guerrero**

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Las Dras. **Dña. Catalina Alarcón de la Lastra Romero**, Profesora Catedrática de Universidad y **Dña. Marina Sánchez Hidalgo,** Profesora Titular de Universidad, adscritas al Departamento de Farmacología de la Facultad de Farmacia de la Universidad de Sevilla,

CERTIFICAN:

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En Sevilla, a 31 de Octubre de 2019

VºBº de las directoras

Fdo.: **Dra. Catalina Alarcón de la Lastra Romero**

Fdo.: **Dra. Marina Sánchez Hidalgo**

Abreviaturas

A

ACPAs	Anti-citrullinated protein antibodies
AIN	American Institute of Nutrition
AINE	Anti-inflamatorio no esteroideo
AO	Aceite de oliva
AOVE	Aceite de oliva virgen extra
AP-1	Activator protein - 1
AR	Artritis reumatoide
ARE	Antioxidant responsive elements
ASC	Apoptotic speck protein containing a caspase recruitment domain

C

C1q	Componente del complemento 1q
CD	Cluster of differentation
CII	Type II Collagen
CIA	Collagen-induced arthritis / Artritis inducida por colágeno tipo II
COMP	Cartilage oligomeric matrix protein / Proteína oligomérica de la matriz del cartílago
COX-2	Cyclooxigenase-2 / Ciclooxigenasa-2
CXCR4	Motif chemokine receptor type 4/ Receptor de quimiocinas CXC tipo 4

D

DC	Dendritic cells / Células dendríticas
DCF	2,7-dichlorofluorescein
DCFH-DA	2,7-dichlorofluorescein-diacetate
DMARDs	Disease-modifying antirheumatic drugs
DMEM	Dubelcco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNASE1L3	Desoxirribonucleasa 1 similar a 3
DSS	Dextran sulphate sodium

E

EDTA	Ethylenediaminetetraacetic acid
EII	Enfermedad inflamatoria intestinal
ELISA	Enzyme-linked immunoassay / Ensayo por inmunoabsorción ligado a enzima
EP4	Prostaglandin E_2 receptor 4
ERK	Extracellular signal-regulated kinases / Cinasa regulada por señal extracelular
EVOO	Extra virgin olive oil

F

FAME	Fármacos antirreumáticos modificadores de la enfermedad
FBS	Fetal bovine serum
FI	Fracción insaponificable
FOXP3	Forkhead box P3 / Factor nuclear FOXP3
FP	Fracción polifenólica
FS	Fibroblastos sinoviales
	G
GE10H	Geraniol 10-hydrolase enzime
GES	Geraniol synthase
GM-CSF	Granulocyte-macrophage colony-stimulating factor/ Factor estimulante de colonias de granulocitos-macrófagos
GT	Glycosyltransferase
	H
H	hours
HBV	Hepatitis B virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H&E	Haematoxylin and Eosin
HIF-1	Factor inducible por hipoxia - 1
HIV	Human immunodeficiency virus
HO-1	Heme oxigenase-1
HRP	Horseadish peroxidase
HTy	Hydroxytyrosol
HTy-Ac	Hydroxytyrosol acetate / Acetato de hidroxitirosol
	I
IBD	Inflammatory bowel disease
ICs	Immune complexes / Inmunocomplejos
ICAM–1	Intercellular adhesión molecule-1 / Adhesinas endoteliales
IFN	Interferon / Interferón
Ig	Immunoglobulin / Inmunoglobulina
IκB-α	Inhibitor of NF-κB / Inhibidor de NF-κB
IL	Interleukin / Interleucina
IMID	Enfemedades inflamatorias inmunomediadas
iNOS	Inducible nitric oxide synthase / Óxido nítrico sintasa inducible
i.p.	Intraperitoneally / Intraperitoneal
IP-10	Proteína inducida por interferón γ -10
IRF	Factor regulador del interferón
	J

JAK-STAT	Janus kinase-signal transducer and activator of transcription / Janus cinasas-transductor de señal y activador de la transcripción
JNK	c-Jun NH_2- terminal kinase / C-Jun NH_2- terminal cinasa
	K
Keap-1	Kelch-likeECH-associated protein 1
	L
LDL	Low-density lipoprotein
LES	Lupus eritematoso sistémico
LPS	Lipopolysaccharide / Lipopolisacárido
	M
MAPK	Mitogen-activated protein kinases / Proteínas cinasas activadas por mitógeno
MCP-1	Monocyte chemoatractant protein-1/ Proteína quimiotáctica de monocitos 1
MMP	Metalloproteinases / Metaloproteasas
mPGES- 1	Microsomal prostaglandin E synthase – 1 / Prostaglandine E sintasa microsomal 1
MPO	Myeloperoxidase / Mieloperoxidasa
mRNA	Messenger RNA
MS	Multiple sclerosis
MUFA	Monounsaturated fatty acid / Ácidos grasos
MTX	Metotrexato
	N
NF-κB	Nuclear transcription factor-kappa B / Factor nuclear kappa B
NLRP3	NLR family pyrin domain-containing 3 inflammasome / Inflamasoma NLRP3
NO	Nitric oxide / Óxido nítrico
NOD	Nucleotide-binding oligomerization domain
Nrf2	Nuclear factor E2-related factor 2 / Factor nuclear eritroide 2
NSAIDs	Non-steroidal anti-inflammatory drugs
	O
OA	Osteoarthritis
OL	Oleuropein / Oleuropeína
	P
PAF	Factor activador de plaquetas
PAS	Periodic acid Schiff
PBMC	Peripheral blood mononuclear cells / Células mononucleares de sangre periférica

PBS	Phosphate buffered saline
Per-HTy	Peracetylated-hydroxytyrosol / Hidroxitirosol peracetilado
Per-OL	Peracetylated-oleuropein / Oleuropeína peracetilada
PG	Prostaglandin / Prostaglandina
PGE_2	Prostaglandin E_2
	R
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species / Especies reactivas de oxígeno
	S
SD	Standard diet
SDF1	Factor derivado de células estromales 1
SDS	Sodium dodecyl sulfate
S.E.M.	Standard error of the mean
SF	Sinovial fibroblasts
SLE	Systemic lupus erythematosus
SLS	Secologanin
SRB	Sulforhodamine B / Sulforhodamina B
STAT	Signal transducer and activator of transcription / Transductor de señal y activador de la transcripción
	T
Th	Helper T cells / Células T cooperadoras
TLR	Toll like receptor / Receptor tipo Toll
TNF-α	Tumor necrosis factor alpha/ Factor de necrosis tumoral alfa
Treg	Regulatory T cells / Células T reguladoras
TREX1	Exonucleasa de reparación de 3 cepas 1
Ty	Tyrosol / Tirosol
	U
UC	Ulcerative colitis
	V
VHSV	Hemorrhagic septicemia rhabdovirus
VSMC	Vascular smooth muscle cells

Índice

Abstract

Olea europea L. is an ancient tree originally native from Asia Minor and Siria, which today is mainly cultivated in the entire Mediterranean area. The major producers of olives and olive oil are Spain, Italy and Greece. Concretely, Spain was able to produce around 127100 tons of olive oil in the last year according to the Agency of Olive Oil (Romani *et al.*, 2019). The fruit and compression extracted oil have a widespread of therapeutic and culinary uses.

Olive oil is considered an excellent source of lipids, phenolic constituents and squalene, among others, being associated with the primary and secondary prevention of cardiovascular diseases outcomes and it has also been linked to a reduced incidence of inflammatory diseases and cancer particularly skin, colon and breast (Cárdeno, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2013; Marcelino *et al.*, 2019). More recently, it has been well established the potential observed immunomodulatory effects of extra virgin olive oil (EVOO) in different autoimmune experimental animal models such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Cardeno *et al.*, 2014; Aparicio-Soto *et al.*, 2016; Rosillo *et al.*, 2016a).

In addition to olive oil, there are also other interesting beneficial components in the olive tree. This is the case of the olive tree leaf and fruits, which are an abundant source of biophenols of particular interest as food supplements and various industrial applications in cosmetic and pharmaceutical industries (Benincasa *et al.*, 2019).

The phenolic composition of olive leaf and fruit varies according to plant variety, harvesting season and method, maturity and extraction method. Normally, the main chemical compound in unprocessed olive leaves and drupes is oleuropein (OL), a secoiridoid, ester of elenolic acid and 3,4-dihydroxyphenyl ethanol. Its degradation results in the formation of hydroxytyrosol (HTy) in olive oil. In addition , other polyphenols such as caffeic acid, chlorogenic acid , flavan-3-ols (catechin) and flavonoids, including luteolin 7-O glucoside, kaempherol-3-O-glucoside, rutin, quercetin 3-O-glucoside and apigenin-7-O-glucoside are also present (Herrero *et al.*, 2011; Castejon *et al.*, 2019; Gonçalves *et al.*, 2019).

OL has catechol functionality and exerts potent and well-stablished antioxidant activity, mostly correlated to its ability to enhance radical stability through the formation of an intramolecular hydrogen bond between the free hydrogen of the hydroxyl group and its phenoxyl radicals (Visioli, Bellomo and Galli, 1998). OL also possesses a well-documented anti-atherogenic, anti-cancer, anti-angiogenic, neuroprotective, antimicrobial and antiviral**,** gastroprotective, hepatoprotective, antidiabetic, antiobesity and radioprotective activities, among others (Hassen, Casabianca and Hosni, 2015).

Interesting several studies have reported important anti-inflammatory properties of this secoiridoid (Barbaro *et al.*, 2014; Marcelino *et al.*, 2019). In addition, it has the ability to scavenge nitric oxide (NO) and it also promotes the expression of the inducible nitric oxide synthase (iNOS) in cells (de la Puerta *et al.*, 2001).

Besides, OL was able to suppress lipopolysaccharide (LPS)-stimulated RAW 264 (Ryu *et al.*, 2015). Also OL exerted a significantly protective effect on cartilage slowing down the progression of osteoarthritic lesions in Guinea pigs (Horcajada *et al.*, 2015). Likewise, OL-aglycone improved clinical markers and histological status in joints and paws on collagen induced arthritis (CIA) mice model (Impellizzeri *et al.*, 2011). Indeed, OL has been shown to inhibit pulmonary inflammation in

experimental models of interleukin (IL)-4-exposed bronchial BEAS-2B epithelial cells and ovalbumin or cigarette smoke exposed BALB/c mice (Kim *et al.*, 2018).

On the other hand, some studies have shown the importance of acetylated derivatives obtained from natural phenols, as a value alternative for improving the properties of the original molecules, for example, bioavailability, cell membrane penetration and enhanced anti-inflammatory and antioxidant activities. So, the acetylation of these natural compounds may improve pharmacodynamics and pharmacokinetic profiles of the natural compounds and it could be an appealing strategy in the management of different inflammatory process (de Araújo *et al.*, 2017; Rizzo *et al.*, 2017).

Immune cell plasticity is mainly involved in the pathogenesis and resolution of chronic inflammatory autoimmune processes such as RA and SLE. Dietary components, especially dietary fatty acids and polyphenols, modulate the immune response and they might be explicit as a pharma-nutritional methodology for the prevention and management of these illnesses (Ricordi, Garcia-Contreras and Farnetti, 2015).

RA is a chronic autoimmune inflammatory disorder characterized by inflammation of the synovial membrane and it courses with a progressive destruction of the cartilage and bone. Its pathogenesis involved the activation of innate and adoptive immune cells, resident cells such as osteoclasts, fibroblast-like synoviocytes and chondrocytes, as well as certain autoantibodies, rheumatoid factor and anti-citrullinates peptide antibodies. Of note, activated T cells release cytokines that activate macrophages to secrete other proinflammatory cytokines, inducing the differentiation of B cells and activate the release of metalloproteinases (MMPs) (Salgado and Maneiro, 2014).

The pharmacological treatment in RA includes non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease-modifying antirheumatic drugs (DMARDs) and biological agents. The objective of conventional pharmacological therapy is to finish or reverse cartilage destruction and diminish the pain devoid of untoward effects. However, current treatments are not efficient in all patients and possess a number of disadvantages, such as high cost, necessity for parenteral administration and potential adverse effects. Consequently, nutritional therapy as complementary and alternative medicine is under development as an innovative strategy in RA management (Gaforio *et al.*, 2019).

Another autoimmune and chronic inflammatory disease currently actively investigated is SLE. Unlike the previous pathology, SLE affects multiple organ systems including joints, skin, kidneys and the brain, among others. One characteristic of SLE is its pathogenic complexity, what makes its diagnosis difficult, among a high number of complications that can affect the quality of life of patients (Petri *et al.*, 2013). In fact genetic, epigenetic and environmental factors, as well as nutrition and infection, are involved concur. Immune complexes, autoantibodies, imbalance of T-helper cell subsets (Th)-1/Th2/Th17 and regulatory T-cells (Tregs), play an important role in SLE tissue damage (Noble *et al.*, 2016).

Typical management of SLE includes the use of low-dose glucocorticoids, NSAIDs and also, antimalarial drugs, immunosuppressive agents and biological drugs (Kuhn *et al.*, 2015; Rosillo, Alarcón-de-la-Lastra and Sánchez-Hidalgo, 2016). Moreover, diet quality in SLE patients is relevant since these patients suffer a higher risk of other pathologies which are directly influenced by diet. Therefore, nutritional therapy by means of diet changes and the use of nutritional supplements could

be a promising tool for SLE due, possibly reducing comorbidities and improving quality of life in SLE patients (Aparicio-Soto, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2017).

Considering this background the **objectives** of this Doctoral Thesis have been the following:

1. To investigate the potential immunomodulatory and anti-inflammatory effects of OL and its semi-synthetic acetyl-derivatives as well as to explore the molecular mechanisms and signaling pathways involved on:

1.a. An *ex vivo* model of murine peritoneal macrophages stimulated with LPS.

1.b. An *in vitro* model of human IL-1β-stimulated synovial fibroblasts cell line (SW982).

2. To explore the effects of OL and peracetylated OL (Per-OL), enriched diets in a model of RA collagen-type II induced arthritis in DBAJ/1 mice and clarify the molecular mechanisms and signaling pathways involved.

3. To study the potential effects of OL and Per-OL enriched diets in a model of SLE induced by pristane (2,6,10,14-tetramethylpentadecane) in BALB/c mice and explore the molecular mechanisms underlying and signaling pathways probably involved.

Results and Discussion

1. Olive secoiridoid Oleuropein and its semisynthetic acetyl-derivatives reduce LPS-induced inflammatory response in murine peritoneal macrophages via JAK-STAT and MAPKs signaling pathways.

This study was designed to investigate the potential anti-inflammatory, antioxidant and immunomodulatory activities of three new acyl derivatives synthesized from OL: Per-OL; 2'', 3'', 4'', 6''-Tetra-*O*-acetyloleuropein and 6''-*O*-Acetyloleuropein in LPS-stimulated mouse peritoneal macrophages in comparison with natural OL.

OL is the main phenolic component of olives leaves, roots and unprocessed olive drupes which is hydrolyzed and form different products, including HTy (Pan *et al.*, 2018). The acetylation of polyphenols may bring improved properties to these molecules, such as, improved bioavailability, cell membrane penetration and enhanced anti-inflammatory and antioxidant activities among others (de Araújo *et al.*, 2017).

Balance disruption of the intracellular reduction-oxidation state has been observed in stimulated macrophages. Our findings showed that the new OL acyl derivatives were able to reduce reactive oxygen species (ROS) levels acting as effective antioxidants. The stimulation of macrophages by LPS induces the transcription of iNOS gene and generation of large amount of nitrites (Li *et al.*, 2012). In our study, we found that OL and its acetylated derivatives prevented the NO mediated induction and reduced iNOS expression induced by LPS. Ciclooxigenase-2 (COX-2) is essential for the inflammatory response and is responsible for the overproduction of prostaglandin E_2 (PGE_2) in inflammation

process. Likewise, PGE_2 modulates a variety of immune processes at sites of inflammation, including production of proinflammatory cytokines (Cardeno *et al.*, 2014). Besides, LPS-stimulated macrophages are closely related to an imbalance of cytokine network. It is well-known that this kind of inflammatory process is characterized by an increase of proinflammatory cytokines, mainly, tumor necrosis factor (TNF)-α, IL-1β, IL-6, IL-17 and interferon (IFN)-γ. According to our results, OL and its derivatives treatments were able to significantly reduce COX-2 protein expression, PGE_2 and proinflammatory cytokines levels in LPS-stimulated macrophages.

Additionally, mitogen-activated protein kinases (MAPKs) pathway is a critical axis essential for both induction and propagation of the inflammatory LPS-activated macrophages response (Radnai *et al.*, 2009). Moreover, MAPKs are also involved in the activation of janus kinase-signal transducer and activator of transcription (JAK/STAT), an important signaling transduction pathway for the biological function of many cytokines (Zhu *et al.*, 2013). Our results showed that the pre-treatment with these phenolic compounds significantly prevented MAPKs and signal transducer and activator of transcription (STAT)-3 phosphorylation in LPS-stimulated macrophages.

Nuclear factor E2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that binds to antioxidant response elements (ARE) located in the promoter regions of many detoxifying/antioxidant genes, including heme oxigenase-1 (HO-1). In inflammatory conditions, HO-1 protein expression could be part of an adaptive response to limit cytoxicity via several mechanisms, regulation of cell proliferation and prevention of apoptosis. Thus, Nrf2 regulates redox status and plays key roles in cellular defense by enhancing ROS remove (Rosillo *et al.*, 2015). Our data showed that OL derivatives were able to increase the protein expression of Nrf2 and HO-1 in LPS-stimulated macrophages.

In conclusion, the new acetylated OL derivatives have an important role in the balance of the inflammatory microenvironment induced by LPS in murine peritoneal macrophages by inhibiting proinflammatory cytokines production, as well as, iNOS and COX-2 overexpression. The mechanisms underlying these protective effects could be related via Nrf2/HO-1 antioxidant pathway activation and inhibition of both JAK/STAT and MAPKs signaling pathways.

2. Oleuropein down-regulated IL-1β-induced inflammation and oxidative stress in human synovial fibroblasts cell line SW982.

RA is a chronic and systemic inflammatory autoimmune disease mainly characterized by aggressive hyperproliferation of synovial fibroblasts (SFs).

As it is has been commented above, OL possesses well-documented pharmacological properties, including antioxidant and anti-inflammatory effects, and it is available as a food supplement in Mediterranean countries. However, to date, anti-arthritic effects of OL on SFs have not been yet elucidated.

The aim of the present study was to investigate the potential effects of OL, on IL-1β-induced production of inflammatory mediators and oxidative stress in the human synovial sarcoma cell line (SW982). In order to gain a better insight into its mechanisms of action, signaling pathways were also explored. OL exerted anti-inflammatory and anti-oxidant effects via down-regulation of MAPKs and nuclear transcription factor-kappa B (NF-κB) signaling pathways and induction of Nrf2-linked HO-1 controlling the production of inflammatory mediators decreasing IL-6 and TNF-α cytokines, MMP-1

and MMP-3 levels and microsomal prostaglandin E synthase-1 (mPGEs-1) and COX-2 overexpression. Thus, OL might provide a basis for developing a new dietary strategy for the prevention and management of RA.

3. Oleuropein and its new peracetylated derivative ameliorate joint inflammation and destruction in a murine collagen-induced arthritis model via activation of the Nrf2/HO-1 antioxidant pathway and suppression of MAPKs and NF-κB activation.

This study was designed to evaluate dietary OL and Per-OL supplementation effects on CIA model in mice.

Three-weeks-old male DBA-1/J mice were randomized into five experimental groups during all experiment period: (i) Naïve group (SD-Naïve) which received a Standard Diet (SD); (ii) CIA control group (SD-CIA) which received a SD; (iii) OL diet group enriched 0.05% (OL-CIA); (iv) Per-OL diet enriched 0.05% (Per-OL 0.05-CIA) and (v) Per-OL diet enriched 0.025% (Per-OL 0.025-CIA). After six weeks, arthritis was induced by type II collagen (CII) (day 0) and on day 21, mice received a booster injection. Mice were sacrificed 42 days after first immunization. Blood was recollected and paws were histological and biochemically processed.

Our data revealed, that OL and Per-OL supplemented diets exhibited preventive effects in the development of inflammation and joint damage in SD-CIA in comparison with animals that were fed with OL and Per-OL enriched-diets. These results were correlated to an improve arthritis score and with a reduction of inflammatory cells infiltration into articular tissue, synovial hyperplasia and cartilage destruction.

Cartilage oligomeric matrix protein (COMP) is non-collagenous component of cartilage with a great potential as a biological marker of cartilage metabolism in RA (Rosillo *et al.*, 2016b). Also, MMP-3 has been reported to be the major enzymes produced by fibroblasts and macrophages in the synovium and it is responsible for the degradation of proteoglycans, cartilage link protein, fibronectin and collagen (Castejón *et al.*, 2017).

Our results showed that the levels of both parameters, COMP and MMP-3 were significantly lowered with dietary OL and Per-OL treatment in CIA mice. In this study, we have demonstrated that OL and Per-OL enriched-diets could be able to control the local levels of proinflammatory cytokines, such as IL-6, IL-1β, IL-17, TNF-α and IFN-γ, which were associated with the progression of RA (Komatsu and Takayanagi, 2012). Besides, the increment of proinflammatory mediators, like COX-2 and iNOS protein expression could contribute to the progression of RA (Zhao *et al.*, 2019). However, the expression of both biomarkers was significantly inhibited by dietary OL and Per-OL treatment.

Abnormal signaling pathways play an important role in the inflammatory process and can lead to a dysregulation of the inflammatory response being crucial in RA pathogenesis. On one hand, MAPKs play important roles in transducing synovial inflammation and joint destruction and they are considered critical molecular targets for therapeutic intervention in RA (Thalhamer, McGrath and Harnett, 2007). Besides, NF-κB is considered a key signaling molecule in the control of synovial inflammation, hyperplasia and matrix generation playing an important role in the development of RA (Chen *et al.*, 2016). Our results suggested that dietary OL and Per-OL treatments suppressed NF-κB activation and MAPKs phosphorylation in CIA-induced arthritis mice. On the other hand, the activation of Nrf2/HO-1 signaling pathway plays a critical role in the prevention and relief of RA

(Fan *et al.*, 2018) since Nrf2 inhibition aggravates cartilage destruction and accelerates the effector phase of RA in mice. However, upregulating the expression of Nrf2 exerts anti-inflammatory effects in RA (Wu *et al.*, 2016). Our data showed that HO-1 and Nrf2 protein expression were decreased in SD-CIA control group, however dietary Per-OL treatments could restore Nrf2 and HO-1 expressions.

OL and Per-OL dietary treatments improved the oxidative events due to Nrf2/HO-1 activation and returned proinflammatory proteins expression to basal levels probably blocking MAPKs and NF-κB pathways. Therefore, OL and Per-OL supplements might provide a basis for developing a new dietary strategy for the prevention of rheumatoid arthritis.

4. Oleuropein and its new acetyl derivative dietary treatments modulate inflammatory response in peritoneal macrophages from pristane-induced SLE mice via regulation of canonical and non-canonical NLRP3 inflammasome.

The present study was designed to evaluate the potential effects of OL and Per-OL dietary treatments on peritoneal macrophages from pristane-induced mice and clarify the mediators and molecular mechanisms involved. Murine macrophages were collected after the sacrificed of mice for further experimental assays.

Our data showed that OL and Per-OL enriched-diets produced a considerable reduction of inflammatory damage in kidneys tissues. Whereas pristane injection induced an increment of levels of proinflammatory cytokines, this increase was effectively prevented after OL and Per-OL dietary treatments. It is has been reported that IL-1β can promote the proliferation, migration and invasion of other cells via activating STAT3. Besides, STAT3 play a crucial role in the Th17 generation and up-regulation of STAT3/IL17 expression had been described in lupus patients. Additionally, in a previous study the reduction of Th1 and Th17 cytokines levels was accompanied by a pSTAT3 down-regulation in LPS-stimulated macrophages from OL and Per-OL treated SLE mice (Chen *et al.*, 2019).

Furthermore, an increment of COX-2 and iNOS protein expression was found in LPS-stimulated peritoneal macrophages from SD-SLE mice in comparison with SD-sham control mice. Both biomarkers protein expression were reduced after OL and Per-OL enriched-diets treatments.

Additionally, NF-κB system induced the expression of various proinflammatory genes, including those encoding cytokines and chemokines such as TNF-α, IL-1β, IL-6, iNOS, COX-2 and also participates in the regulation and development of inflammation disease (Liu *et al.*, 2017). Our studies revealed that dietary OL and Per-OL treatments produced a significantly down-regulation of both signaling pathways in murine macrophages isolated from SLE treated mice.

Inflammasome machinery is dysregulated in SLE and plays an important role in promotion of organ damage. Maturation and secretion of IL-1β and IL-18 translation are mediated by inflammasome activated caspase-1 (Shirato *et al.*, 2017) and also, non-canonical inflammasome, there is an alternative via which has been described to activate caspase-11, and also serve as an additional pathway of maturation and secretion of IL-1β and IL-18 in macrophage-mediate immune response (Kayagaki *et al.*, 2011). Our results showed that OL and Per-OL dietary treatments inhibited canonical and non-canonical activation of NLR family pyrin domain-containing 3 (NLRP3) inflammasome/IL-1β in murine peritoneal macrophages isolated from pristane-SLE mice.

Taken all together, OL and Per-OL exerted a protective effect against inflammatory lupic response in murine peritoneal macrophages from pristane-SLE mice via STAT3 and NF-κB signaling expression coupled with inhibition of canonical and non-canonical NLRP3 inflammasome.

5. Dietary Oleuropein and its new acetyl derivative, attenuate murine lupus nephritis through HO-1/Nrf2 activation and suppressing JAK-STAT, NF-κB and NLRP3 inflammasome signaling pathways.

In this study, we evaluated the effects of dietary OL and its new derivate, Per-OL, in a pristane-induced SLE model.

Three-months-old mice were randomized into four experimental groups: (i) Sham group were fed with SD (SD-Sham); (ii) pristane group received SD (SD-SLE); (iii) pristane OL group were fed with SD supplemented with OL and (iv) pristane Per-OL group were fed with SD supplemented with Per-OL. SLE model was induced and performed by the procedure previously described by Satoh *et al.* (Satoh and Reeves, 1994). Mice received an injection of pristane or saline solution and were fed with experimental diets: enriched with OL and Per-OL.

Our results showed that OL and Per-OL enriched-diets decreased renal abnormalities and renal interstitial fibrosis in comparison with SD-SLE control group. It is well-known that lupus nephritis and its progression might depend on the disintegration of the membrane due to the effects of MMPs activities. Besides, pristane-induced SLE is characterized by an increasing of iNOS expression in kidneys (Botte *et al.*, 2014), and also mPGEs-1 and PGE_2 play an important role in inflammatory kidney injury (Lazarus *et al.*, 2001; Suganami *et al.*, 2003). Our findings showed a significantly reduction of MMP-3 and PGE_2 levels and mPGEs-1 protein expression in OL and Per-OL groups compared with pristane control group.

To recognize whether up-regulation of Nrf2-dependent signaling by OL and Per-OL supplemented diets could diminish renal inflammation in pristane-induced kidney damage, we measured Nrf2 and HO-1 protein expression and we have observed a significantly increment of Nrf2 in comparison with pristane control group in mice which were fed with OL enriched diets, while a significantly up-regulation of both proteins were detected in Per-OL supplemented diet.

To have to delve in the study of mechanisms underlying in the effects of OL and Per-OL diets in SLE model, we observed a markedly increment of p-STAT3 expression in SD-SLE group compared with SD-sham group but this was significantly down-regulated with OL and Per-OL diets. Also, we observed an important degradation of inhibitory protein (IκB-α) in kidney in pristane group mice, but Per-OL diet was able to prevent its degradation after pristane injection. On the other hand, NF-κB-p65 translocation into the nucleus was remarkably increased after pristane injection whereas OL and Per-OL supplemented diets were able to prevent the nuclear migration. Besides, an increment of p38, janus NH_2-terminal kinase (JNK) and extracellular signal-regulated kinase ($ERK_{1/2}$) phosphorylation occur after pristane injection but OL and Per-OL enriched-diets could prevent MAPKs phosphorylation.

Inflammasome complex is dysregulated in SLE, playing an important role in promotion of organ damage and development of lupus pathologies (Kahlenberg and Kaplan, 2014). Our data showed that

OL and Per-OL enriched-diets could inhibit the canonical and non-canonical NLRP3 inflammasome signaling pathways.

In summary, dietary OL and Per-OL supplementation reduced kidney damage and proinflammatory cytokines levels in kidneys from pristane-induced mice, and showed a remarkable blockage of JAK/STAT, MAPKs and NF-κB pathways, showing that OL and Per-OL may offer a new promising strategy to improve different immune-inflammatory markers on this SLE model.

Conclusion

Altogether, our results suggest that OL and Per-OL exert significant anti-inflammatory and immunomodulatory effects on *in vitro*, *ex vivo*, and *in vivo* (RA and SLE) immunoinflammatory experimental models. The mechanisms underlying these protective effects could be related to the regulation of B cell responses, activation of the Nrf2/HO-1 signaling and the inhibition of relevant signaling pathways such as JAK/STAT, MAPKs and NF-κB coupled with inhibition of canonical and non-canonical NLRP3 inflammasome, thus controlling the production of inflammatory mediators including Th1 cytokines and PGE_2

Hence, OL and its acetyl-derivative might provide a basis for potential nutraceutical complements offering a new promising therapeutic strategy in the management of immune-inflammatory diseases just like RA and SLE. Nevertheless, to validate our preclinical obtained results, intervention nutritional studies are guaranteed.

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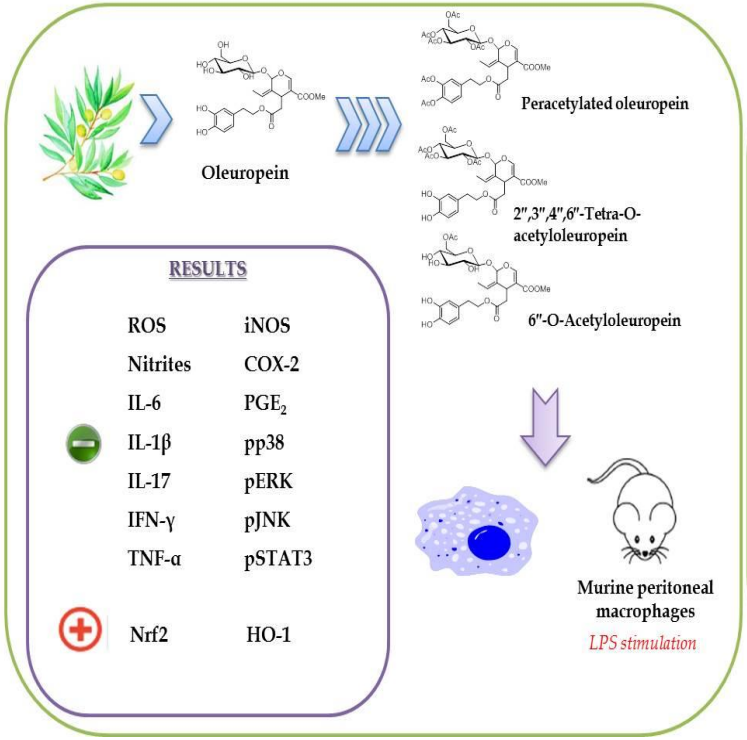
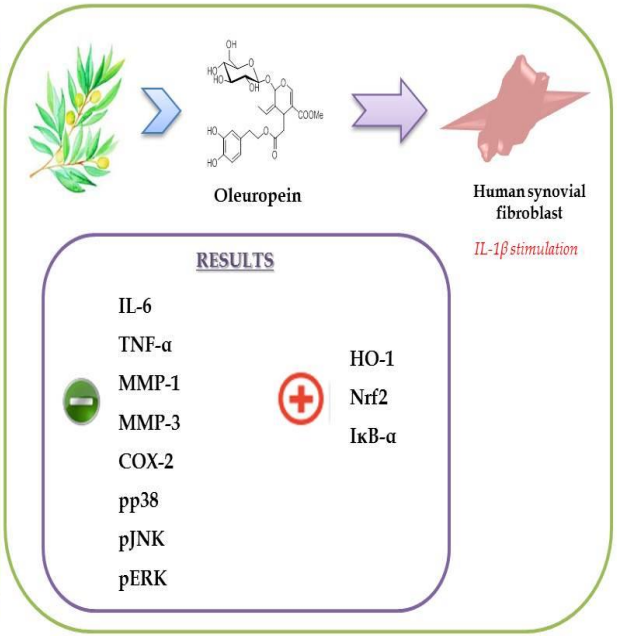
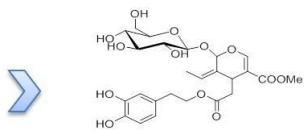
General Results

GENERAL RESULTS

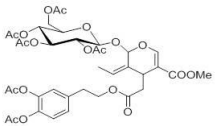




GENERAL RESULTS



Oleuropein



Peracetylated oleuropein

Collagen type II Arthritis Induced (CIA) in DBAJ/c

RESULTS

Serum — MMP-3 COMP

Paw

Arthritic Score
Tissular Damage
IL-17 IL-1β IL-6 TNF-α IFN-γ
COX-2 iNOS
pp38 pJNK pERK
NF-κB-p65 NF-κB-p50

HO-1 IκB-α Nrf2

Pristane induced SLE model in BALB/c mice

RESULTS

Serum — MMP-3

Kidney

Renal abnormalities
iNOS mPGEs-1 PGE_2
pp38 pJNK pERK
NF-κB-p65
Inflammasome (NLRP3 ASC IL-1β IL18 Caspase-1 Caspase-11)

HO-1 IκB-α Nrf2

Murine peritoneal macrophages
LPS stimulation

IL-17 IL-1β IL-6 TNF-α IFN-γ
iNOS COX-2
pSTAT3
Inflammasome (NLRP3 ASC IL-1β IL18 Caspase-1 Caspase-11)

IκB-α

INTRODUCTION

POTENTIAL ROLE OF SECOIRIDOIDS DERIVED FROM OLIVE TREE IN AUTOIMMUNE DISEASES

EFECTO POTENCIAL DE LOS SECOIRIDOIDES DERIVADOS DEL ARBOL DEL OLIVO EN ENFERMEDADES AUTOINMUNES

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RESUMEN

Los iridoides y secoiridoides constituyen un amplio grupo de monoterpenos presentes en plantas e insectos que tienen multitud de propiedades beneficiosas. Principalmente, los secoiridoides han demostrado ejercer importantes efectos farmacológicos. Destacan sus propiedades antidiabética, anti-inflamatoria, inmunosupresora, neuroprotectiva, anticancerosa y antiobesidad. Es por ello, que en los últimos años el interés por profundizar en el estudio de estos compuestos se ha incrementado notablemente. Los secoiridoides están distribuidos por numerosas familias de especies, entre las cuales destacan las siguientes: Oleaceae, Valerianaceae y Gentianaceae, entre otras. Concretamente, *Olea europaea* L. (Oleaceae) es una especie rica en secoiridoides como la oleuropeína (OL), dimetil-OL, ligustrósido así como en productos derivados de sus hidrólisis, como el aglicón de OL, ácido elenoico, hidroxitirosol (HTy) y tirosol (Ty).

Particularmente, las capacidades antioxidantes, antiinflamatorias e inmunomoduladoras de los secoiridoides presentes en el olivo sugieren su potencial aplicación en el manejo de enfermedades de índole inmunoinflamatorio. Existe evidencia que indica que los secoiridoides obtenidos del olivo poseen un potencial terapéutico en enfermedades autoinmunes crónicas como la artritis reumatoide, el lupus eritematoso sistémico, enfermedad inflamatoria intestinal, diabetes mellitus tipo 1 y la esclerosis múltiple. Asi pues,, el objetivo de esta revisión es actualizar los recientes avances en el papel protector de estos secoiridoides (estudios preclínicos y ensayos clínicos) en enfermedades inmunoinflamatorias más prevalentes profundizando en sus mecanismos de acción y vías de señalización moleculares posiblemente involucradas en el proceso general, los datos publicados a pesar de ser escasos, revelan resultados consistentes y satisfactorios. Es por ello que se necesitan estudios adicionales para tratar de dilucidar los procesos bioquímicos y biológicos subyacentes a sus efectos beneficiosos en dichas patologías autoinmunes, así como los perfiles farmacodinámicos y farmacocinéticos de estos compuestos.

POTENTIAL ROLE OF SECOIRIDOIDS DERIVED FROM OLIVE TREE IN AUTOIMMUNE DISEASES

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ABSTRACT

Iridoids and secoiridoids include a wide group of monoterpenes present in plants and insects, which have beneficial health properties. Mainly, secoiridoids have shown large variety of pharmacological effects including anti-diabetic, anti-inflammatory, immunomodulatory,, neuroprotective, anti-cancer and anti-obesity, which increase the interest of studying these types of compounds in depth. These compounds are thoroughly distributed in several families of plants including Oleaceae, Valerianaceae, Gentianaceae, among others. Particularly, *Olea europaea* L. (Oleaceae) is rich in biophenol secoiridoids such as oleuropein (OL), dimethyl-OL, ligstroside, and its hydrolysis derivatives including OL-aglycone, elenolate, oleoside-11-methyl ester, elenoic acid, hydroxytyrosol (HTy) and tyrosol (Ty). These compounds have proved their efficacy in the management of various complex diseases including diabetes, cardiovascular disorders, viral and microbial infections. In fact, the antioxidant, anti-inflammatory and immunomodulatory properties of secoiridoids from olive tree (*Olea europaea* L. (Oleaceae)) have been suggested as a potential application in several immuno-inflammatory diseases. There is evidence indicating that secoiridoids from olive tree has potential as a therapy for a wide variety of chronic autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), diabetes type 1 (T1DM) and multiple sclerosis (MS). Thus, the purpose of this review is summarize recent advances in the protective role of these secoiridoids derived from olive tree (pre-clinical studies and clinical trials) in autoimmune diseases focusing on their mechanisms on most prevalent immunoinflammatory diseases. In general, the published data revealed consistent and very satisfactory results; however, the knowledge is very limitated. . In this sense, further efforts are needed to mechanistically clarify the underlying biochemical and biological activities and pharmacokinetics/pharmacodynamics of secoiridoids from olive tree in additional preclinical and clinical studies of chronic autoimmune diseases.

KEYWORDS: autoimmune diseases; inflammation; oleuropein; olive tree; secoirioids.

1. INTRODUCTION

Iridoids and secoiridoids include a wide group of monoterpenes present in plants and insects, which have beneficial health properties. The name iridoid derived from *Iridomyrmex*, a genus of fornices from which iridomirmecin and iridodial compounds were isolated. These products have been considered as defensive compounds. The biosynthesis of these compounds takes place in the different organisms by similar pathways, being the defense its main role, or in the case of insects, used as sex pheromones (Boros Christie, Stermitz, 1990).

Iridoids were first isolated in the latter part of nineteenth century, but it was not until 1958 that Halpern and Schmid proposed the basic skeleton of the iridoids in their investigation of the structure of plumieride (El-Naggar and Beal, 1980). Particularly, they are secondary metabolites of terrestrial and marine flora and fauna and are found in a large number of plants families usually as glycosides. For this reason, some of them are chemotaxonomically useful as markers of genus in various plant families (Dinda, Debnath and Harigaya, 2007).

Iridoids can have open structures (secoiridoids) or closed structures (really iridoids) and they appear usually as heteroside compounds, in particular as glycosides. In addition they have demonstrated anti-inflammatory, antibacterial, anti-carcinogenic and antiviral activity, and they can be used as antidote in mushroom intoxications by *Amanita* type (López Carreras, Miguel and Aleixandre, 2012). Mainly, secoiridoids have shown a variety pharmacological effects including anti-diabetic, anti-inflammatory, immunosuppressive, neuroprotective, anti-cancer and anti-obesity, which increases the interest of studying these types of compounds in depth (Huang *et al.*, 2019).

This kind of natural compounds included ten carbon atoms in their structure, so they belong to the monoterpenes group. They have acyclopena-[c]-pyrane skeleton, also known as 2-oxabicyclo-[4,3,0]-nonane. When they present a cleavage of cyclopentane ring is known as secoiridoids, while if the cleavage is of pyran ring produces iridoid derivatives. Hence, the ring of cyclopentane is considered as basic skeletal ring of iridoids (Dinda, Debnath and Harigaya, 2007).

The study of biological and pharmacological activities of iridoids and secoiridioids has revealed that both exhibit an extensive variety of bioactivities, and they are used as bitter tonics, sedatives, antipyretics, and cough drugs, remedies for skin disorders and as hypotensive. So, this fact encouraged to investigate the bio-activities of these phytochemicals (Dinda, Debnath and Harigaya, 2007).

1.1. Structure and classification

Given the variety and complexity of this group of molecules, there are several ways of classifying iridoids and secoiridoids that have developed over the years.

According to Hegnauer, iridoids have distributed in three large groups: non-glycosides, glycosides and secoiridoids (Hegnauer and Kooiman, 1978). Non-glycosides and glycosides could have traces of sugars in their structures, so it is their main differential feature. In the other hand, secoiridoids derive from the rupture of the C_7-C_8 and, in turn, they are subdivided into four large groups:

- Molecules, which structure derives from sweroside and morroniside
- Molecules, which their structure is type of oleoside
- Alcaloids, which included pseudoalcaloids and complex alcaloids
- Hydrangesides

In 1975, Sticher and Junod (Sticher and Junod-Busch, 1975) proposed a new classification of iridoids in five large groups:

- Methylciclopentanoids: iridoids with a simple structure
- Iridoids with a complex pattern of oxidation and they could be glucosides or non-glucosides if they have a molecule of glucose in their structure or not
- Secoiridoids
- Monoterpenalcaloids
- Alcaloids with an irioid rest in their structure

From 1980 to date, the bibliographical reviews that order the recently discovered structrures, use the classification proposed by El-Naggar and Beal (El-Naggar and Beal, 1980), which groups these compounds according to the number of carbons included in their structure; thus, the first two groups correspond to nor-iridoids:

- The group 1 is poor numerous and included C_8 iridoids (di-nor-iridoids).
-
- The group 2 integrated C_9 iridoids (nor-iridoids) and it separated in two another groups:

  2.1. Iridoids derivated from loss of C_{10}.

  2.2. This group is the most numerous, and they included iridoids derivated from loss of C_{11}.
- The group 3, the most commonly in the nature, is C_{10}. It included a sugar residue, normally glucose. These compounds are separated in four subgroups:

  3.1. Compounds with a sugar residue in C_1.

3.2. Compounds with a sugar residue in other position in the molecule.

3.3. Iridoids type *Valeriana.*

3.4. Iridoids type *Plumeria.*

- The group 4, included aglycones and some iridoids included in the other three groups but without a sugar residue in their structure.

- The group 5, included a iridoids derivatives. It group included compounds derived from the opening of the pyran ring. It group included two another subgroups:

  5.1. Compounds C_{10} in which pyran ring is opened though the rupture of O_2-C_3 bond.

  5.2. Compounds C_9 bicyclic which are derived from the O_2-C_3 bond rupture, and with a loss of C_{11} and after that, a cyclisation of the C_3-C_4 chain to form a furan ring.

- The group 6, included bis-iridoids like a result of condensation of two monomers, probably with direct form or through a sugar residue.

At the same time, due to the wide range that these kinds of compounds, there are other classifications of secoiridoids according to the presence of these compounds in certain families including Oleaceae family. In fact, a total of 232 secoiridoids (glycosides, aglycones, derivatives and dimers) have been isolated from 9 genus of the family Oleaceae. These genera include *Fontanesia, Fraxinus, Jasminum, Ligustrum, Olea, Osmanthus, Phillyrea, Picconia and Syringa* and the secoiridoids were classified into 5 groups (Huang *et al.*, 2019):

- Simple secoiridoids

Generally, for the simple secoiridoids, positions C_7 and C_{11} have either a free carboxylic acid group or a methyl ethyl ester derivative of the acid.

- Conjugated secoiridoids

This group of compounds constitutes the majority of secoiridoids isolated from the *Oleaceae* family. The name of the class stems from the type of compound that is linked or conjugated to the secoiridoid nucleus. Based on this, the class is further into seven subgroups: aromatic-conjugated, sugar-conjugated, terpene-conjugated, cyclopentane-conjugated, coumarin-conjugated, lignans-conjugated and others secoiridoids. Normally, the conjugations occur in C_7 because this position is usually oxidized to a carboxylic acid and esterified with different groups.

- 10-Oxyderivative of oleoside secoiridoids

This group possesses the oleoside nucleus with distinct structural differences. The C_8 and C_9 positions exist as double bonds or an ester is formed by an oxygen atom with different groups. A total of 40 compounds from this group have been isolated from Oleaceae family.

- Z-Secoiridoids

These compounds present double bond geometry at the C_8 in Z-configuration, for this, only a few compounds isolated from Oleaceae are included in it.

- Secologanosides and oxidized secologanoside secoiridoids

The most of compounds in this class are based on the secologanoside nucleus. The special structure features of these compounds are the positions on the carbon-carbon double bond between C_8 and C_{10} and the level oxidation of C_{10}.

1.2. Main representants

These compounds are thoroughly distributed in plants of class Magnoliopside, concretely of the families Scropulariaceae, Verbenaceae, Lamiaceae, Apocynaceae, Loganiaceae, Bignoniaceae, Plantaginaceae, Rubiaceae, Pedaliaceae, Cornaceae, Acantheceae, Loasaceae, Lentibulariaceae, Gentianaceae, Oleaceae, Nyctanthaceae, Caprifoliaceae, Dispsacaceae and Valerianaceae.

Besides, there are plants, which are commonly used in medicine, whose pharmacological activity is due to these irioids, for example, *Valeriana officinalis* L. (Valerianaceae), *Olea europaea* L. (Oleaceae), *Harpagophytum procumbens* L. (Pedaliaceae), *Genciana lutea* L. (Gentianaceae), *Fraxinus excelsior* L. (Oleaceae) (Dinda, Debnath and Harigaya, 2007).

- ***Valeriana officinalis* L. (Valerianaceae)**

Plants of family of Valerianaceae such as *Valeriana officinalis* L. or *Valeriana jatamansi* L. present in their composition an acylic isoprenoid monoterpene called geraniol. It could be isolated from the essential oils of these kinds of aromatic plants (Lei *et al.*, 2019). Geranoil could be considered a precursor of bicyclic monoterpenes which are name iridoids (López Carreras, Miguel and Aleixandre, 2012). The potential application of geraniol is based on its pharmacological properties, including antitumor and cytotoxic activities in different types of cancer (Lei *et al.*, 2019), anti-inflammatory and antioxidant activities in several inflammatory diseases such as ulcerative colitis (UC) (Medicherla *et al.*, 2015) as well as antimicrobial activity against *Candida albicans* (Singh, Fatima and Hameed, 2016), among others.

Besides their essential oils, the chemical composition of *Valeriana* sp. mainly focused on monoterpenoids, sesquiterpenoids, lignans, flavonoids, alkaloids, etc. Iridoids are the main

chemical components of monoterpenoids. The *Valeriana* plants have been draw much attention for their significant sedation, spasmolysis, antidepression, antitumor, against adenosine A1 receptors and cytoxicity activity and had certain function for cardiovascular diseases treatment (Wang *et al.*, 2016).

- ***Harpagophytum procumbens* L. (Pedaliaceae)**

Harpagophytum procumbens L. is an herbaceous plant, which has been long used in the forms of infusions, decoctions, tinctures, powders and extracts. Its main components are rich in iridoids glycosides such as harpagoside, harpagide and procumbide which are present in plants tubers (Menghini *et al.*, 2019). Also, other components which could be found in this plants are sugars, triterpenoids (mainly oleanolic and ursolic acid), phytosterols (primarly β-sitosterol), aromatic acid (caffeic, cinnamic and chlorogenic acid) and flavonoids (lutoelin and kaemferol).

In others studies has been demonstrated the potential role of *Harpagophytum procumens* L. in the management of different inflammatory and stress oxidative pathologies such as: rheumatoid arthritis (RA), osteoporosis, inflammatory bowel disease (IBD), low-back pain, diabetes and neurodegeneration.

- ***Getiana lutea* L. (Gentiaceae)**

Phytochemicals of the Gentinaceae family includes a wide spectrum of compounds with various chemical structures. Most common are iridoids, flavonoids and xanthones. There are several studies that confirm the potential of some gentians plants for the production of bioactive compounds and validate the ethnomedical use of these species as remedies for the treatment of gastric disorders (lypoacidity) (Yang, Liu and Shi, 2010). Secoiridoidal glycosides are the most important bitter constituents, belonging to *Gentiana* genus. Gentiopicroside, amatogentin are the secoiridoids isolated from *Gentiana lutea*'s root (Aberham *et al.*, 2011). Secoiridoidal glycosides isolated from different *Gentiana* species have several important activities, for example, amarogentin and amaroswerin have the strongest gastroprotective effects among the other secoiridoidals (Niiho *et al.*, 2006). Gentiopicrin and xanthone isogentisin, extracted from leaves and flowers of *Gentiana lutea* L. have considerable antimicrobial activities (Šavikin *et al.*, 2009). Moreover, phytochemical studies on *Gentiana* sp. led to the discovery of other compounds such as xanthones, polyphenols and flavones which are beside secoiridoids responsible for cholinesterase inhibitory, antioxidant, antitumor and vascular smooth muscle cells (VSMC) proliferation inhibitory activities (Isakovic *et al.*, 2008; Senol *et al.*, 2012; Waltenberger *et al.*, 2015).

- ***Fraxinus excelsior* L. (Olaceae)**

Fraxinus, is a member of the Olaceae family, which chemical constituents of Fraxinus plant include various secoiridoids, phenylethanoids, flavonoids, coumarius and lignans; therefore, it is considered as a plant with versatile biological and pharmacological activities (Sarfraz *et al.*, 2017). Metabolites and extracts from these types of plants have been found to possess variety of biological activities such as anticancer, anti-inflammatory, antioxidative, antimicrobial, hepatoprotective, antiallergic, skin regenerating and diuretic (Kostova and Iossifova, 2007).

In fact, *Fraxinus rhynchophylla C* presents a high amount of beneficial compounds such as hydroxyframoside B 2"-hydroxyoleuropein, oleuropein (OL), ligstroside, syringing and esculitin which have reported different properties such as pancreatic lipase inhibitory activity, inhibitor of adypocite differentiation in 3T3-L1 cells, neuroprotective, antidyslipidemia, renoprotective, antioxidant and hepatoprotective (Jiang *et al.*, 2008; Tien *et al.*, 2011; Ahn *et al.*, 2013; Kim *et al.*, 2018).

The specie *Fraxinus excelsior* L. , presents in its seeds active compounds like nuzhenide, ligstroside, oleoside 11-methyl ester, oleoside dimethyl ester, among others, which have beneficial properties such as antihypertensive, antihypertrigliceridemia, antirheumatic, anti-inflammatory and antidiabetic (von Kruedener, Schneider and Elstner, 1995; Maghrani *et al.*, 2004; Montó *et al.*, 2014).

- ***Olea europaea* L. (Olaceae)**

Oleaceae is a family of dicotyledonous flowering plants which is widely distributed in the temperate and tropical regions. This family includes 25 genera with approximately 688 species. Phytochemical studies have demonstrated that the main chemical constituents from this family are flavonoids, monoterpenoids, iridoids, secoiridoids and phenylethanoid glycosides (Huang *et al.*, 2019). The leaves of *Olea europaea* L. are used in Greece for lowering blood pressure (Lawrendiadis, 1961). In the Peninsula Sorrentina (Southern Italy), essential oil extracted from *Olea europaea* L. is used to treat rheumatism and promote blood circulation (De Feo *et al.*, 1992). In the northern and central Oman (Arabia) essential oil extracted from the fruit of *Olea europaea* L. is used as a laxative (Ghazanfar and Al-Al-Sabahi, 1993).

Our main interest focuses on the study of the secoiridoids that mainly found in family *Oleaceae*, due to our extensive experience in the study of beneficial effects of the olive tree and extra virgin olive oil (EVOO), as well as its different components in the prevention and treatment of diverse autoimmune diseases (Cárdeno, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2013; Aparicio-Soto *et al.*, 2016; Rosillo, Alarcón-de-la-Lastra and Sánchez-Hidalgo, 2016). Besides, there several studies which have proved the beneficial effects of EVOO and also, its phenolic

components, such as hydroxytyrosol (HTy) in the reduction of the inflammatory pathway and in the prevention of cardiovascular diseases (Perrone *et al.*, 2019).

Olea europaea L. (Oleaceae) is a small evergreen tree with hoary, rigid branches and a grayish bark. The leaves are opposite, lanceolate, mucronate, shortpetioled, green above, and hoary on the underside. For other hand, the flowers are small, in short, axillary, erect racemes, very much shorter than the leaves, and the fruit is a small drup, smooth, purple or green, with a nauseous, bitter flesh, enclosing a sharp-pointed stone (Ghanbari *et al.*, 2012). *Olea europaea* L. preparations have been traditional used in folk medicine in European Mediterranean area, Arabia peninsula, India and other tropical and subtropical regions, as a diuretic, emollient, hypotensive and for urinary and bladder infections (Somova *et al.*, 2003).

Most of the plants parts of *Olea europaea* L. are used in traditional system of medicine in world. Oil is taken with lemon juice to treat galls stones (Sheth, 2005). Leaves are taken orally for stomach and intestinal diseases and used as mouth cleanser (Bellakhdar *et al.*, 1991). Decoction of dried leaves is taken orally for diabetes (Alarcon-Aguilara *et al.*, 1998). Besides, an extract of the fresh leaves is taken orally to treat hypertension and to induce diuresis (De A. Ribeiro *et al.*, 1986) and the infusion of the fresh leaf is taken orally for as an alternative treatment for inflammatory diseases (Pieroni *et al.*, 1996). Besides, the green olive drups are rich in biophenol secoiridoids such as OL, dimethyl-OL, ligstroside, and their hydrolysis derivatives such as OL aglycone, elenolate, oleoside-11-methyl ester, elenoic acid, HTy and tyrosol (Ty) (Figure 1).

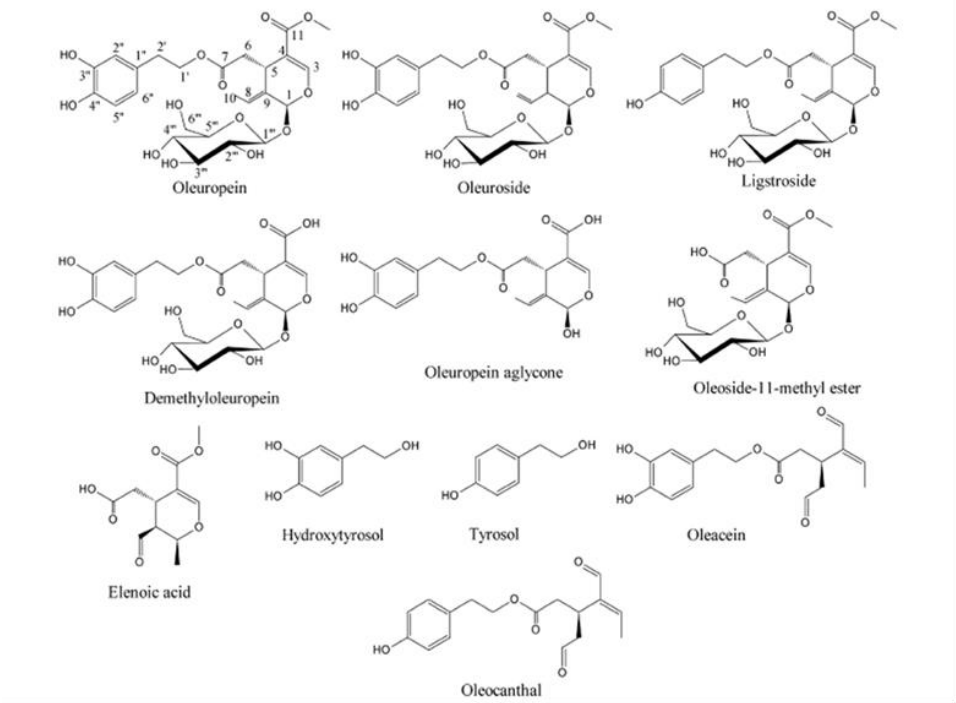


Figure 1. Molecular structures of olive biophenols secoiridoids (*taken by Gentile et al., 2017*).

In conclusion, there are a lot of plants which have been used as medicines since the time of immemorial, concretely *Olea europaea* L. is a specie rich in compounds which have proved their efficacy in the management of various complex diseases including diabetes, cardiovascular disorders, viral and microbial infections but still a lot of work is to be done for exploring the evidences for other traditional uses of the plant.

1.3. Biosynthesis and biotransformation of secoiridoids in olive tree

Amount and distribution of secoiridoids in olive tissues depend of various environmental factors as ripening cycle, geographical origin, and cultivation practices, among others. Besides of this, the content of phenolic glycosides as patterns and the activity of endogenous enzymes can play a role in the quantitative composition of secoiridoids in olive (Termentzi, Halabalaki and Skaltsounis, 2015).

The fact that secoiridoids are mostly present in early stages is due to enzymatic and chemistry reactions in the maturation time. It has been described three different states in maturation of fruit:

growth phase, green maturation phase and black maturation phase, which is characterized by presence of anthocyans.

OL is mainly abundant in early stages, although their levels following decrease during maturation. In black crops, OL decreases quickly. For this reason, some varieties of Oleaceae not present this secoiridoid in black fruits.

The main precursor of OL and ligstroside is oleoside 11-methyl ester (elenolic acid glucoside). Firstly, geraniol synthase (GES) catalyses the transformation of genaryl diphosphate to geraniol, that is converted to 10-hydroxygeraniol by geraniol 10-hydroxylase enzyme (GE10H). The iridioids in Oleaceae must be formed from this point, with 10-hydroxygeraniol as the starting compound, via irididal and iridotrial up to the deoxyloganic acid, which is the known precursor of loganin and loganic acid, secologanin and secologanic acid (Perez-Martinez *et al.*, 2007). From this point, it has been proposed up to five routes to explain the origin of all iridoids found in this family. However, it known that in *Olea europaea L.* most of secoiridoids derived from deoxyloganic acid as common intermediate (Rosendal Jensen, Franzyk and Wallander, 2002; Perez-Martinez *et al.*, 2007). Following this line, NADH deshydrogenase acts on 10-hydroxygeraniol to form deoxyloganic acid algucone. Transfer of glucosyl groups to deoxyloganic acid aglucone (precursor of monoterpene indolic alcaloids and OL is catalysed by glucosyltransferase (GT). Deoxyloganic acid experiments a 7-α-hydroxylation of cyclopentane ring and forms 7-epiloganic acid, that quickly goes to 7-ketologanic acid trough hydroxyl group oxidation by NHDI. Loganic acid methyltransferase catalyse 7-ketologanin syntheses. In this point, secologanin synthase (SLS) oxides ketonic group to form 11-methyl oleoside that immediately is glusylated by GT. Finally, 7-β-1-D-glucopyranosyl-11-methyl oleoside is esterified with Ty to produce ligstroside and then, OL is formed (Perez-Martinez *et al.*, 2007; Obied *et al.*, 2008).

Secoiridoids are distributed all most tissues of the olive tree, but their nature and concentration change between different parts of the plant. Data from literature concerning that biosynthetic or mechanicals transformation through the production are determinatives to quantitative alterations of the bioactive small molecules (Rosendal Jensen, Franzyk and Wallander, 2002). OL is the major secoiridoid constituent of unripe drupes (peel, pulp and seed). The amount of OL decreases with maturation of fruit, and it turn; its aglycon form goes to increasing their levels. OL-aglycone is formed by the cleavage of the glycosidic bond due to β-glucosidades activity. Ligustroside have been described as a common phenolic component in different olive tissues (leaf, fruit pulp, stone) and olive oil, but it has been rarely found in olive seeds (Maestri *et al.*, 2019).

In the course of maturation, OL and ligustroside are considered pattern components. Both of them are present in olive fruit but they are almost non-existent in the olive oil (85-95% reduction) (Gómez-Rico *et al.*, 2009; Kanakis *et al.*, 2013). β-glucosydases act decreasing the levels of OL and ligustroside while their by-products increase. Aglycon forms from OL and ligustroside can be

detected as isomers. It is due to the keto-enolic tautomeric equilibrium of the elenolic acid moiety (Caruso *et al.*, 2000; Obied *et al.*, 2007).

Other dialdehydic structurally related with these secoiridoid precursors are olacein and oleocanthal. Different authors have reported that both olacein and oleocanthal levels increase during ripening due to degradation of OL and ligustroside, respectively (Gutierrez-Rosales *et al.*, 2010). So, they conclude that OL and ligustroside are natural precursors of olacein and oleocanthal as breakdown products resulting from enzymatic activity during extraction process and maturation (Servili *et al.*, 1999; Ryan *et al.*, 2002; Danielle Ryan *et al.*, 2003; Savarese, Demarco and Sacchi, 2007).

OL and ligustroside have been detected in leaves of olive, but not theirs by-products. On the contrary, olacein and oleocanthal levels were augmented in mature fruits, such as the aglycon forms from OL and ligustroside. Nevertheless, such olacein as OL aglycon were more plentiful that oleocanthal and ligustroside aglycon in olive oil (Termentzi, Halabalaki and Skaltsounis, 2015).

2. BIOLOGICAL ACTIVITIES OF SECOIRIDOIDS

2.1. Oleuropein (OL)

Among the different components which are already known in the olive plant, the first one is the OL, the most important component of the glucosidic fraction of the *Olea europaea* from the quantitative point of view and from the historical point of view (Bianco and Ramunno, 2006). This compound is responsible for the bitter taste of the fruits and the leaves of olive plant. Its structure is a secoiridoid glucoside that esterifies a dihydroxy-phenyl-ethyl alcohol. OL may play a role in the prevention of cardiovascular diseases (Visioli and Galli, 1994). Besides, OL has been reported to directly stimulate macrophage activation in laboratory studies (Visioli, Bellosta and Galli, 1998). The active constituent was reported to be OL, with a proposed mechanism of action of potentiation of glucose-induced insulin release, and an increase in peripheral blood glucose uptake (Gonzalez *et al.*, 1992; Bennani-Kabchi *et al.*, 1999). Recently, it has been demonstrated that OL was able to improve postprandial glycaemic profile in healthy subjects (Carnevale *et al.*, 2018).

The antioxidant effect of OL is exerted through different mechanisms, including its ability to improve radical stability through the formation of an intramolecular hydrogen bond between the free hydrogen of the hydroxyl group and its phenoxyl radicals. OL also has a protective effect in counteracting low-density lipoprotein (LDL) oxidation, both *in vitro*, inhibiting in a dose-dependent manner, LDL copper-induced oxidation (Visioli and Galli, 1994; Visioli *et al.*, 1995), and *in vivo*, reducing plasmatic levels of total, free and ester cholesterol in rabbits (Coni *et al.*, 2000). Besides, OL has also the ability to scavenge nitric oxide (NO); in addition, it also promotes the expression of the inducible nitric oxide synthse (iNOS) in cells (De la Puerta et al. 2001). Some previous studies have been demonstrated that OL have anti-inflammatory effects by lipoxygenase activity, production of

leukotriene B4 (De La Puerta *et al.*, 1999), inhibiting biosynthesis of pro-inflammatory cytokines or modulating inflammatory parameters. For example, OL was able to reduce significantly tumor necrosis factor (TNF)-α, interleukin (IL)-1β and NO levels in a mouse model of carrageenan-induced pleurisy (Impellizzeri *et al.*, 2011). Also, it have been demonstrated that OL treatment was capable to attenuate the expression of TNF-α and IL-1β and consequently, the expression of ciclooxigenase 2 (COX-2) and iNOS (Khalatbary and Zarrinjoei, 2012).

OL is endowed also with antithrombotic and anti-atherogenic properties which is dependent of its anti-inflammatory and antioxidant activities. OL has a beneficial effect on some cardiovascular disease via its vasodilatory, anti-platelet aggregation, anti-ischemic and hypotensive properties (Andreadou *et al.*, 2006; Omar, 2010; Bulotta *et al.*, 2014).

Also, OL has been reported to modulate several oncogenic signaling pathways. Both *in vivo* and *in vitro* studies have demonstrated its anti-cancer potentials (Chimento *et al.*, 2014; Hassan *et al.*, 2014; Yao *et al.*, 2014; Yan *et al.*, 2015; Xu and Xiao, 2017). The consumptions of EVOO is inversely correlated with the incidence of some forms of cancer (Barbaro *et al.*, 2014). The beneficial effects of olive oil may in part be attributable to the ability of polyphenolic compounds to induce epigenetic modulation and altered miRNA expression. Inside the minor component of olive oil and olives, OL has been described as the one responsible for the major anti-tumor activity (Hamdi and Castellon, 2005). In fact, there are several previous studies which described the beneficial effects of OL treatment in the prevention of development of skin, soft tissue and breast cancer (Hamdi and Castellon, 2005; Kimura and Sumiyoshi, 2009; Sepporta *et al.*, 2014).

Besides, OL has an hepatoprotective effects on carbon tetrachloride-induced liver damage in mice (Domitrović *et al.*, 2012). Also, there are studies about the capacity of OL supplementation to reduce lipid accumulation in the liver of mice fed with a high fat diet probably it has been associated with down-regulation of hepatic lipogenesis and up-regulation of visceral fat thermogenesis (Barbaro *et al.*, 2014).

OL exerts an antimicrobial activity against both *Gram negative* and *positive* bacteria (Cicerale, Lucas and Keast, 2012), and it has certain antimycoplasmal activity (Furneri *et al.*, 2002) also against *Mycoplasmas* which are resistant to common antibiotic therapy. OL also possesses a wide antiviral activity. It could be effective against hepatitis B virus (HBV), hemorrhagic septicemia rhabdovirus (VHSV) and human immunodeficiency virus (HIV) (Micol *et al.*, 2005; Lee-Huang *et al.*, 2007; Zhao, Yin and Dong, 2009). Its action against HIV has been correlated to its ability to bind and inhibit HIV-1 integrase activity, so OL represents a suitable molecular template for HIV-1 integrase inhibitors (Lee-Huang *et al.*, 2007).

In order to evaluate the potential neuroprotective activity of OL, there are studies which shows that OL administration improved the antioxidant enzymes activities in midbrain in aged rats (Sarbishegi, Mehraein and Soleimani, 2014), providing a new hope for prevention/attenuation

damage associated with Parkinson's disease. Also, there are others studies such as the work of Park et al., (Park *et al.*, 2017) that validate the potential neuroprotective of OL because of it revealed that OL close to hydroxyframoside A, fraxamoside and jaspolyoside exhibited potent stimulation of NGF release in a C6 rat glioma cell line. OL was also indicated as a molecule with a therapeutic potential against Alzheimer's disease, because is an inhibitor of Tau, a microtubule-associated protein known to aberrantly from amyloid-positive aggregates characteristic of Alzheimer's disease (Daccache *et al.*, 2011).

2.2. Oleocanthal

Since the moment when oleocanthal was discovered in 1993 (Montedoro *et al.*, 1993), it has attracted several attentions. For this reason, there are a lot of studies about it and their healthy beneficial properties. Beauchamp et al. (Beauchamp *et al.*, 2005) reported the anti-inflammatory actions of this secoiridoid, for first time, compared with ibuprofen, a non-steroideal anti-inflammatory drug. This work reported that oleocanthal was able to inhibit cyclooxygenase (COX)-1 and COX-2 in a dose-dependent *in vitro* assay. Oleocanthal exhibited better anti-inflammatory action than ibuprofen (Beauchamp *et al.*, 2005). In this line, this phenolic compound can act in characteristic inflammation mediators as iNOS and NO production in ATDC-5 and J774A.1 lipopolysaccharide (LPS)-stimulated, such as pro-inflammatory cytokines (IL-1β, IL-6, MIP-1α, TNF-α) (Iacono *et al.*, 2010; Scotece *et al.*, 2012). These actions of oleocanthal have been related with possible signalling pathways: MAPKs (p38, $ERK_{1/2}$, JNK), JAK/STAT and inflammasome NLRP3 in LPS-stimulated cells (Iacono *et al.*, 2010; Montoya *et al.*, 2019).

Nevertheless, antioxidants effects have been described in assays with this bioactive compound. Montoya et al. (Montoya *et al.*, 2019) have reported that murine peritoneal macrophages LPS-stimulated and treated with oleocanthal presented a modulation of NO, reactive oxygen species (ROS) and iNOS expression. Besides of that, Nrf-2/HO-1 signaling pathway was postulated as possible main mechanism implicated in antioxidant activity of oleocanthal, and protein expression of both of them was up-regulated (Montoya *et al.*, 2019).

In this line, oleocanthal repressed nicotinamide adenine dinucleotide phosphate oxidase activity and controlled intracellular superoxide anion level in isolated human monocytes (Takashima *et al.*, 2014).

In terms of antimicrobial activity, oleocanthal exhibited action against *Escherichia coli, Salmonella enterica, Listeria monocytogenes* and *Helicobacter pylori, Staphylococcus aureus* and *Enterococcus faecalis* (Romero *et al.*, 2007; Pang and Chin, 2018).

Oleocanthal as anticarcinogenic agent has been the most studied property. There are a large amount of evidential studies about different types where this secoiridoid inhibited migration, angiogenesis and metastasis, and induced apoptosis of cancerous cell lines from breast, prostate,

colon, liver cancer or melanoma, among others (Mohyeldin *et al.*, 2016; Pei *et al.*, 2016; Ayoub *et al.*, 2017; Cusimano *et al.*, 2017; Gu, Wang and Peng, 2017). Though, there is not available an enormous variety of *in vivo* studies. However, oleocanthal has been postulated as a natural promise in this field to future treatments and prevention (Elnagar, Sylvester and El Sayed, 2011).

2.3. Oleacein

Oleacein is maybe one of the most unknown secoiridoid of olive. It is not exist high variety of studies about this compound; however, today it is a natural agent promise. Antioxidant, anti-inflammatory, antimicrobial and antitumor activities are some newsworthy beneficial effects from oleacein.

It inhibits oxidant-activated MMP-9 and IL-8 production in human neutrophils (Czerwińska, Kiss and Naruszewicz, 2014). Paiva-Martins et al. supported the free-radical scavenging action of oleacein in human erythrocyte, where demonstrated a marked role against ROS-induced oxidative injury than HTy or OL (Paiva-Martins and Pinto, 2008).

In terms of anti-inflammatory activity, in human macrophages, oleacein enhanced CD163 receptor expression, an anti-inflammatory gene, increasing IL-10 and heme oxygenase-1 (HO-1) expression (Filipek *et al.*, 2015). Oleacein decreased MMP-9, at the same time HO-1 and IL-10 release increased (Filipek *et al.*, 2017). Besides, this compound has shown to be more effective inhibitor of 5-LOX enzyme than oleocanthal (Vougogiannopoulou *et al.*, 2014).

In vitro, anticancerous activity was related by promoting cell cycle arrest and apoptosis in human multiple myeloma (Juli *et al.*, 2019).

Antibacterial activity was weaker compared to oleocanthal, while oleacein only has shown bactericide effect on *Helicobacter pylori* (Romero *et al.*, 2007).

2.4. Ligstroside aglycon

Ligstroside aglycon is a by-product of ligstroside. Thought, not exits a lot of studies about them, there are more data about aglycon form.

Medina et al., concluded that among others polyphenols of EVOO, ligstroside aglycon presented a marked bactericidal potential against a broad spectrum of microorganism (Medina *et al.*, 2006). In terms of antioxidant activity, various authors have reported this effect on plasma LDL (Tripoli *et al.*, 2005). Ligstroside aglycon showed strong tumoricidal effects in HER-2 overexpression breast carcinomas in a dose-and time-dependent manner (Menendez *et al.*, 2008).

Particularly, the antioxidant, anti-inflammatory and immunomodulatory properties of secoiridoids from olive tree (leaves and fruits) have been suggested as a potential application in

several immunoinflammatory diseases. Thus, the purpose of this review is summarize recent advances in the protective role of these secoiridoids derived from olive tree (pre-clinical studies and clinical trials) in autoimmune diseases focusing on their mechanisms on RA, SLE, IBD and MS.

3. POTENTIAL EFFECTS OF SECOIRIDOIDS FROM *Olea europea* L. IN AUTOIMMUNE DISEASES

Autoimmune diseases are heterogeneous groups of diseases which condition is that your immune system mistakenly attacks your body. I normal state, the immune system is able to difference between foreign cells, such as viruses and bacteria, and own cells. However, in an autoimmune disease, the immune system recognize our own cells like foreign cells and it releases proteins called autoantibodies that attack healthy cells. These types of diseases could appear at any stage of life with higher or lower severity. For example, there are types of autoimmune disease whose target only one organ, such as type 1 diabetes mellitus (T1DM) which damages the pancreas, and there are other diseases, like SLE which affect the whole body.

The National Institutes of Health (NIH) estimates up to 23.5 million of American suffer from autoimmune disease and that the prevalence is rising in 2019. For this reason, autoimmune diseases are recognized like a major health problem. Nowadays, researchers have identified 80-100 different autoimmune diseases and suspect at least 40 more diseases of having an autoimmune basis. Also, these diseases are chronic and can be life-threatening. These kinds of diseases are one of top 10 leading causes of death in female children and women in all age groups up to 64 years ago (Lerner, Jeremias and Matthias, 2016).

The most characteristics examples of these kinds of autoimmune diseases are:

- **RA**

RA can be defined as a chronic inflammatory disease with systemic autoimmune component, and is mainly characterized by aggressive synovial hyperplasia, synovitis, progressive destruction of cartilage, and bone erosion with painful swelling of small joints, fatigue, prolonged stiffness and fever caused by immune responses and specific innate inflammatory processes (Rosillo *et al.*, 2016). Worldwide, the annual incidence of RA is approximately 3 cases per 10000 populations, and the prevalence rate is approximately 1% increasing with age and peaking between the ages of 35 and 50 years. RA affects all populations, through it is much more prevalent in some groups (e.g. 5-6% in some Native American groups) and much less prevalent in others (e.g. black persons from the Caribbean region) (Smolen, Aletaha and McInnes, 2016).

- **SLE**

SLE can be defined as a chronic inflammatory and autoimmune disease that can affect multiple organ system, including skin, joints, kidneys and the brain among others (Noble *et al.*, 2016). SLE is characterized by a deposition of immune complexes, formed in large amounts as antinuclear antibodies bind to the abundant nuclear material in blood and tissues, along with disturbances in both innate and adaptive immunity and T-cell signaling. Also, SLE is characterized by its clinical and pathogenic complexity, difficult diagnosis and the high number of complications that can affects the patient's quality of life (Aparicio-Soto *et al.*, 2017). There are worldwide differences in the incidence and prevalence of SLE that vary with sex, age, ethnicity and time. The highest estimates of incidence and prevalence of SLE were in North America 23.2/10000 persons/years and 24/10000 people respectively. The lowest incidences of SLE were reported in Africa and Ukraine (0.3/10000 persons/years) and the lowest prevalence was in Northern Australia (0 cases in sample of 847 people). Women were more frequently affected those men for every age and ethnic group. Incidence peaked in middle adulthood and occurred later for men. People of black ethnicity had the highest incidence and prevalence of SLE, whereas those with while ethnicity had the lowest incidence and prevalence. These appeared to be an increasing trend of SLE prevalence with time (Rees *et al.*, 2017).

- **IBD**

Crohn's disease and Ulcerative Colitis (UC) are important chronic inflammatory disorders of the gastrointestinal system that contributes to the inflammatory bowel conditions, that includes diarrhea with or without blood, abdominal pain, fever, weight loss, inflammation and ulcers. These diseases have uncertain etiology but can be associated with multifactorial conditions in terms of immunity, genetics and non-immune conditions such as environmental factors (Lopes de Oliveira *et al.*, 2019). The total number of new cases of Crohn's disease diagnosed each year (incidence) was 10.7 per 100000 people or approximately 33000 new cases per year. The total number of new cases of UC diagnosed each year was 12.2 per 100000 people or approximately 38000 new cases per year (Shivashankar *et al.*, 2017).

- **T1DM**

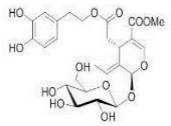
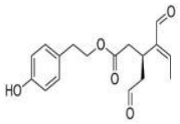
T1DM is considered an autoimmune disease which results from the destruction of pancreatic β-cells that is mediated by the immune system. This is caused by an autoimmune reaction where the body's defense system attacks the cells that produce insulin. As a result, the body produces very little or no insulin. Multiple genetic and environmental factors found in variable combinations in individual patients are involved in the development of T1DM. Genetic

risk is defined by the presence of particular allele combinations, which in the major susceptibility locus (the HLA region) affect T-cell recognition and tolerance to foreign and autologous molecules. T1DM can affect people at any age but usually develops in children or young adults. Around 10% of all people with diabetes have type 1 diabetes (Ilonen, Lempainen and Veijola, 2019).

Due to the high prevalence and rising incidence of this kind of disease, nowadays, there is a requirement to investigate to develop palliative remedies or treatments that help us improve the symptoms and management of these types of diseases to improve the quality of life of patients. A new source of news alternative for autoimmune diseases is based to use a natural compounds obtain from natural resources, such as *Olea europaea* L., which is used traditionally as diuretic, hypotensive, emollient, laxative, febrifuge, skin cleanser, and also used for the treatment of urinary infections, gallstones, bronchial asthma and diarrhea due to its beneficial effects which have been allowed to distinguish it from other species.

The studies of the effects of secoiridoids from olive tree are summarized in following tables:

Table 1. Effective mechanisms and concentrations of bioactive secoiridoids in *in vitro* models of immunoinflammatory diseases

Phenolic compound	Cell line	Concentration	Effects	References
Oleuropein 	LPS-stimulated murine peritoneal macrophages.	25 and 50 µM	Oleuropein reduces pro-inflammatory cytokines levels such as IL-1β, IL-17, IL-6, TNF-α and IFN-γ, as well as iNOS and COX-2 over expressions. It was able to prevent p38, JNK and ERK protein phosphorylation, which was accompanied by an upregulation of Nrf-2 and HO-1 expression.	(Castejon et al. 2019)
	Human synovial fibroblasts cell line (SW982)	50 and 100 µM From olive leaves from *Olea europaea* L	Oleuropein pre-treatment down-regulates MAPKs and NF-κB and induction of Nrf2-linked HO-1 signalling pathways controlling the production of IL-6, TNF-α, MMP-1 and MMP-3 levels as well as mPGES-1 and COX-2 overexpression.	(Castejón et al. 2017)
Oleocanthal 	LPS-stimulated murine peritoneal macrophages.	25-100 µM	Oleocanthal showed a potent reduction of ROS, nitrites and pro-inflammatory cytokines levels, such asIL-1β, IL-6, IL17, INF-γ, and TNF-α . It was able to downregulate the protein expression of iNOS, COX-2 and mPGES-1 and prevented p38, JNK and ERK protein phosphorylation, which was accompanied by an upregulation of Nrf-2 and HO-1 expression. Moreover, oleocanthal inhibited canonical and noncanonical inflammasome signaling pathways. Particularly, oleocanthal treatment reduced NLRP-3 and IL-18 protein expression as well as both forms of caspase-1 and caspase 11 activations.	(Montoya et al. 2019)
	J774 LPS-stimulated macrophages	50 µM	Oleocanthal inhibits LPS-induced NO production without affecting cell viability. Moreover, it inhibits both MIP-1α and IL-6 mRNA and protein expressions as well as the protein synthesis of IL-1β, TNF-α and GM-CSF.	(Scotece et al. 2012)

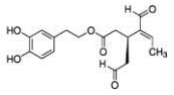
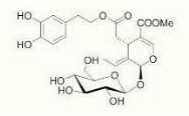
Oleacein 	Human macrophages from healthy donors	10 and 20 µM alone from *Ligustrum vulgare* L. (Oleaceae) or with complexes of haemoglobin (Hb) and haptoglobin 1-1 (Hp11) or haptoglobin 2-2 (Hp22) during 1, 2 and 5 days	Oleacein together with complexes HbHp11 or HbHp22 stimulated the expression of CD163 (30-100-fold), IL-10 (170-300-fold) and HO-1 secretion (60-130-fold) after 5 days of coincubation. The 2-fold (24 h), 4-fold (48 h) increase of CD163 mRNA level and its final (72 h) decrease was also observed.	(Filipek et al. 2015)

Table 2. Effective mechanisms and concentrations of bioactive secoiridoids in *in vivo* models of immunoinflammatory diseases

Phenolic compound	Animal model	Doses	Mechanism/s of Action	References
Oleuropein 	Five week old male C57BL/6JolaHsd mice. Fed with high-fat diet (HFD).	Oral gavage 20 mg/kg/day of OL during five weeks.	Oleuropein reduced insulin levels and ameliorated insulin sensitivity. In addition, prevented the hepatic enlargement, as evident by the liver weight values and the development of HFD-dependent hepatic steatosis, and reduced the protein expression of SREBP-1, FAS, p-ERK in liver. Reduced increase in body weight was observed in oleuropein-treated mice mainly due to a lesser increase in liver tissue. Total cholesterol levels were significantly corrected whereas levels of tryglicerides were not affected, and glycemia was only slightly modified. Oleuropein reduced lipid deposits in the liver and this reduction paralleled the decrease in p-ERK and SREBP-1 protein expression.	(Lombardo *et al.*, 2018)
	Diabetes type I model induced by subcutaneous alloxan monohydrate injection in Sprague-Dawley male rats	Oral gavage 15 mg/kg/day of OL	OL significantly decreased leucocyte infiltration and glomerulosclerosis. OL decreased the levels of urea, nitrite and creatinine and OL significantly decreased MPO activity in the treated group compared with the diabetic untreated group.	(Ahmadvand *et al.*, 2017)
	Experimental autoimmune myocarditis (EAM) model inducedby porcine cardiac myosin in Lewis rats	Oral gavage 20 mg/kg/day of OL	OL treatments improve cardiac functions and attenuate inflammatory cell infiltration and cytokine expression levels (TNF-α, IL-1, IL-6) in a rat model of EAM. These effects may be attributed to its suppression of cardiac NF-κB and MAPK signaling pathways.	(Zhang *et al.*, 2017)
	Chronic colitis model induced by DSS (1% in first and second cycles and 2% in third and fourth cycle) in female C57BL/6mice(6-8 weeks at age weighting 18-20 g)	Diet supplemented with 0.25 % Oleuropein	Dietary treatment of OL exhibited a decrease of inflammatory symptoms, such as improvement of disease activity index and histopathological changes. Also, OL decreased inflammatory cell recruitment and the release of inflammatory cytokines IL-1β and IL-6 with increased IL-10 levels in colon tissue. Colon expression of COX-2 and iNOS was also reduced and it probably was associated with the suppression of the phosphorylation of p38 mitogen-activated protein kinase and by the up-regulation of annexin A1.	(Giner *et al.*, 2013)

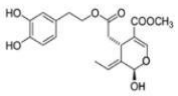
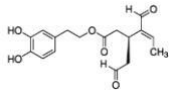
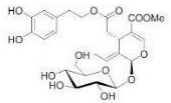
	Acute colitis model induced by DSS (5%) for 7 days in BALB/c mice (6-8 weeks at age weigthing 18-20 g)	Diet supplemented with 1 % Oleuropein	Oral administration of OL notably attenuated the extent and severity of acute colitis and reducing neutrophil infiltration, production of NO, IL-1β, IL-6 and TNF-α; expressions of iNOS, COX-2 and MMP-9 and the translocation of the NF-κ-p65 subunit into the nucleus in colon tissue.	(Giner *et al.*, 2011)
Oleuropein aglycone 	Collagen type II-induced arthritis in three-week-old male DBA-J/1	Oral gavage 40 mg/kg/day of OL	OL prevented joints inflammation and reduced COX-2 and iNOS overexpression and leucocytes infiltration. Also, OL reduced significantly TNF-α, IL-1β and IL-6 plasma levels in CIA mice.	(Impellizzeri *et al.*, 2011)
Oleacein 	Five-week old C57BL/6JOlaHsd mice feed with High-fat diet (HFD)	Oral gavage 20 mg/kg/day during five and eight weeks.	Oleacein treatment prevented the increase of adipocyte size of HFD-oleacein treated mice, and reduced the inflammatory infiltration of both macrophages and lymphocytes. mRNA expression of fibroblast-specific protein 1, FSP1, a marker of fibrosis, was lower. The expression levels of FAS and SREBP-1, were reduced whereas the expression levels of adiponectin were increased. In addition, a higher positive immunostaining of Glut-4 was observed in abdominal fat and muscle tissues of oleacein treated mice.	(Lepore *et al.*, 2019)
	Five week old male C57BL/6JolaHsd mice. Fed with high-fat diet (HFD).	Oral gavage 20 mg/kg/day of oleacein during five weeks.	Oleacein reduced insulin levels and ameliorated insulin sensitivity. In addition, prevented the hepatic enlargement, as evident by the liver weight values and the development of HFD-dependent hepatic steatosis, and reduced the protein expression of SREBP-1, FAS, p-ERK in liver. Reduced increase in body weight was observed in oleacein-treated mice mainly due to a lesser increase in liver tissue. Total cholesterol levels were significantly corrected whereas levels of tryglicerides were not affected, and glycemia was only slightly modified. Oleacein reduced lipid deposits in the liver and this reduction paralleled the decrease in p-ERK and SREBP-1 protein expression.	(Lombardo *et al.*, 2018)

Table 3. Effective mechanisms and concentrations of bioactive secoiridoids in *in vitro* preclinic models of immunoinflammatory diseases

Phenolic compound	Cells	Concentration	Effects	References
Oleuropein 	14 outpatients biopsies with Ulcerative Colitis	3 µM OL from olive leaves from *Olea europaea* L.	The expression of COX-2 and IL-17 were significantly lower in samples treated with OL. Histologically, OL treatment ameliorated inflammatory damage with reduced infiltration of CD3, CD4 and CD20 cells, while CD68 numbers increased	(Larussa et al. 2017)

4. CONCLUSION

There is evidence indicating that secoiridoids from olive tree has potential as a therapy for a wide variety of chronic autoinmune diseases. The remarkable anti-inflammatory and immunomodulatory effects of these bioactive compounds have been reported in several *in vitro* and experimental models of RA, SLE, IBD, T1DM and MS. Particularly, OL, oleocanthal and oleacein may exert a remarkable inmmunodulatory and antiinflammatory effects reducing the induced inflammatory response in murine macrophages and human fibroblasts. This was accompanied by an amelioration on production of essential proinflammatory cytokines involving in the regulation of the immune system response through prevention of mitogen-activated protein kinases (MAPKs) and nuclear transcription factor-kappaB (NF-κB) pathways activation. Moreover, OL and oleacein secoiridoids were effective in preventing the induced immuno-inflammatory response in animal experimental models of colitis, MS, T1DM and SLE. In general, the published data revealed consistent and very satisfactory results; however, the knowledge is very limitated, especially in clinical trials. In this sense, further efforts are needed to mechanistically clarify the underlying biochemical and biological activities and pharmacokinetics/pharmacodynamics of secoiridoids from olive tree in additional preclinical and clinical studies of chronic autoinmune diseases.

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Justificación y Objetivos

Las enfermedades inflamatorias inmunomediadas (IMID) son un grupo clínicamente heterogéneo de enfermedades crónicas y altamente incapacitantes que comparten secuencias inflamatorias comunes y la desregularización del sistema inmune. Las IMID dan lugar a sustanciales niveles de morbilidad, una importante reducción de la calidad de vida y muertes prematuras. Algunas de las más comúnmente incluidas en este grupo son la artritis reumatoide (AR) y el lupus eritematoso sistémico (LES) (Puig *et al.*, 2019) .

La AR es una enfermedad crónica inflamatoria autoinmune de distribución universal. Cursa con una sinovitis erosiva simétrica, en la que el tejido de granulación del sinovio (*pannus*) degenera, invadiendo y erosionando al cartílago y al hueso de las articulaciones diartrodiales. Comprende una amplia gama de características, desde la enfermedad progresivamente crónica con grados variables de destrucción articular hasta las manifestaciones extraarticulares clínicamente evidentes.

En España tiene una prevalencia del 0.5 % que aumenta con la edad y es unas 3 veces mayor en mujeres que en hombres. Su incidencia es de 0.08 a 0.20 casos/ 1000 personas-año (Andrade *et al.*, 2017). El 80 % de los pacientes suelen iniciar la enfermedad entre los 35 y 50 años (González Cernadas, Rodríguez-Romero and Carballo-Costa, 2014). La AR está asociada con una disminución de la calidad de vida y con un exceso de mortalidad del 85% en comparación con la población general. Además la AR representa un problema de salud relevante tanto para el propio paciente como para la sociedad y el Sistema Nacional de Salud, ya que es responsable de hasta un 5% de las incapacidades laborales en España (Fauci and Langford, 2010).

La etiología de AR se desconoce aunque factores genéticos, infecciosos (infección viral, e.g. Virus Epsein-Barr, Proteus, rubeola, etc.), ambientales (tabaco, situaciones de estrés y traumatismo, obesidad, dieta) y hormonales parecen están involucrados en vías relacionadas y complejas (Suzuki and Yamamoto, 2015). Se han identificado múltiples genes que contribuyen a la predisposición y susceptibilidad a la enfermedad fundamentalmente el gen *HLA-DRB1* del complejo mayor de histocompatibilidad, *PADI4, PTPN22, CCR6 FCRL3* (Suzuki and Yamamoto, 2015), TIM-3 rs1036199 (Razi *et al.*, 2019) así como el gen *UBASH3A* y sus polimorfismos (rs1893592 and rs3788013) (Yang *et al.*, 2019)

Aunque no existe consenso sobre la naturaleza del autoantígeno(s) o factores ambientales que inician la desregulación de la respuesta inmune, el proceso inflamatorio es orquestado por macrófagos, células T reguladoras (Tregs), células T reguladoras (Th)-1 y Th17 y células T citotóxicas que generan un desbalance de la producción de citocinas Th1: interferón (IFN)-γ, factor de necrosis tumoral (TNF)-α y las interleucinas (IL)-1β, IL-2 , IL-4, IL-6, IL-13 , IL-15 IL-6, IL-23, IL-27, IL-35 ; Th17 (IL-17, IL-21) y, de citocinas Th2: (IL-10, IL-20, IL-22), quimioquinas (receptor de quimiocinas CXC tipo 4 (CXCR4), proteína inducida por IFN-γ (IP)-10, factor 1 derivado de células estromales (SDF1), proteína quimiotáctica de monocitos (MCP)-1), y metaloproteinasas (MMP-3 y MMP-9) capaces de digerir el cartílago (Brzustewicz and Bryl, 2015). Las vías de señalización molecular comprometidas incluyen los factores nucleares de transcripción*:* NF-κB, FOXP3 y las proteínas cinasas activadas por mitógenos (MAPKs): c-Jun NH_2 – terminal cinasa (JNK) y p38 cinasa fundamentalmente (Han *et al.*, 2001; McInnes and Schett, 2011; Nie *et al.*, 2013).

Por otro lado, los fibroblastos sinoviales (FS) son el componente celular más importante que residen en la membrana sinovial de las articulaciones, y juegan un papel fundamental en la patogenia y desarrollo en la AR (Firestein, 1996). Los FS son inicialmente activados por el microambiente local y posteriormente adquieren un fenotipo pseudomaligno con activación de ciertos oncogenes, que secretan citocinas, quimiocinas, MMPs y catepsinas colaborando en el proceso inflamatorio crónico y catalizando la destrucción articular (Chang, Gu and Brenner, 2010).La identificación de las vías de señalización molecular implicadas en las alteraciones de los FS es la base para el desarrollo de terapias alternativas de la inflamación crónica y daño articular dirigidas al sistema inmunitario, y constituyen un método de estudio validado para la AR.

El abordaje terapéutico de la AR es multidisciplinar, y está dirigido principalmente a la supresión inespecífica del proceso inflamatorio con el objetivo de mitigar los síntomas y signos de la enfermedad englobando tratamientos médico, fisioterápico y otro grupo de métodos dentro del que se incluyen las medidas dietéticas (Fauci and Langford, 2010).

La terapia farmacológica de la AR debe iniciarse de forma temprana y está destinada a mejorar el controlar el curso de la enfermedad y prevenir la progresión del taño tisular. La primera línea terapéutica incluye fármacos que suprimen la inflamación como los anti-inflamatorios no esteroideos (AINE) que se deben utilizar a la menor dosis efectiva y el menor tiempo posible y los glucocorticoides a dosis bajas asociados a fármacos antirreumáticos modificadores de la enfermedad (FAME), en periodos cortos para aliviar los síntomas.

Los FAME son fármacos de acción relativamente lenta que además de proporcionar control sintomático de los síntomas, han demostrado evitar la progresión del daño estructural e inducir la remisión de la enfermedad. En la actualidad, los FAME se dividen en tres grandes grupos: FAME convencionales (metotrexato (MTX), leflunomida, hidroxicloroquina o sulfasalazina), FAME biológicos (agentes anti-TNFα [infliximab, etanercept, adalimumab, certolizumab y golimumab], bloqueante de la activación de linfocitos T [abatacept], fármacos anti-IL-6 [tocilizumab y sarilumab], anti-CD20 [rituximab], inhibidor de la IL-1 [anakinra], y FAME sintéticos dirigidos (baricitinib y tofacitinib) (Rosillo, Alarcón-de-la-Lastra and Sánchez-Hidalgo, 2016; Terap, Il- and Americano, 2018).

Las últimas recomendaciones para el abordaje y tratamiento de la AR publicadas por GUIPCAR (2017) (Díaz, 2017) recomiendan el uso de tratamiento biológico cuando no se alcanza el objetivo terapéutico deseado con un primer FAME (preferiblemente MTX) o la combinación de varios y además existen factores de mal pronóstico o elevada actividad de la enfermedad. En muchas situaciones el tratamiento basado en el uso de FAME se debe suspender por ineficacia, intolerancia, presencia de efectos adversos o contraindicaciones por comorbilidades (Ruiz-Esquide and Sanmarti, 2018).

Por todo ello, aunque en la actualidad existen nuevos fármacos desarrollados para la AR, ninguna combinación de ellos proporciona una alternativa de tratamiento eficaz que consiga controlar la enfermedad de manera continua, y que por otro lado sean seguros para los pacientes de uso crónico.

Junto a la AR, otra enfermedad IMID de creciente interés en la actualidad, debido a los condicionantes que suponen para los pacientes en su calidad de vida es el LES.

El LES es una enfermedad autoinmune inflamatoria crónica cuya afectación puede alcanzar diversos órganos, provocando un deterioro generalizado y progresivo que compromete ampliamente a la calidad de vida de los pacientes. Está considerada como una enfermedad rara según la denominación de esta por la Comisión Europea "Regulation on Orphan Medicinal Products". Es una enfermedad de etiopatogenia muy compleja, cuyo diagnóstico es muy difícil y que supone numerosas complicaciones en el seguimiento y control de los individuos que la padecen (Noble *et al.*, 2016). LES presenta un curso muy variable, con periodos alternados de exacerbaciones y remisiones, que pueden afectar a múltiples sistemas del organismo. Sus manifestaciones clínicas más comunes son fatiga, pérdida de peso y de apetitivo, afectaciones cutáneas , renales y del sistema nervioso, dolores articulares, y así como manifestaciones hematológicas (citopenias), y un aumento del riesgo de sufrir eventos adversos vasculares (Lisnevskaia, Murphy and Isenberg, 2014; Herrmann *et al.*, 2015; Choi *et al.*, 2017; Marín *et al.*, 2019).

La prevalencia de esta enfermedad oscila entre 20 y 150 casos por cada 100.000 habitantes de la población mundial, con una amplia variabilidad geográfica y étnica. Existe una mayor incidencia en mujeres en edad fértil que en hombres, y aunque los síntomas pueden llegar a aparecer a cualquier edad, la mayoría de los casos se concentran entre los 25 o 35 años (Vina *et al.*, 2014). En España, se estima actualmente una prevalencia media de 0.13% en hombres y de 0.32 % en mujeres (Puig *et al.*, 2019)

La etiología del LES es desconocida, aunque factores genéticos, medioambientales (dieta, estrés extremo, exposición a rayos ultravioleta, infecciones), la administración de ciertos medicamentos (algunos antidepresivos y antibióticos) y hormonales (estrógenos) parecen están involucrados en vías relacionadas y complejas (Cai *et al.*, 2014; Mak and Tay, 2014; Ruiz-Larrañaga *et al.*, 2016; Lewis and Jawad, 2017). En este contexto, los factores ambientales dietéticos específicos, incluyendo vitaminas, elementos minerales, ácidos grasos y polifenoles, parecen tener un papel importante en la modulación de la respuesta inmune (Somers and Richardson, 2014). Así, en una reciente revisión llevada a cabo por nuestro equipo hemos analizado el papel de la dieta en la prevención y manejo del LES, y de cómo ciertos componentes de la dieta pueden mejorar o bien constituir un factor de riesgo etiopatogénico importante (Aparicio-Soto, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2017).

El papel de los factores genéticos en la patogénesis del LES es evidente por la alta heredabilidad (43.9%) y el riesgo relativo en familiares de primer grado de pacientes con LES. Aunque la enfermedad puede desarrollarse a partir de una deficiencia de un solo gen, como la del componente del complemento 1q (C1q) subcomponente A (C1qA), C1qB, C1qC, la exonucleasa de reparación de tres cepas 1 (TREX1) o la desoxirribonucleasa 1 similar a 3 (DNASE1L3), en la mayoría de los casos, la enfermedad es el resultado de una combinación de múltiples efectos de variantes genéticas. Existen multitud de *loci* humanos relacionados con funciones del sistema inmunitario deterioradas en el LES: por ejemplo, el factor regulador del interferón (IRF)-5, IRF7, el transductor de señal y activador de la transcripción (STAT)-4, receptor tipo Toll (TLR)-7, TLR8 y TLR9 están involucrados en la detección de ácidos nucleicos y la producción de (IFN)-I por las células presentadoras de antígenos, incluidas las células dendríticas (DC) contribuyendo a la generación de un ambiente proinflamatorio característico de la enfermedad (Moulton *et al.*, 2017).

Desde el punto de vista patológico, LES es una patología inflamatoria crónica que se caracteriza por una alteración de la respuesta inmunitaria definida por la pérdida de la autotolerancia de las células T y B con la consiguiente hiperactividad linfocítica, la producción de autoanticuerpos (antinuclear, antiDNA de doble cadena, anti-Ro, antihistona, anti-Smith y anti-ribonucleoproteina, RNP Anti-protéina P ribosomal, entre otros), la formación de inmunocomplejos aberrantes (ICs), y la generación de una respuesta inflamatoria polisistémica. La generación de los ICs así como una sobreproducción de IFN-α son características del LES (Herrada *et al.*, 2019).

Los anticuerpos e ICs, así como el desbalance en la producción de células T (Th1/Th2/Th17/Th9/Th22) y células Treg contribuyen al daño tisular y parecen estar involucrados en la aparición de la respuesta inflamatoria multisistémica, especialmente en la fase activa de la enfermedad (Dolff *et al.*, 2011). Estudios recientes sugieren que las células T γδ pueden estar involucradas en la regulación del LES debido a sus efectos en la producción de citocinas, presentación de antígenos y su papel en la producción de anticuerpos mediante la estimulación de células B (Wu *et al.*, 2016). Pacientes con LES activo presentan típicamente incrementados los niveles de citocinas proinflamatorias como IFN-γ, IL-6, IL10, IL-12, IL-17, IL-18, IL-21 y IL-23, al contrario que la IL-2 que se caracteriza por encontrarse a niveles más bajos comparados con pacientes sanos (Comte, Karampetsou and Tsokos, 2015; Zhao *et al.*, 2016; Jiang *et al.*, 2019).

Las vías de señalización molecular comprometidas en estos procesos incluyen a los factores de transcripción nuclear NF-κB, FOXP3, las MAPKs ($ERK_{1/2}$, JNK y p38) y Janus quinasas-transductor de señal y activador de la transcripción (JAK/STAT), y más recientemente la vía del inflamasoma NLRP3 y la vía de factor nuclear eritroide 2 (Nrf2) y la enzima antioxidante hemo oxigenasa-1 (HO-1) (Bloch *et al.*, 2014; Marina Aparicio-Soto *et al.*, 2016; Aparicio-Soto, Sánchez-Hidalgo, *et al.*, 2017; Yi, 2018). La secreción de citocinas proinflamatorias a sangre produce una amplificación de la respuesta inflamatoria, produciendo una expresión clonal de linfocitos B sobre todo del tipo inmunoglobulina G (IgG), y una intensa reacción inflamatoria vía receptores *Toll-like* acompañada de una liberación de citocinas, mediadores lipídicos como el factor activador de plaquetas (PAF), leucotrienos, prostaglandinas (PGs) y una activación de los neutrófilos. Los procesos de adhesión y extravasación, y la posterior activación de los neutrófilos se deben a un incremento en la expresión de las adhesinas endoteliales (ICAM-1), selectinas e integrinas leucocitarias. La secreción de proteasas, especies reactivas de oxígeno (ROS) y mieloperoxidasa (MPO) por parte de los neutrófilos contribuyen también al daño celular inducido por el LES (Thieblemont *et al.*, 2016). Igualmente, los niveles de MMP-2, MMP-3, MMP-7, MMP-9 y del factor inducible por hipoxia-1 (HIF-1) se encuentran elevados en el suero de los pacientes con lupus, por lo que contribuyen también a la patogénesis de la enfermedad (Gheita *et al.*, 2015; Phillips *et al.*, 2017; Vira *et al.*, 2017; Mao *et al.*, 2018; Zhao *et al.*, 2018).

En la actualidad, no existe un tratamiento eficaz, seguro y efectivo para el LES, por lo que el abordaje terapéutico utilizado se basa sobre todo en el control de las manifestaciones clínicas derivadas de la enfermedad las cuales son extremadamente heterogéneas y multisistémicas, incluidos síntomas como fiebre y malestar general, manifestaciones dermatológicas, musculoesqueléticas, renales, respiratorias, hematológicas y neurológicas (Kamal, 2014). Hasta hace muy poco, el tratamiento del LES se basaba principalmente en el consumo de AINE, glucocorticoides, fármacos antipalúdicos (hidroxicloroquina y

cloroquina) así como fármacos inmunosupresores (micofenólico, ciclofosfamida, MTX, ciclosporina o azatioprina) (Kuhn *et al.*, 2015). Otros fármacos que se utilizan en el LES son los agentes biológicos belimumam y rituximab. Belimumab se emplea en manifestaciones leves a moderadas como artritis, serositis o si hay afectación cutánea y el rituximab se usa cuando además hay afección articular resístete a tratamiento convencional, hematológica, del sistema nervioso central o nefritis resistente (Cardiel *et al.*, 2019).

Esta farmacología es muy complicada debido a la importante idiosincrasia que aparece entre los invididuos, así como la falta de eficacia y seguridad. Es por ello, que actualmente los principales objetivos de la investigación del LES se basan en la búsqueda de terapias menos tóxicas y eficaces, que mejoren el curso de la enfermedad.

La terapia nutricional, incluyendo modificaciones en el dieta y el uso de complementos nutricionales, podría ser una interesante alternativa en el control de enfermedades IMID, como la AR y el LES por su posible contribución a la reducción de comorbilidades y a incrementar la calidad de vida de los pacientes, ya que además de su soporte dietético, podría ejercer efectos profilácticos y terapéuticos carentes de los efectos indeseables que acompañan a la farmacoterapia clásica (Paolino *et al.*, 2019).

El estado nutricional y la ingesta de ciertos alimentos en pacientes con AR o LES podría interferir en el curso de la enfermedad y por tanto, una dieta equilibrada podría mejorar el pronóstico y curso de las mismas (Somers and Richardson, 2014; Forsyth *et al.*, 2018). En este sentido, diversos estudios recomiendan una dieta rica en fibra derivada de frutas, verduras, cereales integrales, vitaminas, minerales, ácidos grasos mono- y poliinsaturados y polifenoles, con un consumo reducido de calorías y sodio, para el control de marcadores proinflamatorios y la prevención de síntomas y comorbilidades asociadas a estas patologías (Lima *et al.*, 2016; Forsyth *et al.*, 2018; Khan *et al.*, 2019).

En este sentido, recientes estudios epidemiológicos y clínicos han confirmado que el consumo habitual del aceite de oliva (AO), alimento funcional de primera magnitud, dentro del contexto de la dieta mediterránea, es eficaz en la prevención de la enfermedad cardiovascular y sus complicaciones (Martínez-González *et al.*, 2015; Salas-Salvadó, Mena-Sánchez and Jordi Salas-Salvadó, 2017). Además, se han observado efectos beneficiosos en otras patologías como las de índole inflamatorio (Sánchez-Fidalgo *et al.*, 2013; Rosillo *et al.*, 2016), en el envejecimiento (Fernández del Río *et al.*, 2016) y en diversos tipos de cáncer (Cardeno *et al.*, 2014; Kwan *et al.*, 2017).

La modulación de la respuesta inmune ejercida por dietas que contienen AO se traduce en una menor supresión de la proliferación de linfocitos, mayor producción de citocinas anti-inflamatoria esenciales en la regulación de la respuesta inmune y en definitiva, una mayor capacidad fagocítica de los macrófagos/monocitos, característica esencial para la eliminación de los agentes patógenos. Por lo tanto, y teniendo en cuenta los antecedentes, el AO como un componente de la dieta puede ejercer un efecto inmunomodulador (Puertollano *et al.*, 2010; Aparicio-Soto, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2017).

Previamente, nuestro equipo ha evidenciado el funcionalismo del aceite de oliva virgen extra (AOVE) en patologías autoinmunes mediante proyectos en los que se ha valorado el potencial

antiinflamatorio crónico e inmunomodulador del AOVE en modelos experimentales de AR y de LES, con resultados muy satisfactorios. Particularmente, se ha puesto de manifiesto cómo el AOVE fue eficaz en la prevención de la AR y LES inducidas experimentales a través de la modulación de diversos elementos del sistema inmune involucrados en la respuesta inmuno-inflamatoria (M Aparicio-Soto *et al.*, 2016; Marina Aparicio-Soto *et al.*, 2016; Rosillo *et al.*, 2016).

En los últimos años, las propiedades beneficiosas del AOVE, está siendo atribuidas no sólo a su fracción saponificable, caracterizada por el alto contenido en ácidos grasos monoinsaturados (ácido oleico), sino también a las fracciones insaponificables (FI) y polifenólica (FP) que, aun siendo minoritarias, están constituidas por un amplio número de compuestos de alto valor biológico. Interesantes publicaciones de nuestro grupo así lo avalan (Cardeno *et al.*, 2014; Parkinson *et al.*, 2016; Aparicio-Soto, Sánchéz-Hidalgo, *et al.*, 2017).

Actualmente, existen más de 30 compuesto fenólicos en el olivo, aunque su composición cuali- y cuantitativa depende de la variedad de la aceituna, el grado de maduración y la climatología. Pueden clasificarse en varios grupos: ácidos fenólicos, alcoholes fenólicos, secoiridoides, hidroxi-isocromanos, flavonoides y lignanos.

Los polifenoles pertenecientes al grupo de los secoirioides se encuentran mayoritariamente en las plantas pertenecientes a la familia Oleaceae dentro de la cual se incluye la especie *Olea europea* L. Son los compuestos más abundantes y se han relacionado con atributos característicos del AO como son el amargor, la astringencia o el picante. Estos compuestos proceden del metabolismo secundario de los terpenos, siendo sintetizados a partir del ácido mevalónico. Se caracterizan por la presencia en su estructura de un fenil etil alcohol (3,4 DHPEA o *p*-HPEA) unido al ácido elenólico o sus derivados, y generalemente se encuentran glicosilados (Tasioula-Margari and Tsabolatidou, 2015; Parkinson *et al.*, 2016). Entre estos compuestos destacan la Oleuropeína (OL) y el ligustrósido presentes en diferentes órganos del olivo (*Olea europea* L.) (hoja, madera o fruto), la olaceína, el oleocantal y los aglicones de la oleuropeína y del ligrustrósido que se encuentran en la FP del AO.

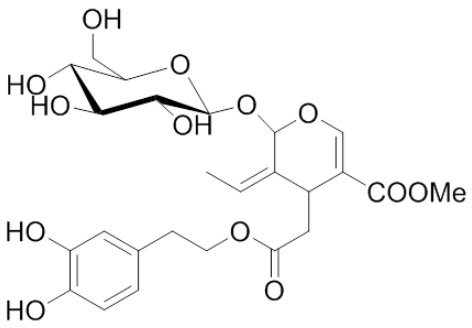

Figure 1. Estructura química de la OL.

La OL tiene funcionalidad de catecol y ejerce una potente actividad antioxidante relacionada principalmente con su capacidad para mejorar la estabilidad de los ROS mediante la formación de un

enlace intramolecular entre el hidrógeno libre del grupo hidroxilo y sus radicales fenoxilos (Visioli, Bellomo and Galli, 1998). Además, la OL tiene la capacidad de eliminar el óxido nítrico (NO) y promover la expresión de la enzima óxido nítrico sintasa inducible (iNOS) en células (de la Puerta *et al.*, 2001). También posee una ampliamente validada actividad anti-aterogénica, anticancerosa, anti-angiogénica, neuroprotectora, antimicrobiana y antiviral, gastroprotectora, hepatoprotectora, antidiabética, antiobesidad y radioprotectora, entre otras (Hassen, Casabianca and Hosni, 2015).

Estudios previos han puesto de manifiesto la remarcable actividad anti-inflamatoria de la OL (Barbaro *et al.*, 2014; Marcelino *et al.*, 2019). OL fue capaz de suprimir la respuesta inflamatoria producida en fibroblastos RAW264 estimulados con lipopolisacárido (LPS) (Ryu *et al.*, 2015). También se ha observado un importante efecto protector ante la pérdida de cartílago en lesiones osteoartríticas de cerdos guineanos (Horcajada *et al.*, 2015). El aglicón de la OL mejoró los niveles de marcadores clínicos y promovió la mejora del tejido sinovial de las articulaciones en un modelo murino de artritis inducida por colágeno (CIA) (Impellizzeri *et al.*, 2011). Además, OL ha demostrado su capacidad para reducir la inflamación pulmonar en modelos experimentales con células epiteliales bronquiales BEAS-2B expuestas a IL4 y en ratones BALB/c expuestos a cigarrillos y a ovoalbúmina (Kim *et al.*, 2018). Además, OL mejoró considerablemente el daño colónico agudo en un modelo murino inducido por DSS, así como minimizó los efectos inducidos en un modelo murino de colitis ulcerosa asociado a cáncer colorrectal (Giner *et al.*, 2011, 2013, 2016).

Quizás uno de los principales inconvenientes de los fenoles es su termo y fotolabilidad, así como su inestabilidad frente a la oxidación. Asimismo, presentan unas características biofarmacéuticas que limitan su absorción oral, debido a sus deficientes capacidades de permeabilidad a través de las membranas, la baja solubilidad de su forma libre en los fluidos acuosos, así como importantes efectos de metabolización presistémica intestinal y eflujo mediado por la glicoproteína P, ocasionando valores de biodisponibilidad muy bajos (Bohn, 2014). Por ello, numerosos estudios han remarcado la importancia de los derivados acetilados sintetizados a partir de compuestos fenólicos naturales con el fin de mejorar las propiedades de las moléculas originales, así pues como mejorar su biodisponibilidad, la penetración a través de la membrana celular y mejorar así sus propiedades antiinflamatorias y anti-oxidantes. De esta manera, la acetilación de las moléculas de los compuestos naturales nos proporciona una posibilidad de mejorar los perfiles farmacocinéticos y farmacodinámicos de los compuestos naturales y podría ser una nueva estrategia para el tratamiento de enfermedades de índole inmunoinflamatorio (de Araújo *et al.*, 2017; Rizzo *et al.*, 2017).

A pesar del creciente interés de este secoiridoide, no existen hasta la fecha, estudios experimentales en modelos de inmunoinflamación *in vitro*, *ex vivo* e *in vivo* que validen el potencial antirreumático de OL, así como los mecanismos bioquímicos y vías de señalización implicados.

Por ello, el **objetivo principal** de esta Tesis Doctoral es el estudio preclínico de OL, un secoiridoide del olivo (*Olea europea* L.) y de sus derivados acetilados en modelos experimentales de inmunoinflamación de AR y LES. Concretamente, nos hemos centrado en una serie de objetivos específicos:

1. Investigar los posibles efectos inmunomoduladores y antiinflamatorios de la OL y sus derivados semisintéticos, así como explorar los mecanismos moleculares y vías de señalización involucradas en el proceso, en:

a. Un modelo ex vivo de macrófagos peritoneales murinos estimulados con LPS.

b. Un modelo in vitro de una línea celular de fibroblastos sinoviales humanos (SW982) estimulados con IL-1β.

2. Evaluar los efectos de dietas enriquecidas con OL y la OL peracetilada (Per-OL) en un modelo de artritis inducida por colágeno tipo II en ratones DBAJ/1 y explorar los mecanismos moleculares y las vías de señalización posiblemente implicadas.

3. Estudiar los efectos potenciales de dietas enriquecidas con OL y Per-OL en un modelo de LES inducido por pristano (2,4,6,10,16- tetrametilpentadecano) en ratones BALB/c y explorar los mecanismos moleculares subyacentes y las vías de señalización probablemente involucradas en el proceso.

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CHAPTER I

OLIVE SECOIRIOID OLEUROPEIN AND ITS SEMISYNTHETIC ACETYL-DERIVATIVES REDUCE LPS-INDUCED INFLAMMATORY RESPONSE IN MURINE PERITONEAL MACROPHAGES VIA JAK/STAT AND MAPKS SIGNALING PATHWAYS

LA OLEUROPEINA Y SUS NUEVOS ACETIL-DERIVADOS MODULAN LA RESPUESTA INFLAMATORIA INDUCIRDA POR LPS EN MACRÓFAGOS PERITONEALES MURINOS

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RESUMEN

La oleuropeína (OL) es un glucósido secoiridoide extraído de la hoja de *Olea Europaea* L., con importantes propiedades antioxidantes y antiinflamatorias.

El objetivo principal de este estudio fue comprobar la posible actividad antiinflamatoria de tres nuevos derivados semisintéticos desarrollados a partir de la OL natural extraída de la hoja con un mejor perfil farmacocinético/farmacodinámico, en un modelo *ex vivo* de macrófagos peritoneales de ratón estimulados con lipopolisacárido bacteriano (LPS), comparando su efecto con el producido por la molécula natural y clarificar los mecanismos de señalización molecular involucrados.

Los macrófagos peritoneales obtenidos fueron tratados con OL y sus acetil-derivados en presencia o ausencia de LPS. La viabilidad celular fue determinada usando la técnica de la Sulforhodamina B (SRB). Los niveles de producción de óxido nítrico (NO) fueron analizados por el método de Griess. La producción de citoquinas pro-inflamatorias fue evaluada mediante la técnica de ELISA, y los cambios en la expresión proteica de parámetros bioquímicos de inflamación y señalización celular fueron determinados por Western Blotting.

Los derivados de OL redujeron significativamente los niveles de nitritos, los niveles de producción de citoquinas proinflamatorias Th1, Th2 y Th17, así como la sobreexpresión de las enzimas ciclooxigenasa 2 (COX-2) y óxido nítrico sintasa inducible (iNOS). Además, los derivados de OL redujeron significativamente la fosforilación del factor de transducción de la señal y activador de la transcripción (STAT)-3 inhibiendo así la vía de señalización Janus quinasas-transductor de señal y activador de la transcripción (JAK/STAT), y de las proteínas MAP quinasas (MAPKs). Por último, se observó un aumento significativo en la expresión de hemo oxigenasa-1 (HO-1) y el factor nuclear eritroide-2 (Nrf2), mostrando mejores resultados que el compuesto natural.

Estos nuevos acetil-derivados muestran un mejor efecto antiinflamatorio que la molécula natural, que puede deberse a la modificación de su estructura. Los mecanismos subyacentes a estos efectos beneficiosos pueden ser consecuencia de la activación de la vía antioxidante Nrf2/HO-1 y la inhibición de las vías de señalización protagonizadas por JAK/STAT y MAP quinasas. Estos

nuevos derivados semisintéticos podrían constituir una alternativa interesante en el abordaje terapéutico de diversas patologías de índole inflamatorio.

OLIVE SECOIRIDOID OLEUROPEIN AND ITS SEMISYNTHETIC ACETYL-DERIVATIVES REDUCE LPS-INDUCED INFLAMMATORY RESPONSE IN MURINE PERITONEAL MACROPHAGES VIA JAK/STAT AND MAPKs SIGANLING PATHWAYS

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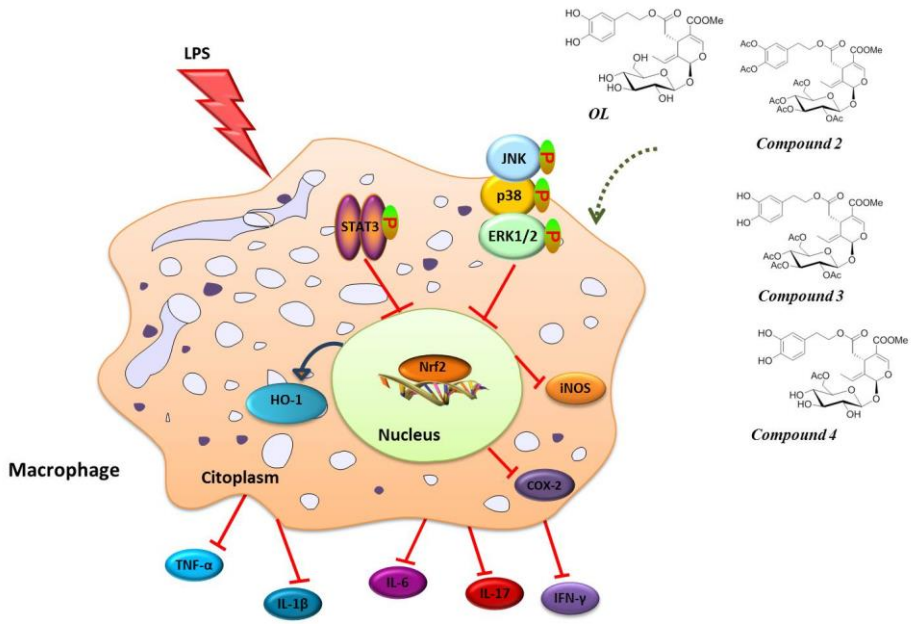
ABSTRACT

The present study was designed to investigate the potential anti-inflammatory, antioxidant and immunomodulatory activities of three new derivatives from OL in lipopolysaccharide (LPS)-induced mouse peritoneal macrophages in comparison with natural OL. Nitrites levels were analyzed by Griess method and intracellular reactive oxygen species (ROS) by fluorescence analysis. The levels of cytokines were evaluated by ELISA. The protein expression of cyclooxigenase-2(COX-2), inducible nitric oxide synthase (iNOS), janus kinase/signal transducer activator of transcription (JAK/STAT) pathway, heme oxygenase-1 (HO-1), nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and mitogen-activated protein kinases (MAPKs) activation were determined by Western Blotting.

The mechanisms underlying these protective effects could be related to the activation of Nrf2/HO-1 pathway and inhibition of both JAK/STAT and MAPKs pathways. We conclude that OL and new OL acetylated-derivatives exhibited immunomodulatory effects and thus, may offer a new promising therapeutic strategy in the management of immune mediated-inflammatory diseases.

KEYWORDS: HO-1; LPS; macrophage; Nrf2; oleuropein; ROS



1. INTRODUCTION

Macrophages are main components of the innate immune system and play a crucial function in the inflammatory process, recognizing an extensive variety of pathogen-associated molecular patterns such as lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria, via pattern recognition receptors, including the Toll-like receptor (TLR) family of proteins (Kawai and Akira 2011). LPS exposure drives macrophages to an activated state that induces an excess of inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), as well as proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and interferon (IFN)-γ. Moreover, LPS induces the expression of different enzymes such as inducible nitric oxide synthase (iNOS) and cyclo-oxygenase 2 (COX-2), which are regulated by the activation of mitogen-activated protein kinases (MAPKs) and the nuclear transcription factor kappa B (NF-κB) (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, and Alarcon-de-la-Lastra 2014; Aparicio-Soto et al. 2014). Besides, the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), the crucial regulator in antioxidant response element (ARE)-driven gene expression in response to oxidative stress, leads to the expression of several antioxidant enzymes, such as heme oxygenase -1 (HO-1), which are regarded as essential anti-inflammatory targets (Kang and Kim 2013; Li et al. 2014).

Recently, accumulating experimental, clinical and epidemiological data have provided support to the traditional beliefs of the beneficial effect provided by olive derivatives (Battino et al. 2018; Pedret et al. 2018). In this regard, particular attention has been drawn to the secoiridoid oleuropein (OL), the main phenolic component of olive leaves, roots and unprocessed olive drupes (accounting up to 14% of total dry weight), which is hydrolysed during fruit maturation and forms different products, including hydroxytyrosol (HTy) (2-(3,4-dihydroxyphenyl)ethanol) (Pan et al. 2018).

OL has been reported as a well-established antioxidant, anti-thrombotic and anti-atherogenic, hepatoprotective, antiviral, antimicrobial and potential anti-cancer activities (Castejón et al. 2017; Janahmadi et al. 2017; Kremastinos 2008). Besides, OL has been also identified as a potential molecule with a therapeutic utility against neurodegenerative diseases, including Parkinson and Alzheimer diseases (Barbaro et al. 2014; Pantano et al. 2017). Moreover, some studies have documented that OL elicited anti-inflammatory effects inhibiting biosynthesis of proinflammatory cytokines, lypoxigenase activity and production of leukotriene B4. In fact, OL regulated LPS-stimulated RAW 264.7 inflammatory response by the suppression of both iNOS and COX-2 expressions (Ryu et al. 2015). As well, OL exerted gastrointestinal benefits preventing ethanol-induced gastric ulcers via elevation of antioxidant enzyme activities in rats (Alirezaei et al. 2012) and decreasing the extent and severity on dextran sulphate sodium (DSS)-induced acute colitis, reducing neutrophil infiltration, NO, iNOS, COX-2, proinflammatory cytokines and metalloproteinase (MMP)-9 protein production, via inhibition of NF-κB p65 subunit nucleus translocation (Barbaro et al. 2014). Nevertheless, although OL is rapidly absorbed and distributed in animals, its bioavailibity is low because it is fast metabolized into more polar compounds, mainly, HTy (Edgecombe, Stretch, and Hayball 2000).

In this line, some studies have shown the importance of acetylated derivatives of natural phenols since their lipophilic nature allows them to cross the cytoplasmic cell membranes and their uptake by cells, offering a possible protection of membrane components. Acetylation of polyphenols may bring improved properties to these molecules, such as, improved bioavailability, cell membrane penetration and enhanced anti-inflammatory and antioxidant activities among others (de Araújo et al. 2017; Hoang, Huynh, and Nguyen 2015; Rizzo et al. 2017). Therefore, the synthesis of OL derivatives with better pharmacodynamic/pharmacokinetic profiles than natural OL could be an appealing strategy in the management of the inflammatory process, due to the well-established anti-inflammatory activity of the natural compound.

In this context, the present study was designed to investigate the potential anti-inflammatory activity of three new acetylated derivatives synthesized from OL (1): Peracetylated OL (Per-OL); (2); 2'', 3'', 4'', 6''-Tetra-*O*-acetyloleuropein (3); and 6''-*O*-Acetyloleuropein (4), in LPS-

induced inflammatory response in murine peritoneal macrophages in comparison with natural OL, and to clarify the potential underlying molecular mechanisms involved in their effects.

2. MATERIALS AND METHODS

2.1. Reagents

OL (1)

The extraction of OL (1) was performed following the reported literature (Stamatopoulos, Chatzilazarou, and Katsoyannos 2013). Then, the extract was purified by column chromatography (CH2Cl2-MeOH 10:1→5:1) to give the product as a yellow solid (Figure 1A).

Per-OL (2)

OL (1 g, 1.85 mmol) was solved and stirred in a mixture of 1:1 (v/v) pyridine/acetic anhydride (8 ml) at 0º C for 10 min. Then, the reaction was kept at room temperature overnight. After hydrolysing the acetic anhydride, the solution was concentrated to dryness resulting a residue that was purified by column chromatography (EtOAc-C. Hexane 1:1) to give the product (quant.) as a brown solid. Spectroscopy data was identical to reported literature (Procopio AS M.; Costa, N.; Nardi, M. 2008) (Figure 1B).

2'', 3'', 4'', 6''-Tetra-O-acetyloleuropein (3)

To a solution of (2) (200 mg, 0.25 mmol) in MeOH (5 ml), imidazole (68 mg, 1.0 mmol) was added and the solution was stirred at 60º C for 20 h. Then, the solution was concentrated to dryness resulting a residue that was purified by column chromatography (CH_2Cl_2-MeOH 30:1) to give the product (115 mg, 65%) as a brown solid (Figure 1C). $[\alpha_L^{22}]$= -98. R_F 0.7 (CH_2Cl_2-MeOH 10:1). ^{1}H-RMN (300 MHz, CD_3OD): δ 7.50 (s 1H, H-3), 6.70 (d 1H, $J_{7',8'}$ = 8.0 Hz, H-7'), 6.66 (d 1H, $J_{4',8'}$ = 2.0 Hz, H-4'), 6.54 (dd 1H, $J_{8',7'}$ = 8.0 Hz, $J_{8',4'}$ = 2.0, H-8'), 5.98 (c 1H, $J_{8,10}$ = 7.7 Hz, H-8), 5.79 (m 1H, H-1), 5.34 (t 1H, J = 9.4 Hz, H-3''), 5.18 (d 1H, $J_{1'',2''}$ = 8.0 Hz, H-1''), 5.08 (t 1H, J = 10.0 Hz, H-4''), 5.05 (dd 1H, $J_{2'',4''}$ = 9.5 Hz, $J_{2'',1''}$ = 8.0 Hz, H-2''), 4.32 (dd 1H, $J_{6''a,6''b}$ = 12.4 Hz, $J_{6''a,5''}$ = 4.4 Hz, H-6''a), 4.25-4.06 (m 3H, H-6''b, H-1', H-5), 3.99-3.94 (m 1H, H-5''), 3.73 (s 3H, OMe), 2.75 (t 2H, $J_{2',1'}$ = 7.1 Hz, H-2'), 2.72 (dd 1H, $J_{6a,6b}$ = 14.0 Hz, $J_{6a,5}$ = 4.6 Hz, H-6a), 2.43 (dd 1H, $J_{6b,6a}$ = 14.0 Hz, $J_{6b,5}$ = 9.2 Hz, H-6b), 2.02, 2.00 and 1.99 (3s 12H, 4 Ac), 1.67 (dd 3H, $J_{10,8}$ = 7.1 Hz, $J_{10,1}$ = 1.4 Hz, H-10). ^{13}C-RMN (75.5 MHz, CD_3OD): δ 172.9 (C-7), 172.4, 171.6, 171.3 (4 CO Glc), 168.5 (*C*OOMe), 154.8 (C-3), 153.3 (C-5' y 6'), 130.7 (C-3'), 130.0 (C-9), 125.3 (C-8), 121.4 (C-8'), 117.0 (C-4'), 116.5 (C-7'), 109.8 (C-4), 98.9 (C-1''), 95.8 (C-1), 74.0 (C-5''), 73.2 (C-3''), 72.4 (C-2''), 69.6 (C-4''), 66.9 (C-1'), 62.9 (C-6''), 52.0 (C-COO*Me*), 41.1 (C-6), 35.4 (C-2'), 31.8 (C-5), 20.6 y 20.5 (4 Ac), 13.6 (C-10). α (22 ºC): -98. HRLSI-MS *m/z* calc. for $C_{33}H_{40}O_{17}Na$ ($[M + Na]^+$): 731.2158, found: 731.2133.

6''-O-Acetyloleuropein (4)

To a solution of (1) (100 mg, 0.18 mmol) in ^{t}BuOH (6 ml) vinyl acetate (313 µl, 3.40 mmol) and lipase-supported in Immobed *Thermomyces Lanoginosus* (300 mg) were added. The mixture was stirred at 60º C for 20 h. Then, it was filtered and concentrated to dryness resulting a residue that was purified by column chromatography (CH_2Cl_2-MeOH 30:1) to give the product (60 mg, 57%) as a yellow solid *(Figure 1D).* $[\alpha_L^{22}]$= -96. R_F 0.3 CH_2Cl_2-MeOH 10:1). ^{1}H-RMN (300 MHz, CD_3OD): δ 7.52 (s 1H, H-3), 6.69 (d 1H, $J_{7',8'}$ = 8.0 Hz, H-7'), 6.64 (d 1H, $J_{4',8'}$ = 2.0 Hz, H-4'), 6.53 (dd 1H, $J_{8',7'}$ = 8.0 Hz, $J_{8',4'}$ = 2.0, H-8'), 6.07 (c 1H, $J_{8,10}$ = 7.1 Hz, H-8), 5.83 (m 1H, H-1), 4.80 (d 1H, $J_{1'',2''}$ = 7.7 Hz, H-1''), 4.36 (dd 1H, $J_{6''a,6''b}$ = 12.0 Hz, $J_{6''a,5''}$ = 2.2 Hz, H-6''a), 4.25-4.06 (m 3H, H-6''b, H-1'), 3.96 (dd 1H, $J_{5,6a}$ = 9.2 Hz, $J_{5,6b}$ = 4.5 Hz, H-5), 3.71 (s 3H, OMe), 3.65-3.36 (m 4H, H-2'', H-3'', H-4'', H-5''), 2.73 (t 2H, $J_{2',1'}$ = 7.1 Hz, H-2'), 2.72 (dd 1H, $J_{6a,6b}$ = 13.9 Hz, $J_{6a,5}$ = 4.5 Hz, H-6a), 2.40 (dd 1H, $J_{6b,6a}$ = 13.9 Hz, $J_{6b,5}$ = 9.2 Hz, H-6b), 1.99 (s 3H, Ac), 1.65 (dd 3H, $J_{10,8}$ = 7.1 Hz, $J_{10,1}$ = 1.4 Hz, H-10). ^{13}C-RMN (75.5 MHz, CD_3OD): δ 172.9 (C-7), 172.8 (CO Glc), 168.6 (*C*OOMe), 155.1 (C-3), 146.2 (C-5'), 144.9 (C-6'), 130.7 (C-3'), 130.2 (C-9), 125.1 (C-8), 121.3 (C-8'), 117.0 (C-4'), 116.5 (C-7'), 109.4 (C-4), 101.2 (C-1''), 95.5 (C-1), 77.7 (C-5''), 75.5 (C-3''), 74.6 (C-2''), 71.4 (C-4''), 66.8 (C-1'), 64.6 (C-6''), 51.9 (C-COO*Me*), 41.2 (C-6), 35.4 (C-2'), 31.8 (C-5), 20.7(Ac), 13.5 (C-10). HRLSI-MS *m/z* calcd for $C_{27}H_{34}O_{14}Na$ ([M + Na]$^+$): 605.1841, found: 605.1836.

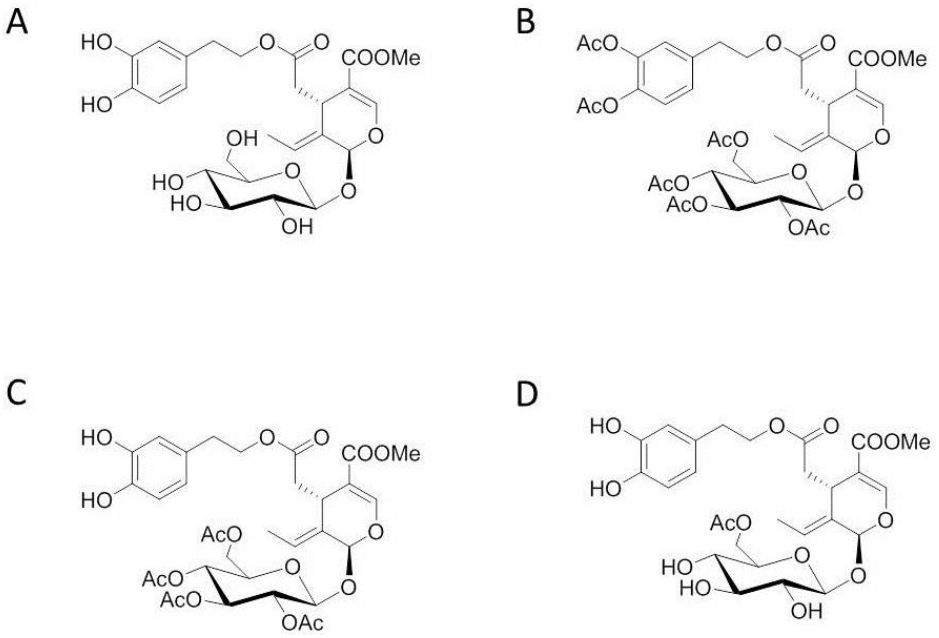


Figure 1. A) Chemical structure of OL (1), **(B)** Per-OL (2), **(C)** 2'', 3'', 4'', 6''-Tetra-O-acetyloleuropein (3) and **(D)** 6''-O-Acetyloleuropein (4).

2.2. Cell membrane permeability and lipophilicity of OL and its acetylated-derivatives

ClogP has been extensively used to evaluate the lipophilicity of organic molecules, and to estimate cell membrane permeability (Lipinski et al. 2001). The calculated logP, the logarithm of the partition coefficient between octan-1-ol and water, were obtained by using ChemBioDraw® Ultra 14.0 (Perkin-Elmer Informatics Waltham, MA), one of the best commercially available chemical softwares, preferred to HyperChem and ACD/log P (Rice 2014).

2.3. Animals

Twenty eight-ten-weeks-old female Swiss mice (20-30 g) were provided by Harlan Interfauna Ibérica® (Barcelona, Spain), randomly distributed in cages (5 mice/cage) and used as sources of peritoneal macrophages. Mice were maintained under constant conditions of temperature (20-25 °C) and humidity (40-60%) with a 12 h light/dark cycle and fed with standard rodent chow (Panlab® A04, Seville, Spain) and water *ad libitum* throughout the whole experiment in our Animal Laboratory Center (Faculty of Pharmacy, University of Seville, Spain). After one-week acclimation, mice were intraperitoneally injected with 1 ml of sterile thioglycollate medium (3.8% w/v) (BD Difco®, Le Pont de Claix, France). After thioglycollate injection, behavioral observation, water and food consumptions, loss of body weight and survival were daily monitored until sacrifice. After 3 days, mice were euthanized in the laboratory surgical area by CO_2 exposure.

All animal care and experimental procedures followed a protocol approved by the Animal Ethics Committee of the University of Seville, and all experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Counsel 2010/630/EU).

2.4. Isolation and culture of murine peritoneal macrophages

Peritoneal cells were obtained following the protocol described by Aparicio-Soto et al., 2015 (Aparicio-Soto et al. 2015). Cells were washed with PBS and resuspended in RPMI 1640 medium (PAA®, Pasching, Austria) supplemented with 10% heat-inactivated foetal calf serum (FCS) (PAA®, Pasching, Austria), L-glutamine (2 mM), glucose (4.5 g/l), and HEPES buffer (10 mM), in the presence of 100 mg/ml streptomycin and 100 U/ml penicillin (PAA®, Pasching, Austria) and seeded in culture plates for 2 h at 37º C in a 5% CO_2 humidified atmosphere. After 2 h, cells were washed with phosphate buffered saline (PBS) and fresh RPMI 1640 medium without fetal bovine serum (FBS) containing different concentrations of assayed compounds (1-4). After 30 min, murine peritoneal macrophages were stimulated with 5 µg/ml of LPS from *Escherichia Coli* (Sigma-Aldrich®, St Louis, MO, US) in presence or absence of OL or its derivatives (2-4) (25 and 50 µM) for 18 h.

OL, (2), (3) and (4) were always freshly prepared as stock solutions in dimethylsulfoxide (DMSO) (Panreac®, Barcelona, Spain) and diluted to the desired concentration in the culture medium. The final concentration of DMSO in the culture medium was ≤ 1% in all experiments and it had not significantly influence cell response.

2.5. Cell viability

Cells (1x10^5 cells/well) were incubated in presence or absence of OL or its derivatives for 18 h. The effects of OL and (2), (3) and (4) on cell viability were analyzed by sulforhodamine B (SRB) assay (Skehan et al. 1990). After 18 h, macrophages were fixed *in situ* by adding 50 µL of 50% (w/v) cold trichloroacetic acid (Sigma-Aldrich®, St. Louis, MO, USA) and incubated for 60 min at 4°C. Supernatants were discarded, and plates were washed four times with deionized water and dried. After that, 50 µL of SRB (Sigma-Aldrich®, St. Louis, MO, USA) solution (0,4% w/v) in 1% acetic acid (Panreac®, Barcelona, Spain) was added in each well and incubated for 30 min at room temperature. Then, plates were washed with 1% acetic acid and air dried. Finally, 100 µL per well of 10 mmol/L Tris base pH 10,5 (Sigma-Aldrich®, St. Louis, MO, USA) were added, and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) reader at 510 nm (BioTek®, Bad Friedrichshall, Germany). Cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells). In each experiment, viability was always ≥ 95 %.

2.6. Measurement of intracellular ROS

Intracellular reactive oxygen species (ROS) production was measured using 2,7-dichlorofluorescein-diacetate (DCFH-DA). DCFH-DA penetrates into the macrophages and is hydrolyzed by intracellular esterases to the nonfluorescent 2,7-dichlorofluorescein (DCFH), which can be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS. Isolated murine peritoneal macrophages were seeded at 10^6 cells per well in dark bottom 24-well plates and were incubated in the absence or presence of OL and its derivatives (2-4) and 30 min later, cells were stimulated with LPS for 18 h. After the incubation time, cells were treated with 25 µM DCFH-DA at 37° C for 45 min and washed once with buffer. The fluorescence intensity was measured with a plate reader (BioTek®, Bad Friedrichshall, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The results were expressed as the intracellular ROS production percentage compared with LPS-DMSO control cells. H_2O_2 (50 µM) (Sigma Aldrich®, Barcelona, Spain) was used as the pro-oxidant positive control. The fluorescence intensity was measured as described previously by Cárdeno et al., 2014 (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, Sanchez-Fidalgo, et al. 2014).

2.7. Measurement of nitrites production

Cells in 24-well plate were treated with different concentrations of OL or its derivatives (2-4) (25 and 50 μM), and 30 min later stimulated with LPS for 18 h. The culture supernatants were transferred into a 96-well assay plate, mixed with Griess reagent (Sigma- Aldrich®, St Louis, MO, USA) and incubated for 15 min at room temperature. The amount of nitrite, as an index of NO generation, was determined and obtained by extrapolation from a standard curve with sodium nitrite. The absorbance at 540 nm was measured by an ELISA reader (BioTek®, Bad Friedrichshall, Germany). Results were expressed as the nitrite production percentage compared with DMSO-LPS cells (stimulated untreated cells).

2.8. Cytokine assay

The levels of PGE_2 and IL-1β, TNF-α, IL-6, IL-17 and IFN-γ cytokines were determined in cell- culture supernatants using appropriate commercial ELISA kits for the appropriate form of these parameters (Diaclone®, Besançon, Cedex, France; Cayman Chemical Company®, Ann Arbor, MI, USA). The results were expressed in pg/ml.

2.9. Isolation of cytoplasmic and nuclear proteins and immunoblotting detection

Cells (1×10^6 cells/ml) were treated with OL or its derivatives and stimulated with LPS for 18 h. After incubation, cells were rinsed, scraped off and collected in ice-cold PBS containing a cocktail of protease and phosphatase inhibitors and processed as described by Sánchez-Hidalgo et al., 2005 (Sánchez-Hidalgo et al. 2005). Protein concentration was measured for each sample using a protein assay reagent (BioRad®, CA, USA) according to the Bradford´s method and using γ-globuline as a standard. Aliquots of supernatant contains equal amount of protein (15 μg) were separated on 10% acrylamide gel by sodium dodecyl sulfate polyacryamide gel electrophoresis, electrophoretically transferred into a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-COX-2 and rabbit anti-iNOS (Cayman®, Ann Arbor, MI, USA) (1:2500 and 1:1000, respectively), mouse anti-pSTAT3 (Cell Signaling Technology®, Danvers, MA, USA) (1:1000), mouse anti-pJNK, rabbit anti-JNK, mouse anti-pp38, rabbit anti-p38 (Cell Signaling Technology®, Danvers, MA, USA) (1:1000), rabbit polyclonal anti p-ERK and mouse anti-ERK½ (Cell Signaling Technology®, Danvers, MA, USA), rabbit anti-HO1 and rabbit anti-Nrf2 (Cell Signaling Technology®, Danvers, MA, USA) (1:500), overnight at 4ºC. After rinsing, the membranes were incubated with a horseradish peroxidase-labeled (HRP) secondary antibody anti-rabbit (Cayman Chemical®, Ann Arbor, MI, USA) (1:50000) or anti-mouse (Dako®, Atlanta, GA, USA) (1:2000) containing blocking solution for 1-2 h at room temperature. To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Sigma Aldrich®, MO, USA). Immunodetection was performed using enhanced chemiluminescence light-detecting kit

(Pierce®, Rockford, IL, USA). The immunosignals were captured using Amersham Imager 600 from GE Healthcare® (Buckinghamshire,US) and densitometric data were studied following normalization to the house-keeping loading control. The signals were analyzed and quantified by an Image Processing and Analysis in Java (Image J, Softonic®) and expressed in relation to the DMSO-LPS treated cells.

2.10. Statistical evaluation

All values in the figures and text are expressed as arithmetic means ± standard error (S.E.M). Experiments were carried out in triplicate. Data were evaluated with Graph Pad Prism® Version 6.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) using Tukey´s multiple comparisons test as post *hoc* test. *P* values of < 0.05 were considered statistically significant. In the experiments involving densitometry, the figures shown are representative of at least three different experiments performed on different days.

3. RESULTS

3.1. Effects of chemical acetylation in the lipophilicity of OL and its acetylated derivatives

Selective enzymatic monoacetylation of the primary hydroxyl group of OL resulted in a small increase of ClogP values, which go from 0.587 for OL to 0.833 for its monoacetyl derivative. On the contrary, the derivatives with the acetylated glucose moiety showed a considerable increase in lipophilicity, with values of 2.727 and 2.509, for the tetra-O-acetyl and hexa-O-acetyl derivatives, respectively. The increased lipophilicity by acetylation may play significant role in the absorption of a compound in the gastrointestinal track, and in its subsequent metabolism in liver.

3.2. OL and its acetylated derivatives do not compromise murine macrophages survival

We first assessed the impact of OL and its acetylated derivatives on murine peritoneal macrophages by SRB survival assay. After 18 h of incubation with the compounds, the viability of cells treated with (1), (2), (3) or (4) was not significantly compromised at concentrations from 50 to 200 μM (Data not shown). However, concentrations lower than 25 μM produced a decrease of cell viability to less than 80%. Therefore, we decided to assay the compounds at concentrations from 25 to 50 μM in forthcoming experiments.

3.3. OL and its acetylated derivatives inhibit LPS-induced intracellular reactive oxygen species (ROS) production in murine peritoneal macrophages

Free radicals of oxygen are suggested to be signaling messengers in the LPS-mediated inflammatory response. So, we tested the effects of OL and its acetylated-derivatives on LPS-induced intracellular ROS production using a DCFH-SA assay. As shown in Figure 2, cells incubated with acetylated OL derivatives treatments of for 18 h exhibited significant decreases in intracellular ROS production. Similar results were found in OL-treated cells. (***p<0.001 vs. untreated cells; ^{++}p<0.01 and ^{+++}p<0.001 vs. DMSO-LPS control cells).

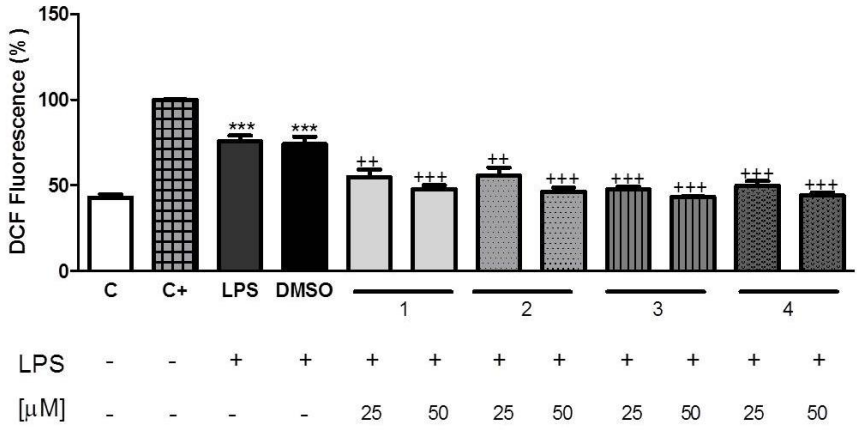


Figure 2. Intracellular ROS generation was reduced by OL and its derivatives. Cells were incubated with OL and its derivatives (25 and 50 μM) for 18 h. Then, cells were harvested and incubated with 25 μM of DCFH-DA for 45 min at 37º C in the dark. Results were expressed as mean fluorescence intensity obtained ± S.E.M. (n=6). ***p<0.001 vs. untreated cells; ^{++}p<0.01; ^{+++}p<0.001 vs. DMSO-treated control cells. H_2O_2 (C+) was used as the pro-oxidant positive control.

3.4. OL and its acetylated derivatives countered nitric oxide production, downregulated iNOS and COX-2 overexpression and production of PGE_2 induced by LPS in murine peritoneal macrophages

Cells were cultured for 30 min in the presence or absence of OL or its derivatives at concentrations of 25 and 50 μM and then stimulated for 18 h. As expected, the stimulation with LPS induced strong production of NO (***p<0.001 vs. untreated cells). On the contrary, pre-treatment with OL or its acetylated derivatives effectively prevented the NO mediated induction (^{++}p<0.01 and ^{+++}p<0.001 vs. DMSO-treated control cells) (Fig. 3A). These data suggested that acetylated OL derivatives could downregulate iNOS enzyme activity, which was further confirmed by measuring iNOS protein expression. Immunoblotting analysis demonstrated that OL derivatives exhibited a significant inhibition of the LPS-induced iNOS expression at both 25 and 50 μM, which a higher

reduction on iNOS protein expression compared with OL (***p<0.001 vs. untreated cells; ^{+}p<0.05; ^{++}p<0.01 and ^{+++}p<0.001 vs. DMSO-LPS control cells) (Fig. 3B).

We investigated the possible effect of OL and its acetylated derivatives on COX-2 and PGE_2 inflammation-related biomarkers. As expected, LPS stimulation remarkably increased COX-2 protein expression (Fig. 3B) and PGE_2 levels (Fig. 3C), whereas OL treatment or its acetylated derivatives (25 and 50 μM) evoked a significant down-regulation of COX-2 expression and amelioration of PGE_2 in LPS-stimulated peritoneal macrophages (***p<0.001 vs. untreated cells; ^{+}p<0.05, ^{++}p<0.01 and ^{+++}p<0.001 vs. DMSO-LPS control cells).

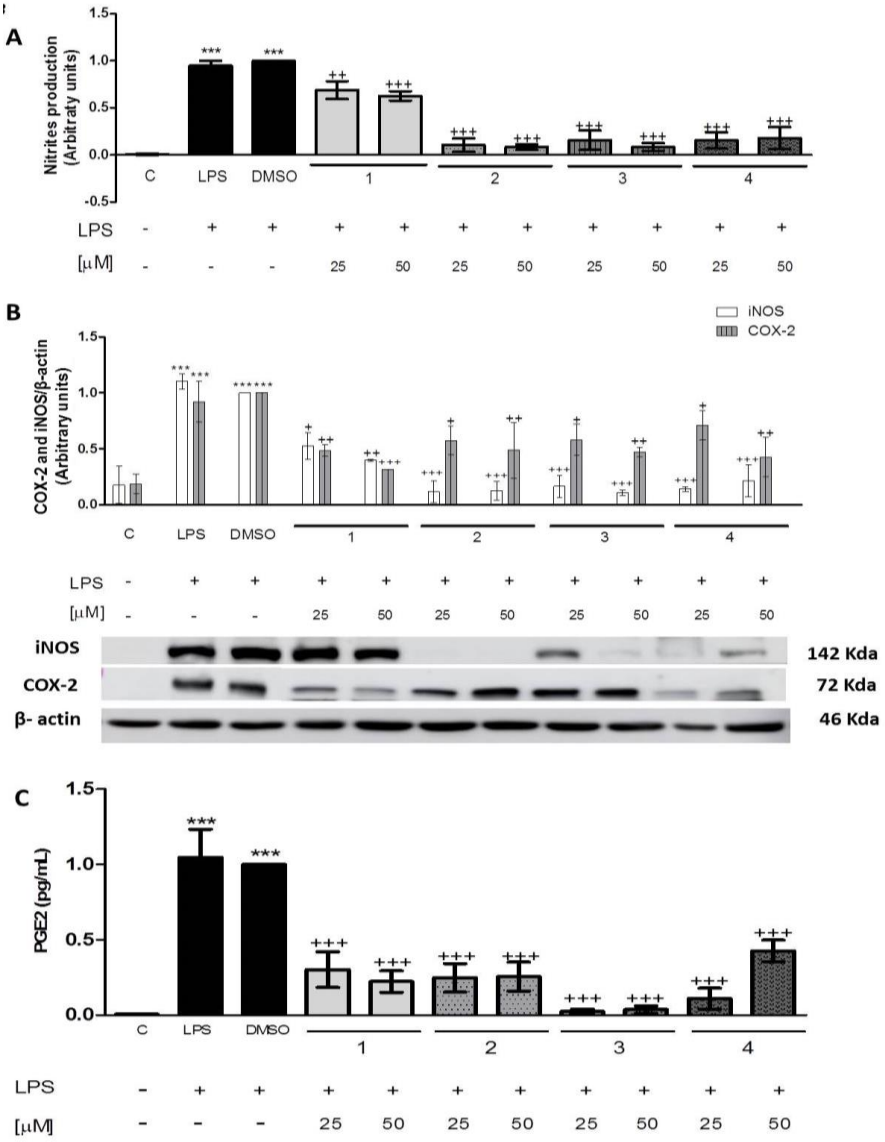
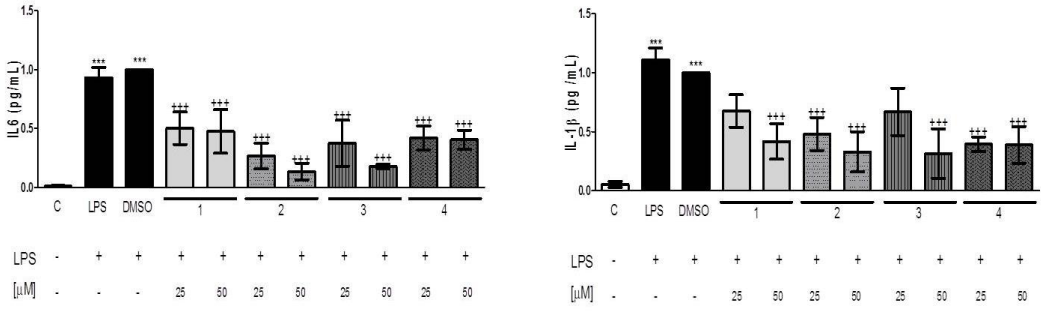


Figure 3. Effect of OL (1), Per-OL (2), 2'', 3'', 4'', 6''-Tetra-O-acetyloleuropein (3) and 6''-O-Acetyloleuropein (4) on LPS-induced NO production (A), iNOs and COX-2 protein expression (B) and PGE_2 levels (C) in mouse peritoneal macrophages. Cells were pre-incubated with the compounds separately, and after 30 min, macrophages were treated with LPS for 18 h. As control, cells were also treated with DMSO (solvent control) and LPS or untreated in absence of LPS. Densitometry analysis was performed following normalization to the control (β-actin housekeeping gene). Each value represents the mean ± S.E.M. for four independent experiments. ***p<0.001 vs. untreated cells; ^{+}p<0.05; ^{++}p<0.01; ^{+++}p<0.001 vs. DMSO-treated control cells.

3.5. OL and its acetylated derivatives on cytokine production

We further explored whether OL or its acetylated derivatives could modify the production of helper T cells (Th)-1/Th2/Th17 cytokines. After 18 h of LPS stimulation, the levels of IL-1β and IL-6 were significantly decreased after OL or its acetylated derivatives treatments (25 and 50 μM) in comparison with LPS-stimulated cells (***p<0,001 vs. untreated cells; ^{+++}p<0,001 vs. DMSO-LPS control cells). However, OL treatment was not able to decrease TNF-α, IFN-γ and IL-17 levels. On the contrary, IL-6, IL-1β , IFN-γ and IL-17 levels were diminished after OL derivative (3) treatment; Similarly, compound (4) could decrease IL-6, IL-1β , TNF-α and IFN-γ levels and finally the derivative (2) significantly reduced the production of all the tested Th1 and Th17 pro-inflammatory cytokines showing the best anti-inflammatory profile at the assayed concentrations (25 and 50 μM) in a dose-dependent manner (Fig. 4) (***p<0.001 vs. untreated cells; ^{+}p<0.05, ^{++}p<0.01 and ^{+++}p<0.001 vs. DMSO-LPS control cells).



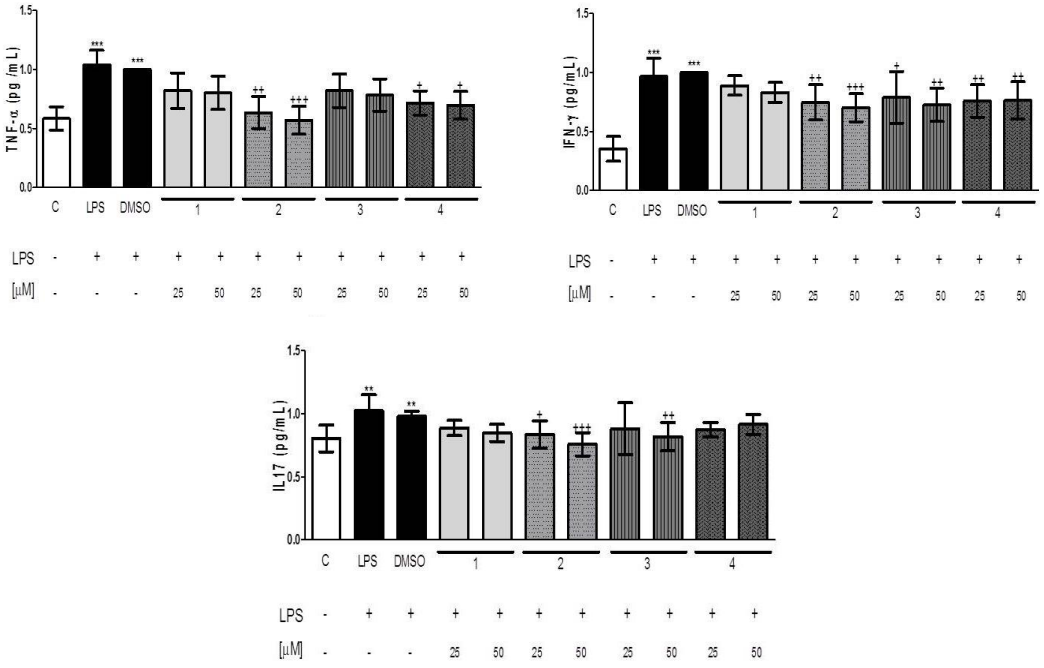


Figure 4. OL (1) and Per-OL (2), 2'', 3'', 4'', 6''-Tetra-O-acetyloleuropein (3) and 6''-O-Acetyloleuropein (4) reduced proinflammatory Th1/Th2/Th17 cytokines production in mouse peritoneal macrophages. Levels of **IL-1β, TNF-α, IL-6, IFN-γ and IL-17** cytokines were measured by ELISA. Data represent mean ± S.E.M. (n=12). **p<0.01; ***p<0.001 vs. non stimulated cells; ^{+}p<0.05; ^{++}p<0.01; ^{+++}p<0.001 vs. DMSO-treated control cells.

3.6. OL and its acetylated derivatives on LPS-induced MAPKS activation in murine peritoneal macrophages

To explore the molecular mechanism underlying the previously observed anti-inflammatory effects, we evaluated whether OL derivatives were able to influence janus NH_2-terminal kinase (JNK), extracellular signal-regulated kinases (ERK), and p38 phosphorylation by western blot. 18 h after LPS stimulation, OL derivatives demonstrated to be able to inhibit significantly ERK, JNK and p38 activation, showing better results in comparison with the natural compound (Fig. 6) (*p<0.05; ***p<0.001 vs. untreated cells; ^{+}p<0.05, ^{++}p<0.01 and ^{+++}p<0.001 vs. DMSO-LPS control cells).

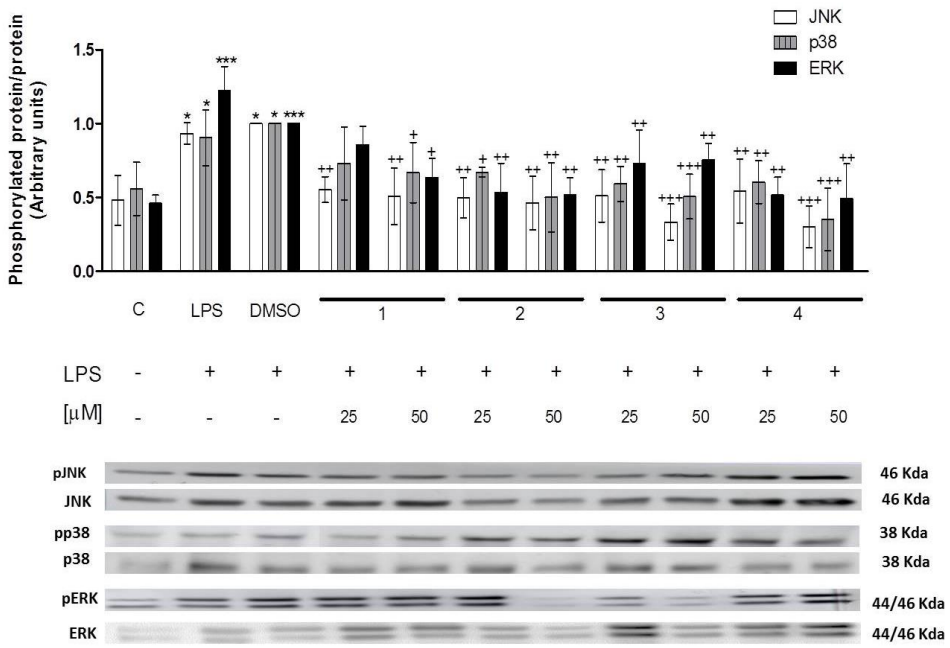


Figure 5. Effects of OL (1), Per-OL (2), 2'', 3'', 4'', 6''-Tetra-O-acetyloleuropein (3) and 6''-O-Acetyloleuropein (4) on p38, JNK and ERK MAPKs activation in mouse peritoneal macrophages. Cells were untreated and treated with 1, 2, 3 and 4 compounds (25 and 50 μM) for 18 h in presence of LPS. As control, cells were also treated with DMSO (solvent control) and LPS or untreated in absence of LPS. Densitometry analysis was performed following normalization to the control (p38, JNK and ERK house-keeping genes, respectively). Each value represents the mean ± S.E.M. for four independent experiments. *p<0.05; ***p<0.001 vs untreated cells; +p<0.05; ++p<0.01; +++p<0.001 vs DMSO-treated control cells.

3.7. OL and its acetylated derivatives on Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway and Nrf2-mediated transcriptional activation and HO-1 induction in LPS murine peritoneal macrophages

In our study, LPS stimulation produced an increase of STAT-3 phosphorylation when compared with non-stimulated cells. On the contrary, (2) and (3) OL derivatives exhibited significant effects in the prevention of the induced phosphorylation of STAT-3, showing better results that those described after treatment with their pattern (Fig. 6A) (*p<0.05 vs. untreated cells; ^{+}p<0.05 vs. DMSO-LPS control cells).

To identify whether acetylated OL derivatives were effective on regulation of Nrf2, we investigated the protein expression of Nrf2 and HO-1 by western blot. As shown in Figure 6B, OL derivatives caused a significantly increase on LPS-induced Nrf2 and HO-1 expression levels, which were

markedly higher compared with data from OL treated cells ($^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ vs. DMSO-LPS control cells). The increment of HO-1 and Nrf2 protein expression was according to the reduction of ROS production that we could observe in Fig. 2. Thus, both parameters highlighted the antioxidant potential of these compounds.

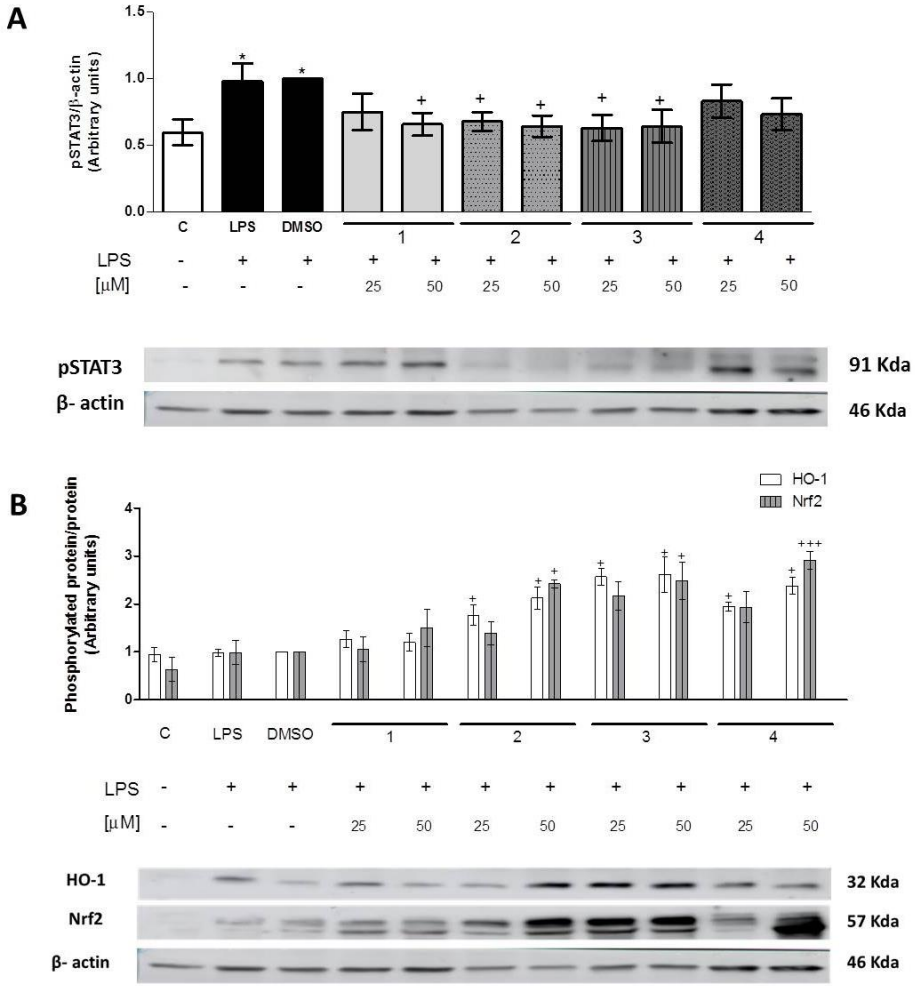


Figure 6. Effect of OL (1), Per-OL (2), 2'', 3'', 4'', 6''-Tetra-O-acetyloleuropein (3) and 6''-O-Acetyloleuropein (4) on JAK/STAT pathway, Nrf2-mediated transcriptional activation and HO-1 induction in mouse peritoneal macrophages. Cells were untreated and treated with (1), (2), (3) and (4) (25 and 50 μM) for 18 h in presence of LPS. As controls, cells were also treated with DMSO (solvent control) and LPS or untreated in absence of LPS. Densitometry analysis was performed following normalization to the control (β-actin housekeeping gene). Each value represents the mean ± S.E.M. for four independent experiments. $^{*}p<0.05$ vs. untreated cells; $^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$ vs. DMSO-treated control cells.

4. DISCUSSION

Currently, natural products still play an essential role in healthcare, providing a countless source of potential components for drug synthesis and development (Cragg and Newman 2013). The results of the present study showed, for the first time, that three new acetylated OL derivatives exhibited significant anti-inflammatory activities and attenuated the oxidative events induced by LPS in murine peritoneal macrophages, exhibiting better results than the observed with original compound OL. Among them, Per-OL (2) displayed the most effective anti-inflammatory behavior. These data are in agreement with previous *in vitro* studies, where OL showed anti-inflammatory effects in LPS-stimulated RAW 264.7 cells (Ryu et al. 2015). Likewise, other olive phenols such as HTy and peracetylated hydroxytyrosol (Per-HTy) (Montoya et al. 2018), hydroxytyrosol acetate (HTy-Ac) and its derivatives with better hydrophilic/ lipophilic balance, exhibited strong anti-inflammatory effects in LPS-stimulated murine peritoneal macrophages (Aparicio-Soto et al. 2015).

A good balance of cell permeability and aqueous solubility, for optimal gastrointestinal absorption of a drug, has been suggested to be in the range of $0 < \log P < 3$. Therefore, OL and its three acetylated derivatives fulfil this requirement, although for compound (2) with the acetylated glucose moiety should have better cell permeability, in accordance with the excellent results observed in *in vitro* assays (Kerns and Di 2008).

Balance disruption of the intracellular reduction-oxidation state has been observed in stimulated macrophages, which leads to oxidative stress characterized by a major shift in the cellular redox balance and usually accompanied by ROS-mediated damage. In fact, the new secoiridoid acetylated derivatives were able to reduce ROS levels acting as effective anti-oxidants. Thus, modulators of ROS production and ROS-induced signaling pathways, especially in macrophages, could represent potential strategies for anti-inflammatory intervention. Our findings are in concordance with other studies in which OL showed a strong antioxidant effects in a similar range tested in our study (50-100 μM). Also, other olive polyphenols like HTy showed important antioxidant effects acting as free radicals scavengers (Granados-Principal et al. 2011).

LPS-stimulated macrophages are closely related to an imbalance of cytokine network. It is well known that this kind of inflammatory process is characterized by an increase of Th1 and Th17 proinflammatory cytokines mainly, TNF-α, IL-1β, IL-6, and IFN-γ and IL-17 respectively.

Our results showed that OL and its derivatives inhibited distinct types of cytokines. In fact, the treatment with OL significantly reduced both IL-6 and IL-1β cytokines in culture medium in agreement with a previous study (Ryu et al. 2015). However, TNF-α, IFN-γ and IL-17 levels were not decreased after OL treatment. On the contrary, IL-6, IL-1β , IFN-γ and IL-17 levels were diminished by OL derivative (3), compound (4) could decrease IL-6, IL-1β , TNF-α and IFN-γ levels and finally, the derivative (2) significantly reduced the production of all tested Th1 and Th17

proinflammatory cytokines showing the best anti-inflammatory profile at the assayed concentrations (25 and 50 μM) in a dose-dependent manner. Hence, it is possible that the acetylation of aliphatic hydroxyl groups in OL leads to an increase of its lipophilic profile, explaining the improvement of the induced cytokines network exhibited after derivatives tested comparison with their natural pattern, OL.

The exposure of peritoneal macrophages to LPS induces the transcription of the iNOS gene and generation of large amounts of NO (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, Sanchez-Fidalgo, et al. 2014). In the present study NO levels and iNOS expression were decreased by OL treatment. Similar data were reported by Ryu et al., 2015 in RAW 264.7 cells (Ryu et al. 2015). Moreover, compounds (2), (3) and (4) at 25 and 50 μM produced a stronger reduction of both NO levels and iNOS protein expression when compared to OL (1).

COX-2 , the inducible isoform of COX is essential for the inflammatory response and is responsible for the overproduction of PGE_2 in inflammation (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, Sanchez-Fidalgo, et al. 2014). Likewise, PGE_2 modulates a variety of immune processes at sites of inflammation, including production of proinflammatory cytokines (Imig, Breyer, and Breyer 2002). Our results showed that LPS stimulus upregulated COX-2 protein expression and increased PGE_2 levels in murine peritoneal macrophages in concordance with previous studies by Aparicio-Soto et al., 2014 (Aparicio-Soto et al. 2014) and Cárdeno et al., 2014 (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, Sanchez-Fidalgo, et al. 2014). However, both parameters were significantly decreased by OL and its derivatives treatment in a dose-dependent manner, at doses assayed (25 and 50 μM).

According to the literature, MAPKs pathway is a critical axis essential for both induction and propagation of the inflammatory LPS-activated macrophage response (Radnai et al. 2009). MAPKs include ERK-1 and -2, JNK and p38 MAPKS. The increase activity of MAPKs as well as their involvement in the regulation of the inflammation mediator synthesis at the transcription level and translation make them potential targets for anti-inflammatory therapy (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, and Alarcon-de-la-Lastra 2014). In effect, MAPKs have been shown to play crucial roles in iNOS and COX-2 upregulation induced by various stimuli in mammalian cells (Guha and Mackman 2001). Our study showed that LPS evoked a significant increment of the p38, JNK and ERK (1/2) MAPKS phosphorylation, in peritoneal macrophages after 18 h of stimulation. In contrast, OL treatment and its acetylated derivatives (25 and 50 μM) reduced MAPKs phosphorylation, which was consistent with previous studies reporting that OL inhibited MAPKs activation in RAW 264.7 cells (Ryu et al. 2015) and in human osteoarthritis chondrocytes (Feng et al. 2017).

Moreover, MAPKs are also involved in the activation of JAK/STAT, an important signaling transduction pathway for the biological function of many cytokines. In fact, the

transcription factor STAT-3 is activated by many cytokines allowing to STAT3 dimers translocate to the nucleus and stimulate transcription mediating the synthesis of inflammatory mediators such as Th1 and Th17 cytokines (Zhu et al. 2013). Our results are in agreement with previous studies reporting that OL treatment attenuated the induced activation of STAT-3 in a murine colitis-associated colorectal cancer model (Giner et al. 2016). Besides, (2) and (3) OL derivatives also exhibited significant effects in the prevention of the induced phosphorylation of STAT-3, showing better results that the described after the treatment with the parent. Altogether, our data suggest that all tested compounds present a potential anti-inflammatory effect mediated in part by STAT-3 inactivation.

Nrf2, is a redox-sensitive transcription factor and binds to ARE located in the promoter regions of many detoxifying/antioxidant genes, including HO-1 (Bang et al. 2012). In the presence of oxidative stress, Nrf2 could migrate to the nucleus, bind to the oxidant response element sequence, and induce phase II gene transcription resulting in a cytoprotective response characterized by up regulation of HO-1 among others, and decreased sensitivity to oxidative stress damage. In inflammatory conditions, HO-1 expression protein could be part of an adaptive mechanism to limit cytotoxicity via several mechanisms including scavenging of ROS and nitrogen species, regulation of cell proliferation and prevention of apoptosis. Thus, Nrf2 regulates redox status and plays key roles in cellular defence by enhancing ROS remove (Rosillo et al. 2015). Our results in concordance with Aparicio-Soto et al., 2015 (Aparicio-Soto et al. 2015), showed that LPS stimulation decreased Nrf2 and HO-1 expression. On the contrary, OL derivatives treatments (25 and 50 μM) were able to increase these reduced expressions of Nrf2 and HO-1 induced by LPS, but we did not observed any significant changes in the expression of HO-1 and Nrf2 after the treatment with the original compound. Altogether, our data suggest that acetylated OL derivatives present also a potential antioxidant effect which is mediated by Nrf2/HO1 antioxidant signaling pathway.

In conclusion, this study showed, for the first time, the new acetylated OL derivatives (2), (3) and (4) have an important role in the balance of the inflammatory microenvironment induced by LPS in murine peritoneal macrophages by inhibiting proinflammatory cytokines production, such as IL-1β, TNF-α, IL-6, IL-17 and IFN-γ, as well as iNOS and COX-2 over expressions. The mechanisms underlying these protective effects could be related via the Nrf2/HO-1 antioxidant pathway activation and inhibition of both JAK/STAT and MAPKs signaling pathways. Furthermore, these new synthetic OL derivatives exhibit a better anti-inflammatory profile that the natural compound OL, that could be due to their better pharmacokinetic/pharmacodynamics profiles related to the modification of their chemical structure. Thereby, these new OL derivatives may offer a new promising therapeutic strategy in the management of inflammatory related pathologies, which needs to be further investigated. It could be a useful option to increase the quality of life of patients with these types of diseases, probably as a dietary supplement.

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CHAPTER II

OLEUROPEIN DOWN-REGULATED IL-1β-INDUCED INFLAMMATION AND OXIDATIVE STRESS IN HUMAN SYNOVIAL FIBROBLASTS CELL LINE SW982

LA OLEUROPEINA SUPRIME LA PRODUCCIÓN DE MEDIADORES PRO INFLAMATORIOS INDUCIDOS POR IL-1B EN FIBROBLASTOS SINOVIALES HUMANOS

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RESUMEN

La Artritis Reumatoide (AR) es una enfermedad crónica del sistema inmune caracterizada por una hiperproliferación de fibroblastos sinoviales (FS) y una infiltración de células inflamatorios immunes que inducen una progresiva degradación de la matriz y la destrucción del cartílago y del hueso a través de la producción de mediadores inflamatorios. En la AR, los FS o sinoviocitos de tipo fibroblástico juegan un papel fundamental en la persistencia de la inflamación crónica y el daño articular siendo unas de las poblaciones celulares más abundantes en el *pannus*, tejido de granulación localmente invasivo implicado en la degradación del cartílago y destrucción ósea.

La oleuropeína (OL) es el compuesto fenólico más abundante en las hojas del árbol del olivo, las semillas y la pulpa y la piel de las aceitunas verdes. Además, la OL es la responsable del característico sabor amargo de las aceitunas cuando aún están sin procesar. Existen numerosas propiedades farmacólogicas que se le atribuyen a la OL, incluida su capacidad antioxidante y anti-inflamatoria, lo que le confiere la capacidad de ser considerada como un suplemento alimentario en los países mediterráneos.

Las dianas de las terapias actuales de la AR se dirigen fundamentalmente contra células T, células B o citoquinas macrofágicas. Con estas terapias se consigue un retardo de la progresión de la enfermedad y de la destrucción articular que produce, sin embargo, presentan limitaciones en eficacia y seguridad para los pacientes con AR. Por ello, en los últimos años surde la necesidad de buscar otras estrategias terapéuticas para el abordaje de la AR, como la terapia nutricional y el uso de nutracéuticos.

En base a estos antecedentes, nos plantemos evaluar el efecto de la OL en la producción de mediadores pro-inflamatorios y estrés oxidativo en una línea celular de fibroblastos humanos (SW982) estimulados con interleucina (IL)-1β y dilucidar los mecanismos de señalización intracelular y vías de señalización que estuvieran posiblemente implicados.

Para llevar a cabo este estudio se utilizó la línea celular de fibroblastos humanos SW982, estimulados con IL-1β (5ng/mL). Los niveles de IL-6, factor de necrosis tumoral (TNF)-α así como

los de metaloproteinasas (MMPs) fueron determinados en el sobrenadante celular mediante la técnica de ELISA. Además, los posibles cambios en la expresión proteica de la enzima ciclo-oxigenasa 2 (COX-2), la prostaglandina E sintasa microsomal (mPGEs)-1, el factor inhibitorio NF-κB (IκB)-α, así como las proteínas cinasas p38, C-Jun NH_2- terminal cinasa (JNK) y Cinasa regulada por señal extracelular (ERK) junto a factor nuclear eritroide 2 (Nrf2) y hemo oxigenasa-1 (HO-1) fueron cuantificadas mediante Western Blotting.

Tras el tratamiento con OL en los fibroblastos SW982 estimulados con IL-1β, se puso de manifiesto una disminución significativa den la producción de los niveles de las citocinas pro-inflamatorias IL-6 y TNF-α y de las proteasas MMP-1 y MMP-3, así como una disminución de la expresión proteica de la enzimas pro-inflamatorias COX-2 y mPEGs-1, todo ello posiblemente asociados a una disminución en la activación de la vía de las proteínas cinasas activadas por mitógenos (MAPKs) y a una prevención en la degradación de la proteína inhibitoria IκB-α. Además, la inducción de la activación de la vía Nrf2/HO-1 puso de manifiesto el poder antioxidante de la OL.

Estos resultados sugirieron que la OL podría ser la base para el desarrollo de una nueva estrategia terapéutica o constituir un complemento nutricional de gran valor en el tratamiento y prevención de la AR.

OLEUROPEIN DOWN-REGULATED IL-1β-INDUCED INFLAMMATION AND OXIDATIVE STRESS IN HUMAN SYNOVYAL FIBROBLASTS CELL LINE SW982

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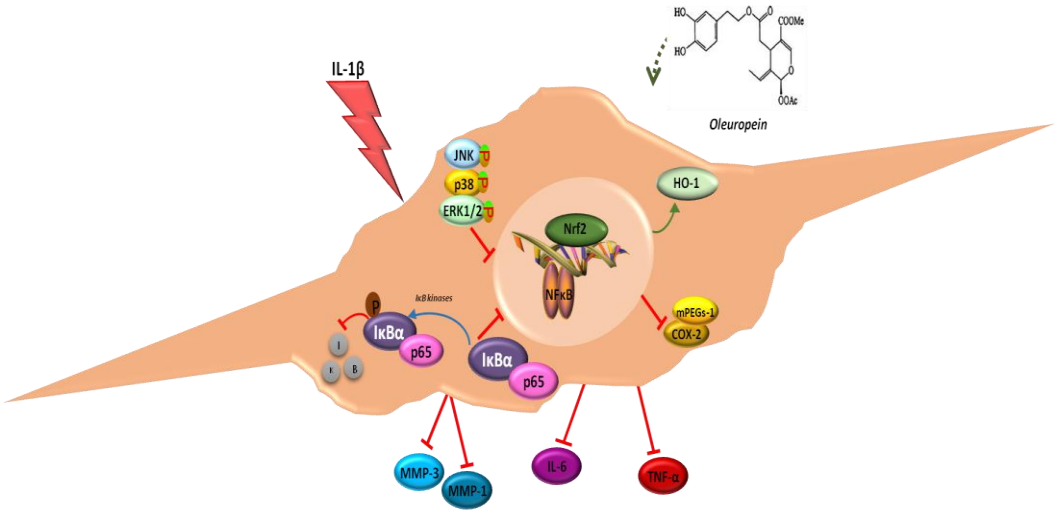
ABSTRACT

Rheumatoid arthritis (RA) is a chronic and systemic inflammatory autoimmune disease mainly characterized by aggressive hyperproliferation of synovial fibroblasts (SFs). It is accompanied by a massive infiltration of inflammatory immune cells inducing progressive matrix degradation, destruction of cartilage and bone erosion through the production of inflammatory mediators. Oleuropein, is the most prevalent phenolic component in olive leaves, seed, pulp and peel of unripe olives and is responsible for unprocessed olives characteristic bitter taste. This secoiridoid possesses a well-documented pharmacological properties, including antioxidant and anti-inflammatory, consequently is available as food supplement in Mediterranean countries. However, at the date, anti-arthritic effects of OL on SFs have not been yet elucidated. Thus, the aim of the present study was to investigate the potential effects of OL, on interleukin (IL)-1β-induced production of inflammatory mediators and oxidative stress in human synovial sarcoma cell line (SW982). In order to gain a better insight into mechanisms of action, signaling pathways were also explored.

Cell viability was determined using sulfhorodamine B (SRB) assay. The expression of inflammatory cytokines IL-6, tumor necrosis factor (TNF)-α, metalloproteinases (MMP)-1 and MMP-3 was evaluated by ELISA. Moreover, changes in the protein expression of cyclooxygenase 2 (COX-2), microsomal prostaglandin E synthase-1 (mPGES-1) as well as mitogen-activated protein kinase (MAPKs), nuclear factor kappa B (NF-κB), and nuclear factor erythroid 2-related (Nrf2) and heme oxigenase-1 (HO-1) signaling pathways were analysed by Western Blotting.

OL exerted anti-inflammatory and anti-oxidant effects via down-regulation of MAPKs and NF-κB signaling pathways and induction of Nrf2-linked HO-1 controlling the production of inflammatory mediators decreasing IL-6 and TNF-α cytokines, MMP-1 and MMP-3 levels as well as mPGES-1 and COX-2 overexpression. Thus, OL might provide a basis for developing a new dietary strategy for the prevention and management of RA.

Keywords*:* HO-1, MAPKs, Nrf2, NF-κB, rheumatoid arthritis, synovial fibroblasts.



1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory joint disease with systemic involvement that affects about 1% of the world's population (Meinecke *et al.*, 2005), which is characterized by leukocyte recruitment and activation, cell proliferation, angiogenesis, and *pannus* formation ultimately resulting in bone erosion, deformity and joint destruction. At a joint level, there are synovial hyperplasia and massive infiltration of immune cells, including cluster of differentiation (CD)4+ T-cells, B-cells, natural killer cells, macrophages, dendritic cells (DC), neutrophils and mast cells (Ganesan and Rasool, 2017).

Although the cause of RA has not been fully elucidated, increasing evidences indicate that synovial fibroblasts (SFs) have an important role in the loss of cartilage tissue integrity (Zhao *et al.*, 2016). In healthy joint, SFs play a homeostatic role, since they are implicated in the maintenance of joint health, controlling the composition of the synovial tissue, extracellular matrix and synovial fluid (Bhattaram and Chandrasekharan, 2017). In arthritic joint, SFs transform from joint-protecting cells into joint-destroying cells, RA-SFs proliferate rapidly, and produce growth factors and pro-angiogenic factors that increase the local vascular network. In addition, they are highly migratory. They attach to the articular cartilage, produce cartilage matrix-degrading enzymes, and once cartilage is eroded, they invade the underlying bone and activate osteoclast-mediated bone resorption. Therefore, SFs constitute one of the major cell populations for the investigation of the pathogenesis and treatment of RA. In fact, controlling oxidative stress and the inflammatory

responses of SFs is a promising strategy for the treatment of RA (Bottini and Firestein, 2013; Lefevre *et al.*, 2014).

SFs produce large amounts of pro-inflammatory mediators, such as interleukin (IL)-1β, IL-6, IL-8, IL-33, tumor necrosis factor (TNF)-α and matrix metalloproteinases (MMPs), which degrade cartilage thereby contributing to joint destruction (Palmer *et al.*, 2009; Cooles and Isaacs, 2011; Kloesch *et al.*, 2013). Among MMPs, MMP-1 and MMP-3 have been reported to be the major enzymes produced by fibroblasts and macrophage-like cells in the synovium, and that the levels of both MMP-1 and MMP-3 are significantly higher in synovial fluids from patients with RA (Yoshihara *et al.*, 2000).

It's well known that pro-inflammatory cytokines such as IL-1β and IL-6 stimulate production of MMPs through the activation of cellular signaling pathways involving mitogen-activated protein kinases (MAPKs) which comprises extracellular signal-regulated kinases (ERK1/2 or p42/p44), c-Jun NH_2-terminal kinases (JNK) 1/2/3. MAPKs phosphorylate selected intracellular proteins, including transcription factors, which subsequently regulate gene expression by transcriptional and posttranscriptional mechanisms (Chen *et al.*, 2016).

In particular, nuclear factor kappa B (NF-κB) plays an important role in MMPs induction and also modulates an extensive range of genes that contribute to inflammation, such as microsomal prostaglandin E synthase-1 (mPGES-1), an efficient downstream enzyme co-localized and functionally coupled with the inducible enzymes cyclooxygenase-2 (COX-2). Both, COX-2 and mPGES-1, are up-regulated and responsible for the overproduction of prostaglandin E_2 (PGE_2) which may affect joint integrity through prostaglan E_2 receptor 4 (EP4) receptor activation (McCoy, Wicks and Audoly, 2002; Xiao *et al.*, 2016).

In addition, in the pathogenesis of RA, there is a close correlation between oxidative stress and progression of inflammation and bone destruction of inflamed joints of RA patients (Jikimoto *et al.*, 2002). Nuclear factor-erythroid 2-related factor2 (Nrf2) is a key transcription factor orchestrator of the induction of several antioxidant enzymes such as heme oxygenase-1 (HO-1). It has been reported that HO-1 deficiency in mice results in a chronic inflammatory state (Rosillo *et al.*, 2014). Also deletion of the Nrf2 gene increases vulnerability to the pathological joint changes associated with adjuvant-induced arthritis (Maicas *et al.*, 2011).

Dietary rich in phenolic compounds, which are typically included in Mediterranean diet through extra virgin olive oil (EVOO), fruits and wine, have shown a wide range of bioactive properties including anti-oxidant, anti-cancer, antimicrobial, antiviral, anti-atherogenic, hypoglycemic, hepatic-, cardiac- and neuro-protective , anti-inflammatory and anti-arthritic effects (Cárdeno, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2013; Rosillo *et al.*, 2016).

Specifically, oleuropein (OL), a secoiridoid, is considered as the most prevalent phenolic component in olive leaves, seed, pulp and peel of unripe olives (up to 14% of the dry weight).

During fruit maturation, OL undergoes hydrolysis, yielding different products, including hydroxytyrosol (HTy) (2-(3,4-dihydroxyphenyl)ethanol) (Ghanbari *et al.*, 2012).

Previous studies have reported important anti-inflammatory properties of this secoiridoid. In fact, OL was able to suppress lipopolysaccharide (LPS)-stimulated RAW 264 by the suppression of inducible nitric oxide synthase (iNOS) and COX-2 (Ryu *et al.*, 2015).

As well, OL have been exerted gastrointestinal benefits preventing ethanol-induced gastric ulcers via elevation of antioxidants enzyme activities in rats (Alirezaei *et al.*, 2012) and decreasing the extent and severity on dextrane sulphate sodium (DSS)-induced acute colitis, reducing neutrophil infiltration, nitric oxide (NO) production, pro-inflammatory cytokines, iNOS, COX-2 and MMP-9 protein expression and NF-κB-p65 subunit translocation to the nucleus (Giner *et al.*, 2011). Moreover, OL has been shown significantly to exert a protective effect on cartilage slowing down the progression of spontaneous osteoarthritis lesions in guinea pigs (Horcajada *et al.*, 2015). Additionally, treatment with OL-aglycone, a hydrolysis product obtained from OL by the action of β-glucosidase on the parent glucoside was able to ameliorate the development of the clinical signs and improved histological status in the joint and paw from collagen-induced arthritis (CIA) mice decreasing the degree of oxidative and nitrosative damage (Impellizzeri *et al.*, 2011).

However, at the date, anti-arthritic effects of OL on SFs have not been yet elucidated. The SW982 human synovial sarcoma cell line, is an useful in vitro tool to study inflammation in arthritis, and is characterized by the expression of inflammatory cytokines, MMPs, and other inflammatory mediators in response to IL-1β (Yamazaki *et al.*, 2003)

Thus taking this background into account, the aim of the present study was to investigate the potential effects of OL, on IL-1β-induced production of inflammatory mediators and oxidative stress in human synovial sarcoma cell line (SW982). In order to gain a better insight into mechanisms of action, signaling pathways were also explored.

2. MATERIALS AND METHODS

2.1. Extraction and purification of OL

The extraction of OL (Figure 1) was performed from leaves samples collected in January 2014 from Picual cultivar (*Olea europaea* L.), cultivated in Seville (Spain). The leaves were dried at room temperature and the extraction of OL was made using aqueous EtOH 70% (v/v) as reported (Stamatopoulos, Katsoyannos and Chatzilazarou, 2014).

The evaporation of the solvent under vacuum gave a powder that was purified by column chromatography in CH_2Cl_2-MeOH (10:1 → 5:1) resulting in OL as a yellow solid. Spectroscopy data was in agreement with those reported literature (Procopio *et al.*, 2009). ^{1}H-NMR (300 MHz, CD_3OD) δ 7.52 (s, 1H, H-3), 6.99 (d, 1H, $J_{7',8'}$ = 8.0 Hz, H-7′), 6.67 (d, $J_{4',8'}$ = 2.0 Hz, H-4′), 6.55 (dd, 1H, H-8′), 6.09 (q, 1H, $J_{8,10}$ = 7.0 Hz, H-8), 5.92 (bs, 1H, H-1), 4.81 (d, 1H, $J_{1'',2''}$ = 7.6 Hz, H-1′′), 4.22, 4.11 (2dt,

1H each, $J_{1'a,1'b}$ = 10.7 Hz, $J_{1',2'}$ = 7.0 Hz, H-1'), 3.98 (dd, 1H, $J_{5,6b}$ = 9.0 Hz, $J_{5,6a}$ = 4.4 Hz, H-5), 3.89 (dd, 1H, $J_{6''a,6''b}$ = 11.5 Hz, $J_{6''a,5''}$ = 1.6 Hz, H-6''a), 3.72 (s, 3H, OMe), 3.70 (dd, 1H, $J_{6''b,5''}$ = 5.4 Hz, H-6''b), 2.78 (t, 2H, $J_{2',1'}$ = 7.0 Hz, H-2'), 2.72 (dd, 1H, $J_{6a,6b}$ = 14.1 Hz, H-6a), 2.45 (dd, 1H, H-6b), 1.67 (dd, 3H, $J_{10,1}$ = 1.4 Hz, H-10) ppm.

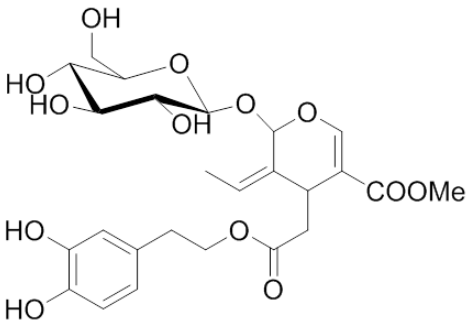


Figure 1. Chemical structure of OL.

2.2. Cell culture

The human synovial cell line SW982 was purchased from the American Tissue Culture Collection (Manassas, VA). SW982 were cultured in Dulbecco's® modified Eagle's medium (DMEM) with 10% FBS, 100 U/mL penicillin, and 100 µL/mL streptomycin at 37ºC in humified air with 5 % CO_2. The medium was changed every 48 hours (h), and cell passage was carried out every 3-4 days. For in vitro experiments, cells were incubated with DMEM for 24 h and then stimulated with IL-1β (Sigma Chemical Co®, St Louis, MO, USA) at 5 ng/mL at the indicated time. The cells were lysed after treatment and used for further experiments.

2.3. Cell viability assay

Cells were seeded in 96-well plates ($1x10^5$ cells $well^{-1}$) and they were incubated in presence or absence of OL for 24 h. At the end of the exposure time, the effect on cell growth/viability was analysed by the sulforhodamine B (SRB) assay (Skehan *et al.*, 1990). After incubation time, adherent cells cultures were fixed in situ by adding 50 µL of 50% (w/v) cold of trichloroacetic acid (AppliChem Panreac®, Darmstadt, Germany) and incubated for 60 min at 4ºC. The supernatant was discarded and plates were washed four times with deionized water and dried. 50 µL of SRB (Sigma-Aldrich®, St Louis, MO, USA) solution (0.4% w/v) in 1 % acetic acid (Panreac®, Barcelona, Spain) was added to each well and incubated for 30 min in dark at room temperature. The supernatant was discarder and plates were washed five times with 1 % acetic acid. Then, plates were air dried and added 100 µL per well of 10 mmol l^{-1} Tris base pH 10.5 (Sigma-Aldrich®, St Louis, MO, USA) and the absorbance of each well was read on an enzyme-linked immunosorbent

assay reader at 492 nm (iMARK Microplate Reader, BioRad®, CA, USA). Finally, cell survival was measured as the percentage compared with that obtained in control cells (non-treated cells).

2.4. Determination of MMPs and pro-inflammatory cytokines by ELISA cytokines by ELISA

SW982 was plated in 24-well plates (10^5 cells per well) for 48 h before treatment. After washing with phosphate buffer solution (PBS) (pH 7.4), cells were incubated at 37ºC with or with-out OL and IL-1β (5 ng /mL) for 24 h in DMEM containing 10% (v/v) FBS in a 5 % CO_2 atmosphere. Culture supernatants were collected and stored at -80ºC. The levels of MMP-1 and MMP-3 were determined in culture supernatant from the above experiments using commercially available ELISA kits essentially according to the instructions of the manufacturer (Ray Bio® Human MMP-1 ELISA kit and Quantikine® Human cytokine MMP-3 immunoassay kit, R&D System). The cytokines (TNF-α and IL-6) concentrations in the medium were measured using an eBioscience ELISA Ready-Set Go® kit.

2.5. Immunoblotting detection

Cells ($1x10^6$ cells/mL) were untreated and treated with OL and stimulated with IL-1β (5 ng/mL) for 18 h. After incubation, cells were rinsed, scraped off and collected in ice-cold PBS containing a cocktail of protease and phosphatise inhibitors and processed as described by Aparicio-Soto et al., (Aparicio-Soto *et al.*, 2014) in order to isolate proteins. Protein concentration was measured for each sample using a protein assay reagent (BioRad®, CA, USA) according to the Bradford's method and using γ-globuline as a standard (Bradford, 1976). Aliquots of supernatant containing equal amount of protein (25 µg) were separated on 10 % acrylamide gel by sodium dodecyl sulphate-polyacriamide (SDS) gel electrophoresis. In the next step, the proteins were electrophoretically transferred into a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-COX_2 and rabbit anti-mPEGs1 (Cayman®, Ann Arbor, MI, USA) (1:1000 and 1:200, respectively), rabbit anti-IκB-α, rabbit anti-HO1, rabbit anti-Nrf2, rabbit anti-pERK and mouse anti-ERK, mouse anti-pJNK, rabbit anti-JNK, mouse anti-pp38 and rabbit anti-p38 (Cell Signaling®, Danvers, MA, USA) (1:1000) overnight at 4ºC. After rinsing, the membranes were incubated with a horseradish peroxidise-labelled (HRP) secondary antibody anti-rabbit (Cayman Chemical®, Ann Arbor, MI, USA) (1:1000) or anti-mouse (Dako®, Atlanta, GA, USA) (1:1000) containing blocking solution for 1 h at room temperature. To prove equal loading, the blots were analysed for β-actin expression using an anti-β-actin antibody (Sigma Aldrich®, MO, USA). Immunodetection was performed using enhanced chemiluminescence light-detecting kit (ThermoFisher Scientific®, Waltham, MA, USA). The immunosignals were captured using Amersham Imaging 600 (GE Healthcare Life Sciences, Barcelona, Spain) and densitometry data

were studied following normalization to the house-keeping loading control. The signals were analysed and quantified by an Image Processing and Analysis in Java (Image J, Softonic®) and expressed in relation to the IL-1β stimulated cells.

2.6. Statistical evaluation

All values in the figures and text are expressed as arithmetic means ± standard error (S.E.M.). Experiments were carried out in triplicated. Data were evaluated with Graph Pad Prism® Version 5.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) followed by Newman-Keuls' test. *P* values of <0.05 were considered statistically significant. In the experiments involving densitometry, the figures shown are representative of at least four different experiments performed on different days.

3. RESULTS

3.1. Effects of OL on cell viability

In order to evaluate the effects of OL, on the viability of human synovial fibroblasts we performed the SRB assay. The SRB assay is an efficient method for the toxicity screening of compounds to adherent cells, based on the measurement of cellular protein content. After 24 h, our data demonstrated that viability of SW982 treated with OL was not significantly reduced at concentrations of 6.25 to 100 µM showing a cell viability >90% (Figure 2).

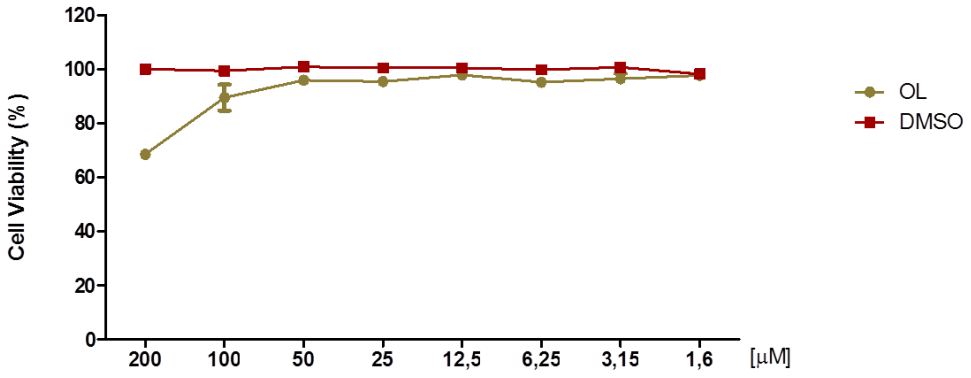


Figure 2. **Effect of OL on cell viability.** The concentrations used in this study did not affect viability of SW982 cells. Cells were treated with OL (1.6-200 µM) for 24 h. Cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells).

3.2. Effects of OL on pro-inflammatory cytokines production

Treatment of SW982 cells with IL-1β increased the levels of proinflammatory cytokines (IL-6 and TNF-α) levels when compared with cells without stimulus (Figure 3). On the contrary, PRE-TREATMENT with OL resulted in a significant inhibition of IL-6 and TNFα production induced by IL-1β.

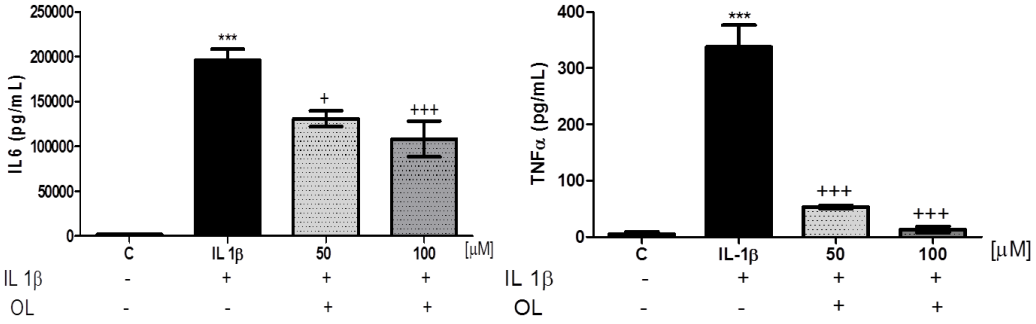


Figure 3. **Inhibitory effects of OL on the production of IL-6 and TNF-α by SW982 cells.** SW982 cells were cultured for 24 h in presence or absence of OL and stimulated with IL-1β (5 ng/mL). As control, cells were untreated in absence of IL-1β. Data are expressed as mean ± SEM (n=6). ***p<0.001; significantly different from Control cells (no stimulated); ^{+}p<0.05 and ^{+++}p<0.001; significantly different from IL-1β-stimulated control cells

3.3. Effects of OL on MMPs production

As shown in figure 4, SW982 cells exhibited an inflammatory response following IL-1β stimulation, which is evidenced by the elevated secretion of MPP-1 and MMP-3 in these cells. By contrast, PRE-TREATMENT with OL at concentrations of 50 and 100 μM for 24 h, significantly reduced both MMP-1 and MMP-3 up-regulation in IL-1β-treated SW982 cells.

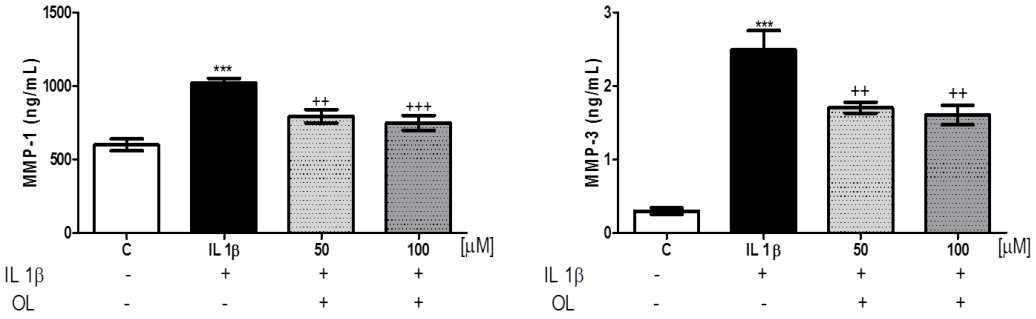


Figure 4. Inhibitory effect of OL on the production of MMP-1 and MMP-3 by SW982 cells. SW982 cells were cultured for 24 h in presence or absence of OL and stimulated with IL-1β (5 ng/mL). As control, cells were untreated in absence of IL-1β. Data are expressed as mean ± SEM (n=6). ***p<0.001; significantly different from Control cells (no stimulated); ^{++}p<0.01 and ^{+++}p<0.001; significantly different from IL-1β-stimulated control cells.

3.4. Effects of OL on mPEGs-1 and COX-2 overexpression

It is well known that both, COX-2 and mPGES-1, are up-regulated and responsible for the overproduction of PGE_2 which may affect joint integrity through EP_4 receptor activation (McCoy, Wicks and Audoly, 2002; Xiao *et al.*, 2016). Thus, we investigated the possible effects of OL on mPEGs-1 and COX-2 inflammation-related enzymes expression. COX-2 and mPEGs-1 protein expression was remarkably increased after 24h-IL-1β stimulation whereas a significant down-regulation on both COX-2 (Figure 5A) and mPEGs-1 expression was seen in SW982 cells treated with OL at 50 and 100 μM (Figure 5B).

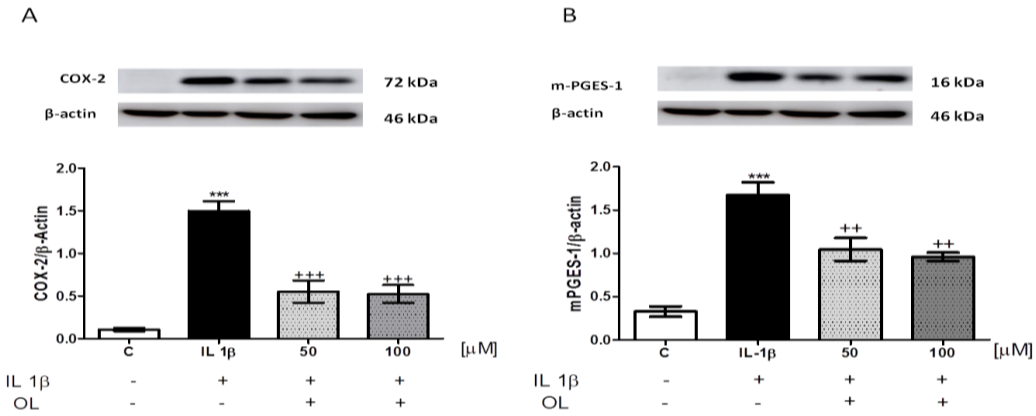


Figure 5. OL inhibit COX-2 (A) and mPEGs-1 (B) protein expression in IL-1β-stimulated SW982 cells. Cells were untreated or treated with OL (50 and 100 μM) for 24 h in presence of IL-1β (5 ng/mL). Control cells were incubated in absence of IL-1β. The plots represent band intensity. B-actin served as an equal loading control for normalization. Data shown are means ± SEM (n=5). ***p<0.001; significantly different from Control cells (no stimulated); ^{++}p<0.01 and ^{+++}p<0.001; significantly different from IL-1β-stimulated control cells.

3.5. OL prevented IL-1β-induced MAPKs activation on SW982 cells

MAPK signaling pathway has been shown to be involved in the expression of inflammatory mediators (Rosillo *et al.*, 2015). To determine whether the effects of OL were attributable to the inhibition of MAPK activation, we evaluated whether both concentrations (50 and 100 μM) were able to modulate JNK, p38 and extracellular signal-regulated kinases (ERK) activation after 30 min of IL-1β (5 ng/mL) stimulation by Western blotting. As shown in Fig. 6, treatment with IL-1β significantly enhanced phosphorylation of p38, JNK and ERK MAPKs in comparison to unstimulated cells. On the contrary pre-treatment with OL (50 and 100 μM) significantly blocked IL-1β-induced phosphorylation (Figure 6).

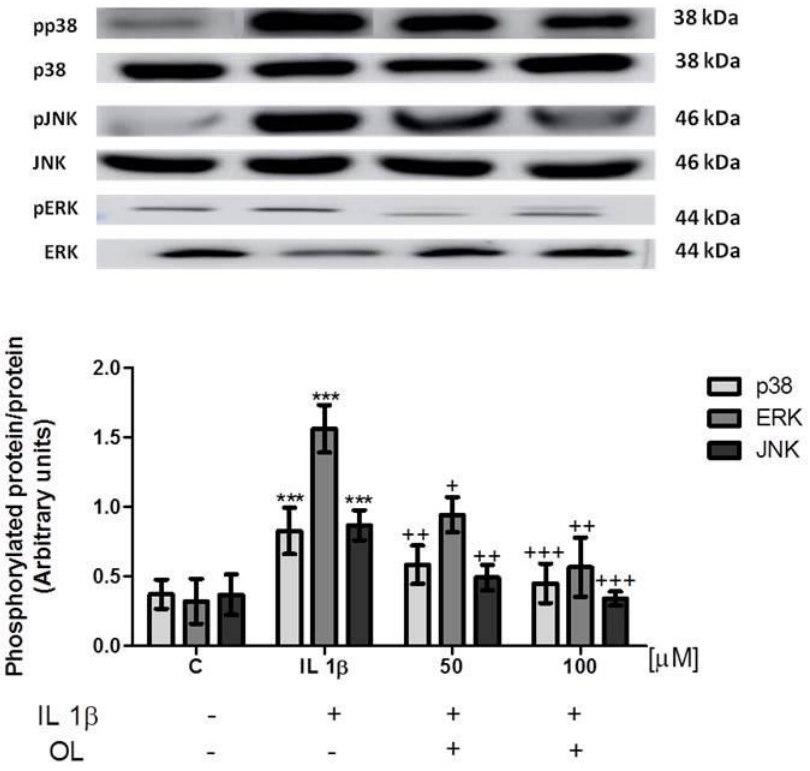


Figure 6. Effects of OL on MAPK signaling pathway in IL-1β-stimulated SW982 cells. Cells were untreated or treated with OL (50 and 100 μM) for 24 h in presence of IL-1β (5 ng/mL) for 30 min. Control cells were incubated in absence of IL-1β. Densitometry was performed following normalization to the control housekeeping genes (JNK, p38 and ERK (1/2). Data shown are means ± SEM (n=5). ***p<0.001; significantly different from Control cells (no stimulated); +p<0.05 ;++p<0.01 and +++p<0.001; significantly different from IL-1β-stimulated control cells.

3.6. Effects OL on IL-1β induced IκB-α degradation on SW982 cells

NF-κB is a pleitropic mediator in the control of several inducible and tissue-specific genes and is one of the key regulators of the cellular responses to oxidative stress in mammalian cells (Helenius *et al.*, 2001). NF-κB activation is initiated by the degradation of IκB-α. After IκB-α is degraded, NF-κB in the NF-κB-IκB-α complex, in free to be translocated into the nucleus, where it can induce the expression of pro-inflammatory genes contributing to the damage. To determine whether pro-inflammatory proteins down-regulation is regulated by the IκB-α pathway, we measured the expression levels of IκB-α in SW982 cells pre-treated with OL for 24 h. As shown in Figure 7, IκB-α was significantly reduced in SW982 cells stimulated with IL-1β whereas PRE-TREATMENT with OL at concentration 50 and 100 μM significantly prevented IKB-α degradation in IL-1β stimulated SW982 cells (Figure 7).

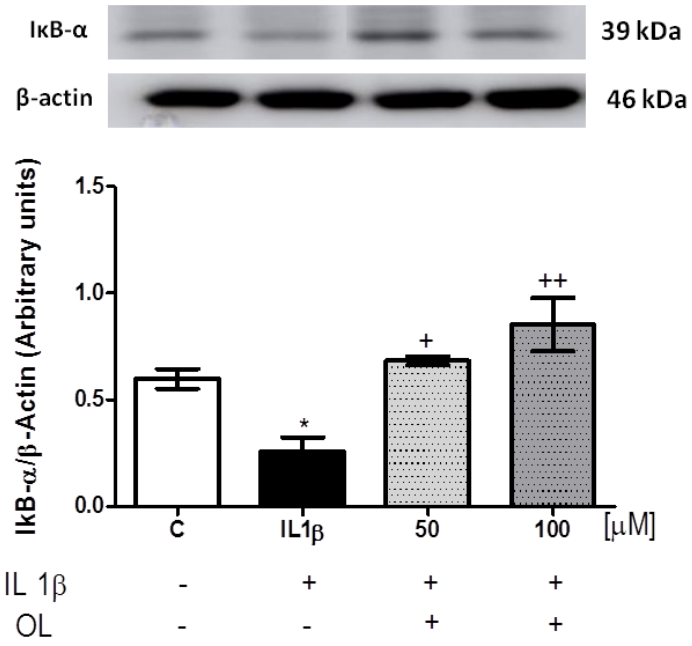


Figure 7. OL pre-treatments prevent IκB-α degradation in IL-1β-stimulated SW982 cells. Cells were untreated or treated with OL (50 and 100 μM) for 24 h and stimulated with IL-1β (5 ng/mL) for 30 min. Control cells were incubated in absence of IL-1β. Densitometry was performed following normalization to the control

(β-actin housekeeping gene). Data shown are means ± SEM (n=5). *p<0.05; significantly different from Control cells (no stimulated); ^{+}p<0.05 and ^{++}p<0.01; significantly different from IL-1β-stimulated control cells.

3.7. Effects OL on HO-1 and Nrf2 protein expression

The expression of the proteins HO-1 and Nrf2 was also evaluated in SW982 cells pre-treated with OL for 24 h. Our data show that HO-1 was significantly down regulated in SW982 cells stimulated with IL-1β in comparison to unstimulated cells. However, pre-treatment with OL at concentration 50 and 100 μM significantly up-regulated HO-1 protein expression. Nrf2 activation has been reported to play an important role in HO-1 expression. According to our results, Nrf2 expression was reduced in IL-1β-stimulated cells, however a significant increase in Nrf2 protein levels was observed in SW982 pre-treated cells with OL at concentration 50 and 100 μM (Figure 8).

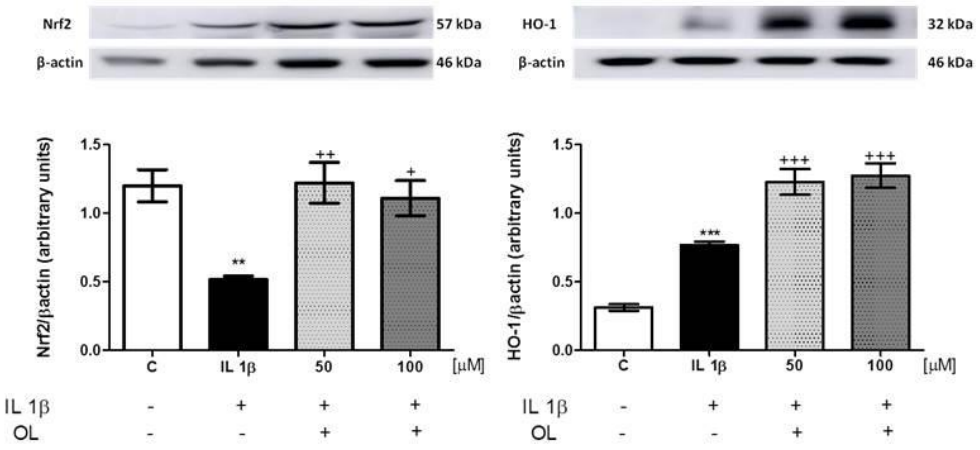


Figure 8. OL pre-treatment up regulated HO1 and Nrf2 protein expression in IL-1β-stimulated SW982 cells. Cells were untreated or treated with OL (50 and 100 μM) for 24 h and stimulated with IL-1β (5 ng/mL) for 30 min. Control cells were incubated in absence of IL-1β. Densitometry was performed following normalization to the control (β-actin housekeeping gene). Data shown are means ± SE (n=5). **p<0.01 and ***p<0.001; significantly different from Control cells (no stimulated); ^{+}p<0.05, ^{++}p<0.01and ^{+++}p<0.001; significantly different from IL-1β-stimulated control cells.

4. DISCUSSION

RA pathology is characterized by chronic synovitis in poly-articular joints. In RA joints, there are hyperplasia and hypertrophy of the synovial lining cells, specifically, SFs. In fact, SFs act as a major cell population in the invasive pannus to participate in the chronic inflammatory

responses (Firestein, 1996). Importantly, our findings have shown, for the first time, that OL, the most prevalent phenolic component in olive leaves, seed, pulp and peel of unripe olives was able to inhibit the activation of SW982 human synovial cells induced by IL-1β, preventing the inflammatory response.

SFs contribute significantly to matrix degradation in RA through the production of inflammatory mediators such as cytokines mainly TNF-α and IL-6 sustaining regulatory feedback loops that induce the production of , enzymes, such as MMPs through the activation of cellular signaling pathways involving MAPKs (McInnes and Schett, 2007; Su *et al.*, 2016).

TNF-α is reportedly involved in early joint swelling, chronic joint inflammation, and the concomitant erosive changes in cartilage and bone (Sommerfelt *et al.*, 2013). On the other hand, IL-6 is a key cytokine inducing a decrease in type II collagen (CII) production, increase in MMPs synthesis, and changes in the subchondral bone layer (Chenoufi *et al.*, 2001). Previous findings proved that IL-6 is responsible for the increase of serum γ-globulin and the emergence of rheumatoid factors (Visser, Smith and Louw, 2010). Besides, high levels of IL-6 have been detected in both sera and synovial fluids from the affected joints of RA patients. In this study, we have demonstrated that treatment with OL caused a significant inhibition of IL-1β-induced TNF-α and IL-6 release in human synovial SW982 cells. These suggest that OL may have the potential to regulate pro - inflammatory cytokines in synoviocytes.

Among MMPs, MMP-1 (colagenase 1) and MMP-3 (stromelysin) have been reported to be the major enzymes produced by fibroblasts and macrophage-like cells in the synovium, and their levels are significantly higher in synovial fluids from patients with RA (Rosillo *et al.*, 2016). MMP-1 is one of the critical neutral proteinases which degrade native fibrillar collagens in the extracellular matrix (Yamanishi and Firestein, 2001). On the other hand, MMP-3 is responsible to degrade proteoglycan, type IV and type IX collagens and denatured type I and type II collagens, fibronectin, gelatin and laminin and is believed to be especially important because, besides of its direct enzyme activity, its activation is necessary for full activation of collagenases (Chen and Mattey, 2012). Our data showed that the production of both MMP-1 and MMP-3 was significantly induced by IL-1β stimulation in human SW982 cells, whereas pre-treatment with OL induced a significant down-regulation of both MMPs levels in IL-1β-stimulated SW982 cells.

COX-2, the inducible isoform of COX and mPGES-1, enzymes responsible for the overproduction of PGE_2 in inflammation, are up-regulated contributing to the progression of RA through EP_4 receptor activation (McCoy, Wicks and Audoly, 2002). We have shown that OL decreased expression of both COX-2 and mPGES-1 in IL-1β-stimulated SW982 cells. Our data are in agreement with those from Impellizzeri et al., (Impellizzeri *et al.*, 2011), who show that OL-aglycone decreased mPEGs-1 and COX-2 expression by immunohistochemical staining and also reduced the levels of the metabolite of COX-2, PGE_2, in the serum of OL-aglycone in mice subjected

to collagen-induced arthritis . Therefore, regulation of these pro-inflammatory biomarkers by OL could represent a potential molecular target susceptible to OL modulation, which has not been demonstrated previously.

Increasing evidence demonstrated that the activation of multiple stress signaling pathways (e.g., MAPKs and NF-κB), which act as pivotal regulators of proliferation, differentiation, and cellular survival in RA, significantly contributes to the pathogenic mechanisms of joint destruction and inflammation in RA (Liu *et al.*, 2016).

In fact, NF-κB has been implicated in cytokine release, activation, autoantibody production, cellular proliferation, inhibition of apoptosis, and numerous other processes associated with RA. Also plays a pivotal role in the development and activation of Th-1 responses and is responsible in addition to MAPKs for COX-2 up-regulation (Makarov, 2001). NF-κB, as a dimeric transcription factor exists in the cytoplasm as an inactive complex with the inhibitory protein IkB-α. An inflammatory signal such as IL-1β induced the activation of IκB-α kinase complex to phosphorylate members of the IkB family. Phosphorylated IkB becomes ubiquitinated and is then targeted for degradation by the proteasome. The NF-κB dimmers can then translocate to the nucleus and activate the transcription and repression of genes (Hayden and Ghosh, 2008; Scheinman, 2013).

MAPK family members, including $ERK_{1/2}$, JNK and p38 MAPKs orchestrate the recruitment of gene transcription, protein biosynthesis, cell cycle control, apoptosis and differentiation and allow cells to respond to oxidative stress and inflammation stimuli (Munoz and Ammit, 2010). MAPKs have previously been shown to play a critical role in the regulation of the synthesis of chemokines, cytokines, adhesion molecules and PGs involved in RA and are considered as the major tyrosine phosphorylation proteins in human synovial stimulated with IL-1β (Barchowsky, Frleta and Vincenti, 2000). Besides JNK MAPK modulates MMPs production by synovial fibroblasts and drives osteoclast differentiation in RA (Han *et al.*, 2001). In particular, p38 MAPK regulates MMP-3 induction in fibroblasts and osteoclast differentiation (Rosillo *et al.*, 2015). Accumulating studies have demonstrated that inhibitors of MAPKs and NF-κB alleviated synovial inflammation, bone destruction, and cartilage damage in animal models of arthritis, including adjuvant arthritis in rats and CIA in mice (Han *et al.*, 2001; Nishikawa *et al.*, 2003).

As expected, the exposure of IL-1β significantly increased the degradation of IκBα and phosphorylation of $ERK_{1/2}$, JNK and p38, indicating NF-κB and MAPKs activation in SW982 cells which was consistent with previous studies reported by Schett et al., and Inoue et al., (Schett *et al.*, 2000; Inoue *et al.*, 2001). However, pre-treatment with OL, at concentration of 50 and 100 μM strongly inhibited the IκBα degradation and prevented the activation of MAPKs. These results indicate that OL inhibit inflammation in IL-1β-treated SW982 cells by regulating the NF-κB and MAPKs pathways.

Nrf2 is a redox-sensitive transcription factor and binds to antioxidant response elements (ARE) located in the promoter regions of many detoxifying/antioxidant genes, including HO-1. In basal conditions, Nrf2 in sequestered in the cytoplasma by Kelch-likeECH-associated protein 1 (Keap1) and degraded by the ubiquitin-dependent 26S proteasome system. In inflammatory and immune conditions, the expression of this protein could be part of an adaptive mechanism to limit cytotoxicity via several mechanisms including scavenging of reactive oxygen or nitrogen species, regulation of cell proliferation and prevention of apoptosis (Rosillo *et al*., 2016). Furthermore, the deficiency of HO-1 in both mice and humans induces the characteristic phenotype of an increased inflammatory state, whereas the induction of HO-1 in animals leads to the protection of the progression of arthritis with decreased levels of matrix MMPs and the prevention of cartilage degradation suggesting the HO-1 plays the key role in RA therapeutic strategy (Su *et al*., 2016).

Our data showed that OL treatment strongly augmented Nrf2 and HO-1 expression conferring a role of HO-1 in the beneficial effects of OL in IL-1β-stimulated SW982 cells. Thus HO-1 could represent a potential molecular target susceptible to modulation with treatment with OL, which has not been demonstrated previously.

In conclusion, our study has demonstrated, for the first time, that OL prevented the inflammatory response and oxidative stress of IL-1β -induced SW982 human synovial cells. The mechanisms underlying these protective effects could be related via down-regulation of MAPKs and NF-κB and induction of Nrf2-linked HO-1 signaling pathways controlling the production of pro-inflammatory cytokines, MMP-1 and MMP-3 levels as well as mPGES-1 and COX-2 overexpression. Thus, OL might provide a basis for developing a new dietary strategy for the prevention and management of RA.

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CHAPTER III

OLEUROPEIN AND ITS NEW PERACETYLATED DERIVATIVE AMELIORATE JOINT INFLAMMATION AND DESTRUCTION IN A MURINE COLLAGEN-INDUCED ARTHRITIS MODEL VIA ACTIVATION OF THE NRF2/HO-1 ANTIOXIDANT PATHWAY AND SUPPRESSION OF MAPKS AND NF-KB ACTIVATION

Capítulo II

LA OLEUROPEÍNA Y SU NUEVO DERIVADO PERACETILADO REDUCEN LA INFLAMACIÓN Y DESTRUCCIÓN ARTICULAR EN UN MODELO MURINO DE ARTRITIS INDUCIDA POR COLÁGENO MEDIANTE LA ACTIVACIÓN DE LA VÍA ANTIOXIDANTE NRF2/HO-1 Y LA SUPRESION DE LA VÍA DE LAS MAPKS Y EL NF-KB

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RESUMEN

La oleuropeína (OL), un secoiridioide extraído a partir de la hoja del olivo, así como su derivado peracetilado (Per-OL), han mostrado multitud de efectos beneficiosos en modelos de inflamación aguda inducida con LPS en macrófagos peritoneales murinos, así como en un modelo experimental murino de lupus eritematoso sistémico (LES). El presente estudio tuvo como objetivo evaluar los beneficios de una suplementación dietética con OL y con Per-OL en un modelo de artritis reumatoide (AR) inducida por colágeno tipo II (CIA).

Los ratones machos DBA-1/J recién destetados fueron alimentados con dietas enriquecidas en OL al 0.05%, o en Per-OL al 0.05% y 0.025%. Tras seis semanas de pre-tratamiento con las dietas experimentales, se indujo la AR (día 0). Pasados 21 días, se realizó una segunda inmunización en estos ratones. El día 42 tras la inducción de la AR, se sacrificaron, se recolectó su sangre y se diseccionaron las patas delanteras y traseras para su posterior determinación histológica y bioquímica.

Las dietas experimentales con OL y Per-OL previnieron el desarrollo del daño articular en la AR, así como redujeron los niveles séricos de la proteína oligomérica de la matriz del cartílago (COMP) y metaloproteinasa 3 (MMP3) y los niveles de citocinas pro-inflamatorias (TNF-α, IL-1β, IL-6, IL-17, IFN-γ) en homogenado de las patas. La activación de las vías de señalización de las proteínas cinasas activadas por mitógenos (MAP) y del factor nuclear kappa-B (NF-κB) fueron reducidas significativamente, mientras que la expresión proteica del factor nuclear eritroide-2 (Nrf2) y de la hemo-oxigenasa-1 (HO-1) se incrementó considerablemente.

Estos resultados ponen de manifiesto, por primera vez, el efecto anti-inflamatorio de una dieta suplementada en OL y en Per-OL en un modelo experimental murino de CIA, el cual se acompañó de una reducción significativa de los marcadores proinflamatorios estudiados (COMP, MMP-3, TNF-α, IL-1β, IL-6, IL-17 e IFN-γ). Los mecanismos implicados podrían estar relacionados con una activación de la vía antioxidante Nrf2/HO-1 y con la inhibición de la activación de las vías de señalización MAP cinasas y NF-κB. Como conclusión, la suplementación dietética con OL y en

Per-OL, podría servir de base para el desarrollo de nuevas estrategias nutricionales para la prevención de la AR.

Chapter I

OLEUROPEIN AND ITS NEW PERACETYLATED DERIVATIVE AMELIORATE JOINT INFLAMMATION AND DESTRUCTION IN A MURINE COLLAGEN-INDUCED ARTHRITIS MODEL VIA ACTIVATION OF THE NRF2/HO-1 ANTIOXIDANT PATHWAY SUPPRESSION OF MAPKS AND NF-κB ACTIVATION

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ABSTRACT

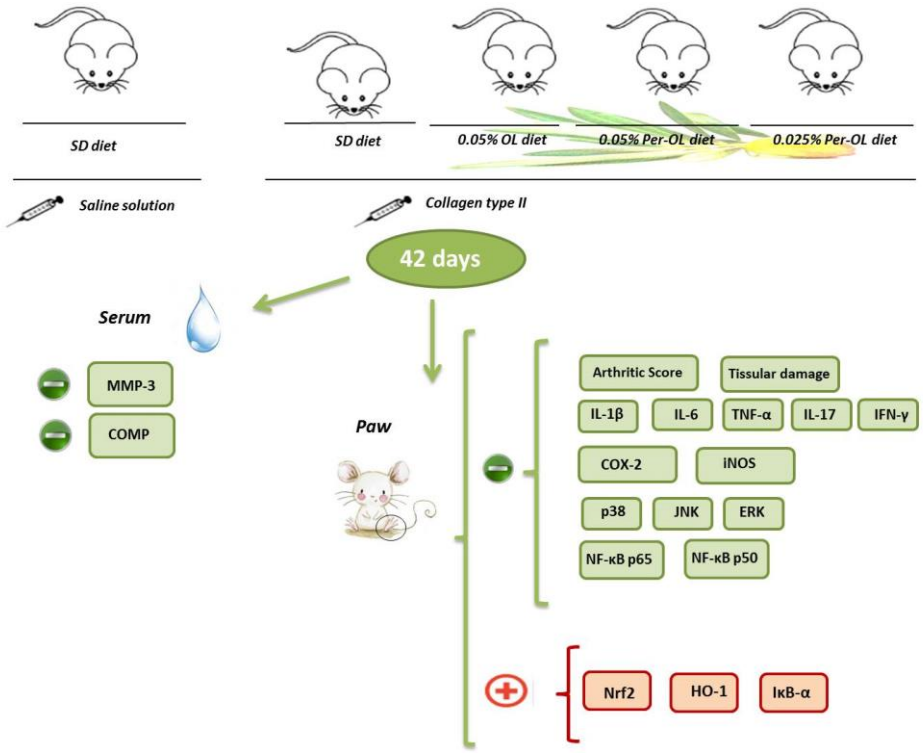
Oleuropein (OL), an olive tree secoiridoid, and its peracetylated derivate (Per-OL) have exhibited several beneficial effects reducing inflammatory responses on LPS-stimulated macrophages and in a murine model of systemic lupus erythematosus (SLE). The present study was designed to evaluate the effects of both dietary OL and Per-OL supplementations on collagen-induced arthritis (CIA) murine model.

Three-weeks-old DBA-1/J male mice were fed from weaning with 0.05% (w/w) OL, 0.05% or 0.025% Per-OL. After six weeks of pre-treatment, arthritis was induced by bovine collagen type II by tail base injection (day 0) and mice received a booster injection on day 21. Mice were sacrificed 42 days after first immunization. Then, blood was recollected and paws were histological and biochemically processed.

OL and Per-OL diets significantly prevented histological damage and arthritic score development. In addition, serum collagen oligomeric matrix protein (COMP) and metalloproteinase 3 (MMP-3) as well as proinflammatory cytokines levels in paw homogenates (including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-17 and interferon (IFN)-γ) were significantly ameliorated in those animals fed with dietary secoiridoids. Mitogen-activated protein kinases (MAPKs) and nuclear transcription factor kappa-B (NF-κB) activations were drastically down-regulated whereas nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) protein expressions were significantly up-regulated in those mice fed with OL and Per-OL diets.

In conclusion, in this study we have demonstrated that dietary OL and Per-OL treatments exert beneficial effects on arthritis CIA in mice improving the oxidative events via Nrf2/HO-1 activation and returning proinflammatory markers to basal levels through blockage of MAPKs and NF-κB signaling pathways reactivation. OL and Per-OL supplements might provide a basis for developing a new dietary strategy for the prevention of rheumatoid arthritis.

Keywords: CIA, Nrf2, OL, Peracetylated-oleuropein, Rheumatoid arthritis.



1. INTRODUCTION

Rheumatoid arthritis (RA) is the most prevalent chronic, painful and debilitating autoimmune disease in the world. It characterized by chronic synovitis leading to the progressive destruction of joints accompanied by systemic inflammation and the production of autoantibodies. In addition, RA includes extraarticular manifestations, such as rheumatoid protuberances, pulmonary involvement or vasculitis and systemic comorbidities (Smolen, Aletaha and McInnes, 2016) affecting to the patient's capacity to perform physical activities compared with the healthy population (Malm *et al.*, 2017).

Although the pathogenesis of RA is multifactorial, substantial insights into RA pathophysiology suggest that various inflammatory pathways lead to an altered immune system and contribute to the joint damage during the disease. Particularly, immune cells mainly B-cells, T-

cells and macrophages and even fibroblast-like synoviocytes play pivotal roles in RA pathogenesis (Moon et al., 2014). Autoreactive B-cells produce rheumatoid factors (RFs) and/or anti-citrullinated protein antibodies (ACPAs) and also mediate T-cell activation through expression of co-stimulatory molecules. According to the T cells, they activate macrophages and fibroblasts and transform them into tissue-destructive cells producing a variety of proinflammatory chemokines and cytokines that perpetuate joint inflammation and aggravate tissue destruction at the chronic disease stage (Yap *et al.*, 2018). Nevertheless, several environmental and other factors such as multiple genetic, geography, socioeconomic status, diet/nutrients, alcohol, smoking, and host microbiome also contribute to the risk of developing RA (Deane *et al.*, 2017; Paolino *et al.*, 2019).

Understanding the mechanisms involved in RA pathogenesis has led in large part to the clinical development of therapeutic drugs targeting specific cells and molecules. In fact, the most successful therapeutic strategy in this disease include disease-modifying-anti-rheumatic drugs (DMARDs), such as methotrexate and cytokine-directed therapies including inhibitors of tumor necrosis factor (TNF)-α and interleukin (IL)-6 (Burmester, Feist and Dörner, 2014; Law and Taylor, 2019). However, these drugs are effective in a fraction of patients only and have other limitations including high cost, the requirement of parenteral administration and important side effects. Thus, the search of novel treatments with a more benign safety profile is needed. In this sense, within the therapeutic approach of RA, nutritional therapy could be relevant because in the last few years, it has been highlighted that determinate foods consumption has influence on health outcomes (Rosillo, Alarcón-de-la-Lastra and Sánchez-Hidalgo, 2016).

The antioxidant and anti-inflammatory properties of biophenolic fraction from olive leaves and fruits from *Olea Europaea* L., have been suggested as a potential application in several *in vitro* and *in vivo* models of inflammation studies (Rosillo, Alarcón-de-la-Lastra and Sánchez-Hidalgo, 2016; Larussa, Imeneo and Luzza, 2019). It is well-known that extra virgin olive oil (EVOO) contains significant amounts of monounsaturated fatty acid (MUFA) (oleic acid) and other minor but highly bioactive components including triterpenic alcohols, sterols, hydrocarbons (squalene), vitamins (α- and γ-tocopherols), β-carotene, phytosterols and numerous functional phenolic compounds (Cárdeno, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2013). Similarly, olive leaves which represent a waste product from olive harvest and pruning of olive trees, contain even higher amount of bioactive polyphenols than olive oil. Thus, it could be an useful font of natural compounds to study in different inflammatory conditions (Da Silva et al., 2019). Particularly, oleuropein (OL) the most abundant and characteristic polyphenolic compound in unprocessed olive leaves (Herrero et al., 2011) is responsible for the major anti-inflammatory effects of olive leaf extract (Qabaha *et al.*, 2018). It has been well-established that OL possess antioxidant and anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages (S. J. Ryu *et al.*, 2015) inhibiting biosynthesis of proinflammatory cytokines, and regulating inflammatory

response by inducible nitric oxide synthase (iNOS) and cyclooxigenase 2 (COX-2) suppression. Besides, it has been described that OL down-regulated TNF-α secretion in freshly isolated human polymorphonuclear cells (PBMCs) culture (Qabaha *et al.*, 2018) and also the IL-1β-induced inflammation and oxidative stress in human synovial fibroblast cell line SW982 (Maria Luisa Castejón *et al.*, 2017).

On the other hand, the synthesis of OL derivatives with better pharmacokinetic/pharmacodynamic profiles than OL could be a strategy in the management of the inflammatory process. In fact, there are some studies which have shown the advantages of acetylated derivatives of natural compounds, since their lipophilic nature allows them to cross the cytoplasmic cell membranes and their uptake by cells, offering a possible protection of membrane components using a LPS-induced acute inflammation model in murine isolated macrophages (Maria Luisa Castejon et al., 2019). More recently, it has been reported that both OL and its new peracetylated derivative, peracetylated-OL (Per-OL) attenuates kidney injury in pristane-induced systemic lupus erythematosus (SLE) model inhibiting proinflammatory biomarkers overexpression via activation of heme oxigenase- 1 (HO-1)/ Nuclear factor E2-related factor 2 (Nrf2) antioxidant pathway and suppression of Janus kinase-signal transducer and activator of transcription (JAK/STAT), nuclear transcription factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) activation (M.L. Castejon et al., 2019).

These data suggest that a wide range of chronic inflammatory diseases such as RA could benefit from these biologically active compounds. Thus, the present study was designed to evaluate, for the first time, the potential protective effects of both dietary OL and Per-OL treatments in a murine collagen-induced arthritis (CIA) model which reproduces the RA pathology and is very useful for drug screening in RA. In addition to macroscopic and histological analyses, we determined the effects of these experimental diets on the production of inflammatory mediators and explored the signaling pathways involved.

2. MATERIALS AND METHODS

2.1. Reagents

OL was extracted from olive leaves according to reported literature (Stamatopoulos, Chatzilazarou and Katsoyannos, 2013), following the purification by column chromatography (CH2Cl2-MeOH 10:1→5:1) to give the product as a yellow solid. The synthesis of Per-OL was carried out from OL. It was solved and stirred in a mixture of 1:1 (v/v) pyridine/acetic anhydride at 8ºC for 10 min. Then, the reaction was kept at room temperature overnight. After hydrolyzing the acetic anhydride, the product was concentrated to dryness obtaining a residue that was purified by column chromatography (EtOAc-C. Hexane 1:1) to give the product (quant.) as a brown solid. Spectroscopy data was identical to reported literature (Procopio AS M.; Costa, N.; Nardi, M., 2008).

2.2. Animals

To evaluate the beneficial effects of OL and Per-OL on CIA model, a total of 60 three-weeks-old male DBA-1/J mice (Janvier®, Le Genest St Isle, France) were maintained in our Animal Laboratory Center under standard conditions (temperature, 24-25ºC; humidity, 70-75%; lighting regimen, 12L/12D). The animals were fed with the corresponded experimental pellets diets and water *ad libitum* during six weeks previous to the CIA induction and also along the all experimental time. Particularly, mice were randomized in five experimental groups (12 animals per group): (i) Naïve group (SD-Naïve); (ii) CIA Control group (SD-CIA), (iii) OL diet group enriched 0.05% (OL-CIA), (iv) Per-OL diet group enriched 0.05% (Per-OL 0.05-CIA) and (v) Per-OL diet group enriched 0.025% (Per-OL 0.025-CIA). Experiments followed a protocol approved by the Animal Ethics Committee of the University of Seville, and all experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive European Counsel 2010/630/EU).

2.3. Induction of CIA

RA was induced in 10-week-old male DBA-1/J mice. Bovine type II collagen (CII) 2 mg/mL in dilute acetic acid (MD Bioproducts®, Zürich, Switzerland) was emulsified in equal volumes of Freund's complete adjuvant (2 mg/mL *Mycobacterium tuberculosis*, strain H37Ra; Difco®, Detroit, Michigan, USA). On day 0, DBA-1/J mice were immunized at the base of the tail with 100 mg of bovine CII. On day 21, mice received an intraperitoneal booster injection of 100 mg of CII dissolved in phosphate-buffered saline (PBS). Joint inflammation was scored visually in each paw, using a scale of 0-2 where 0 = uninflamed, 1 = mild, 1.5 = marked and 2 = severe. Scoring was performed by two independent observers without knowledge of the experimental groups. Animals were euthanized on day 42 by intraperitoneal injection (i.p.) of a mix of ketamine and diazepam (1:2).

2.4. Histological and immunohistochemical analyses

After mice were sacrificed, knee joints were removed and fixed in 4% formaldehyde. After decalcification in 10% ethylenediaminetetraacetic acid (EDTA) specimens were processed for paraffin embedding. Tissue sections (7 µm) were stained with hematoxylin and eosin (H&E) to perform histological analysis. For immunohistochemistry assay of COX-2, the procedure was according to Rosillo et al., 2014 (Rosillo *et al.*, 2014a) using Vectastain Kit®; Vector Lab., Burlingame, CA, USA. Negative controls sections were incubated in the same way but in the absence of primary antibody. Positive cells staining and total cells were observed by two independent observers.

2.5. Study of inflammatory markers

Enzyme-linked immunoassay (ELISA) kits were used to measure serum levels of cartilage oligomeric matrix protein (COMP) (MD Biosciences®, Zürich, Switzerland) and matrix metalloproteinase 3 (MMP-3) (R&D Systems®, Abingdon, UK). Final paws were amputed above the ankle and homogenized in 1 mL of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4. Supernatants were used for determination of IL-17 (Peprotech®, London, UK), TNF-α, IL1-β (BD OptEIA®, San Jose, CA), interferon (IFN)-γ and IL-6 (Diaclone®, Besacon Cedex, France). The results were measured at 450 nm using an ELISA microplate reader (BioTek®, Bad Friedrichshall, Germany). All measurements were carried out in duplicate.

2.6. Isolation of cytoplasmic and nuclear proteins and immunoblotting detection

Frozen final paws were homogenized in liquid N_2. Isolation of cytoplasmic and nuclear proteins was performed according to Rosillo et al., (Rosillo *et al.*, 2016). Protein concentration of the paw's homogenate was measured following Bradford's method (Bradford, 1976). Aliquots of supernatant that contain equal amount of protein (50µg) were used to determine iNOS, pp38, pJNK, pERK, NF-κBp65, NF-κBp50, inhibitory of NF-κB (IκB-α), Nrf2 (Cell Signaling®, MA, USA), mPGES-1 (Cayman Chemical®, Ann Arbor, MI, USA) and HO-1 (Enzo®, Madrid, Spain) proteins expression according by Western Blot (Maria Luisa Castejon *et al.*, 2019). The immunosignals were captured using Amersham Imager 600 (GE Healthcare®, Buckinghamshire, UK) and the signals were analyzed and quantified by an Image Processing and Analysis in Java (Image J®, Softonic).

2.7. Statistical evaluation

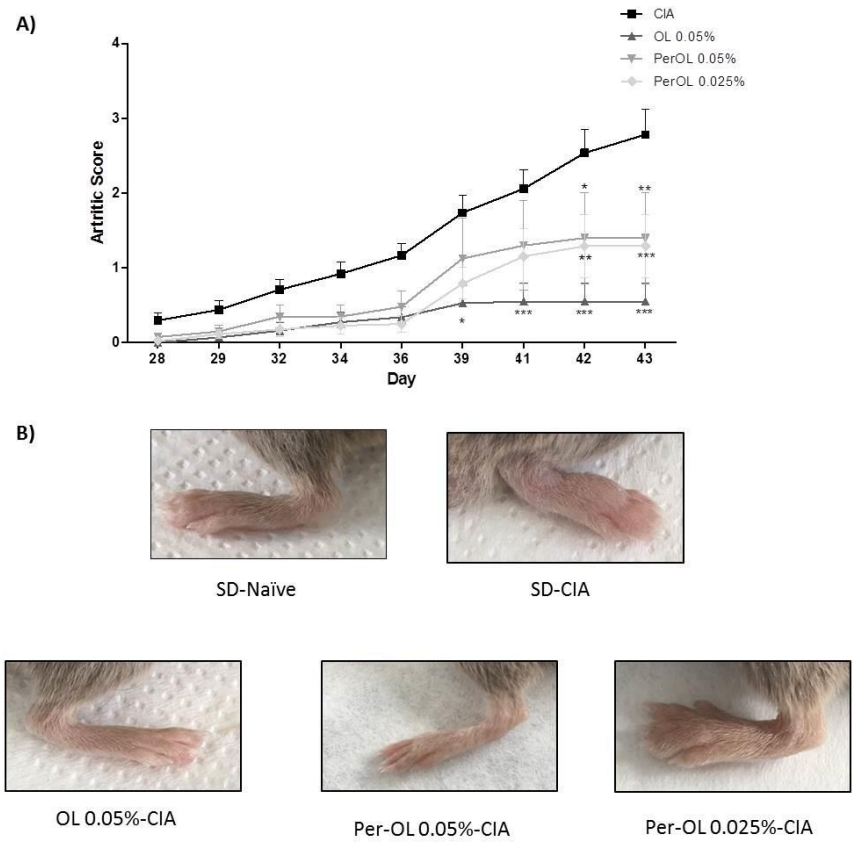
All values in the text and figures are expressed as arithmetic means ± standard error (S.E.M.). Data were evaluated with Graph Pad Prism® Version 6.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance using Tukey's multiple comparisons test as post hoc test. P values of < 0.05 were considered statistically significant. In the experiments involving densitometry, the figures shown are representative of at least three different experiments performed on different days.

3. RESULTS

3.1. Dietary OL and Per-OL treatments alleviated CIA-related symptoms and RA-induced infiltration of inflammatory cells

The development of RA was monitored until day 42. The time course of arthritic score (Fig. 1A) shows that CIA control mice fed with normal standard diet (SD-CIA) presented a progressive development of clinical symptoms. On the contrary, the severity of RA symptoms in those groups which were fed with OL 0.05 % and Per-OL 0.05 and 0.025 % experimental diets was lower than CIA control group from days 30 to 42. These results suggested that dietary OL and Per-OL could have a therapeutic effect on going inflammatory arthritis. Fig. 1B shows representative photographs of hind paws from the different experimental diets animals groups.

In addition, H&E staining revealed that histological features of the joint from sham animals were typical of normal structure with synovial membrane composed of synovial cells, collagen and a clear synovial space. On the contrary, joints from SD-CIA mice exhibited histological changes indicative of severe arthritis, characterized by an extensive inflammatory cells infiltration into articular tissues, exudation into the synovial space, hyperplasia and cartilage erosion. These histological features were less evident in those arthritic mice fed with OL and Per-OL experimental diets (Fig. 1C).



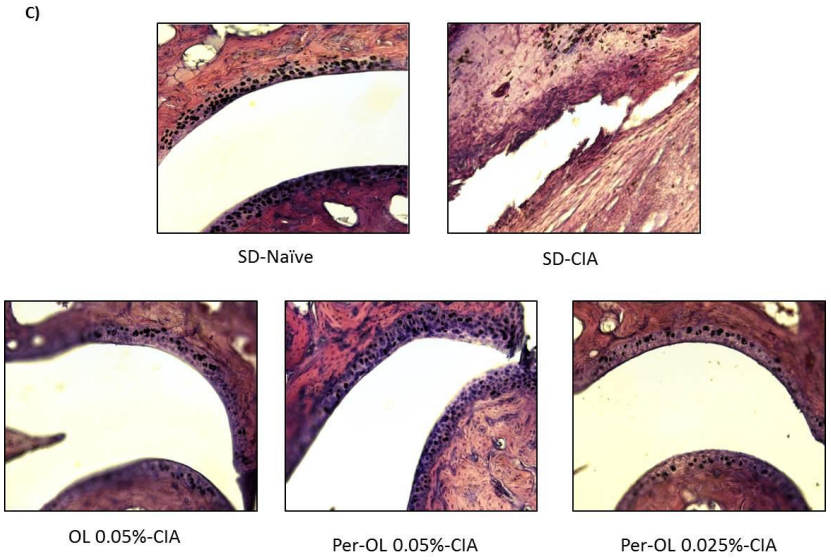


Figure 1. Time course of the arthritis macroscopic score **(A)**, representative pictures **(B)** of hind paws at the end of the experiment (day 42). Histological analysis of the sections of knee joints stained with H&E on day 42 **(C)**. Original magnification x20. SD-Naïve, non-arthritic mice feed with SD; SD-CIA, control arthritic group feed SD; CIA-OL 0.05%, arthritic group feed with enriched-diet with OL 0.05% w/w; CIA-Per-OL 0.05% and CIA-Per-OL 0.025 %, arthritic group feed with enriched-diet with Per-OL 0.05 or 0.025 % w/w, respectively. Pictures show less damage at the cartilage. The images are representative of at least five experiments. Data represent mean ± S.E.M. n= 12. *p<0.05; **p<0.01 and ***p<0.001 vs. SD-CIA control group.

3.2. Effects of dietary OL and Per-OL treatments on serum proinflammatory biomarkers levels in CIA model

MMP-3 is well-known as a predictor for joint destruction in RA. We described an increment of circulating MMP-3 levels in SD-CIA control group mice in comparison to SD-Naïve control group (**p<0.01 vs. SD-Naïve control group) whereas these levels were significantly reduced after OL and Per-OL dietary treatments at all doses assayed ($^{\#}p$<0.05; $^{\#\#}p$<0.01 vs. SD-CIA control group) (Fig. 2A). Similarly, serum levels of the cartilage degradation marker, COMP, were significantly increased in SD-CIA mice when compared to SD-Naïve control group (***p<0.001 vs. SD-Naïve control group). However, OL and Per-OL enriched diets significantly decreased serum COMP levels in CIA mice reaching similar levels to those observed in SD-Naïve control group ($^{\#\#\#}p$<0.001 vs. SD-CIA control group) (Fig. 2B).

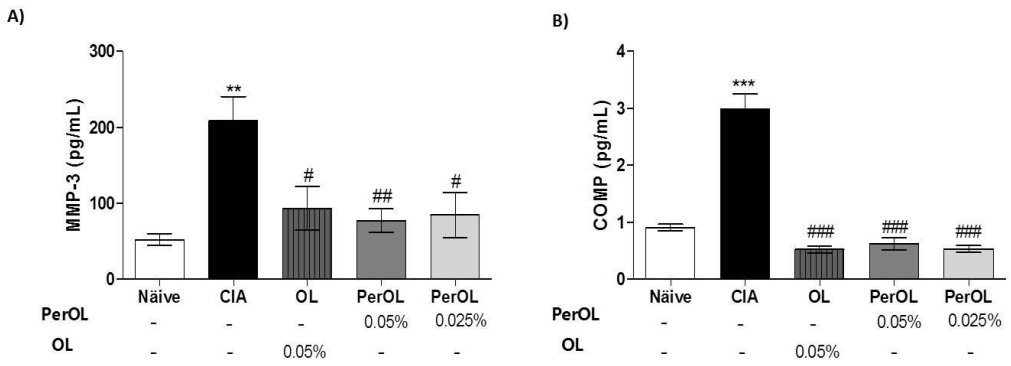


Figure 2. Measurement of serum levels of MMP-3 **(A)** and COMP **(B)**. These proinflammatory markers were measurement by ELISA commercial kits. Data represent mean ± S.E.M., n=12. $^{**}p<0.01$ and $^{***}p<0.001$ vs. SD-Naïve control group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. SD-CIA control group.

3.3. Dietary OL and Per-OL treatments suppressed the production of proinflammatory cytokines in CIA model

We studied if dietary OL and Per-OL treatments were capable to reduce the proinflammatory cytokines levels, which were involved in the pathogenesis of RA. We examined these proinflammatory cytokines levels in paws homogenates by ELISA. We could observe a higher increase on IL-1β, IL-6, IL-17, IFN-γ and TNF-α levels in paw homogenates from SD-CIA control group ($^{**}p<0.01$; $^{***}p<0.001$ vs. SD-Naïve) when compared with SD-Naïve mice. Nevertheless, these levels were reduced significantly on those mice were fed with0.05%) OL and 0.05% or 0.025% Per-OL experimental diets ($^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. SD-CIA control group) (Fig. 3).

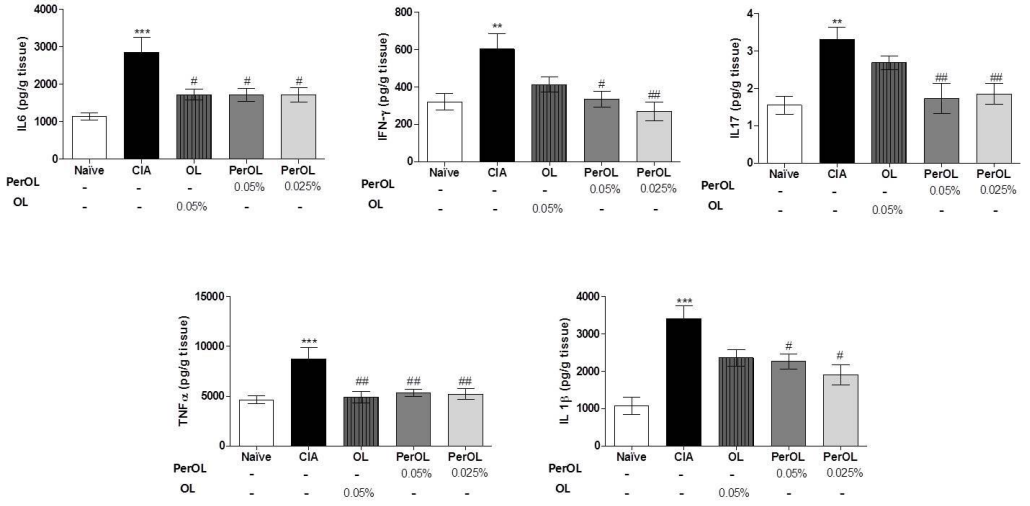


Figure 4. Measurement of proinflammatory cytokines levels (TNF-α, IL-1β, IL-6, IL-17 and IFN-γ) were determined by ELISA kits in hind paws homogenates. Data represent mean ± S.E.M., n=12. **p<0.01 and ***p<0.001 vs. SD-Naïve control group, $^{\#}p$<0.05 and $^{\#\#}p$<0.01 vs. SD-CIA control group.

3.4. OL and Per-OL experimental diets reduced COX-2 and iNOS overexpressions

The effects of OL and Per-OL dietary treatments on CIA-induces COX-2 activation were determined by immunohistochemistry on knee joint sections. We could observe a significant overexpression of COX-2 positive cells in SD-CIA control group, whereas OL and Per-OL experimental diets reduced notably the immunoreactivity for this proinflammatory enzyme (Fig. 4A). In addition, the protein expression of iNOS was significantly upregulated in paws homogenates from in SD-CIA mice in comparison to SD-Naïve control group (*p<0.05 vs. SD-Naïve control group) whereas, the protein expression of this proinflammatory enzyme was down-regulated in paws from arthritic mice fed with 0.05 % and 0.025 % Per-OL experimental diets ($^{\#}p$<0.05; $^{\#\#}p$<0.01 vs. SD-CIA control group) (Fig. 4B).

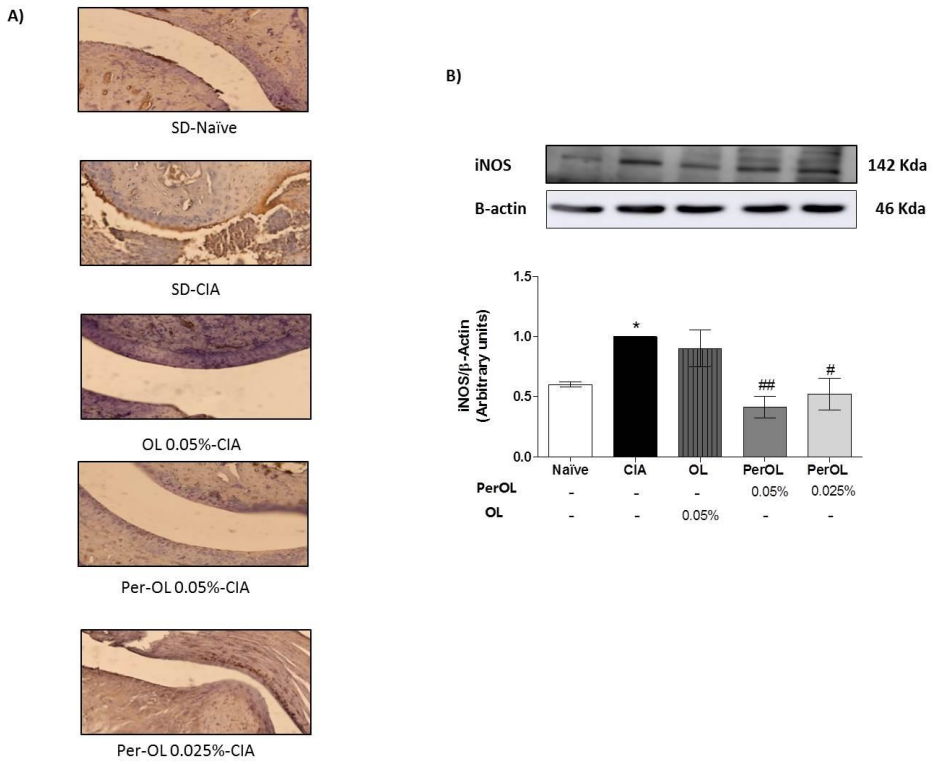


Figure 4. Expression of proinflammatory enzymes: COX-2 **(A)** and iNOS **(B)** in hind paws. COX-2 was determined by immunohistochemistry. Original magnification x20. iNOS protein expression was determined by Western Blot, quantified by densitometry analysis and normalized with respect to a house-keeping, β-actin. Data represent mean ± S.E.M., n=5. *p<0.05 vs. SD-CIA control group; #p<0.05 and ##p<0.01 vs. SD-CIA control group.

3.5. Effects of dietary OL and Per-OL treatments on MAPKs, NFκβ and Nrf2/HO signaling pathways

We investigated the effects of OL and Per-OL experimental diets on NF-κB, MAPKs and Nrf2/HO-1 signaling pathways in paw homogenates.

As shown in Fig. 5, the expression of cytoplasmatic IκB-α was significantly reduced in SD-CIA control group when compared with SD-Naïve control group (***p<0.001 vs. SD-Naïve control group) which was accompanied by an overexpression of nuclear NF-κBp65 and NF-κBp50 subunits protein expressions in SD-CIA control group (**p<0.01; ***p<0.001 vs. SD-Naïve control group). On the contrary, dietary OL and Per-OL treatments prevented the IκB-α degradation (#p<0.05; ##p<0.01 vs. SD-CIA control group) and prevented the nuclear translocation of p50 and p65 subunits (#p<0.05; ###p<0.001 vs. SD-CIA control group).

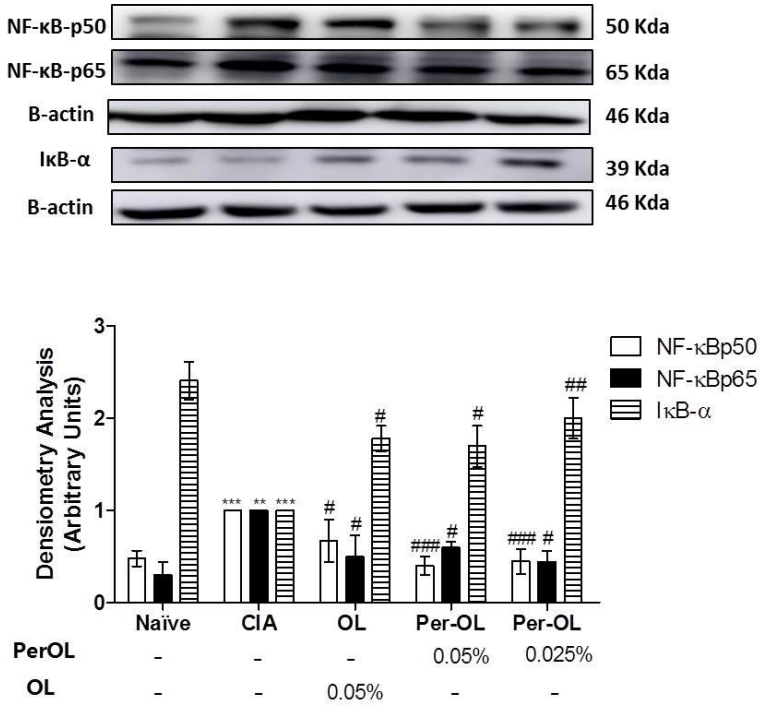


Figure 5. Protein expression of nuclear NF-κB-p65 and –p50 subunits and cytoplasmatic IκB-α fraction was determinate in hind paws. The expression was determined by Western Blot, quantified by densitometry analysis and normalized with respect to a house-keeping, β-actin. Data represent mean ± S.E.M., n=5. ***p*<0.01 and ****p*<0.001 vs. SD-Naïve control group, #*p*<0.05, ##*p*<0.01 and ###*p*<0.001 vs. SD-CIA control group.

MAPKs signaling pathways play a key role in the establishment of inflammation process. We investigated the effects of dietary OL and Per-OL treatments on MAPKs (extracellular signal-regulated kinases ($ERK_{1/2}$), c-Jun NH2-terminal kinase (JNK) and p38) signaling pathway activation in CIA mice. We observed a significant increase on $ERK_{1/2}$, JNK and p38 phosphorylation in SD-CIA control group in comparison with SD-Naïve control group (***p<0.001 vs. SD-Naïve control group). Nevertheless, the phosphorylation degree of these MAPKs proteins was significantly ameliorated in those arthritic mice fed with all experimental diets assayed (#*p*<0.05; ##*p*<0.01; ###*p*<0.001 vs. SD-CIA control group) (Fig. 6).

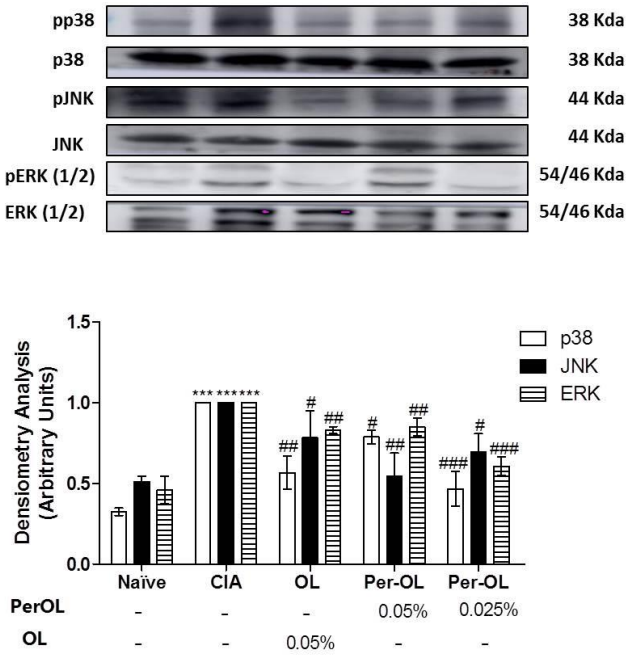


Figure 6. Effects of OL and Per-OL treatments on MAPKs phosphorylation in hind paws. The expressions of phosphorylated proteins (JNK, p38 and ERK $_{(1/2)}$) were expressed related to the expression of corresponding total protein. Data represent mean ± S.E.M., n=5. ***p<0.001 vs. SD-Naïve control group, #p<0.05, ##p<0.01 and ###p<0.001 vs. SD-CIA control group.

Finally, the effects of dietary OL and Per-OL treatments on Nrf2 and HO-1 antioxidant pathway activation were also explored. We could observe a remarkable downregulation of both Nrf2 and HO-1 protein expressions in SD-CIA mice (*p<0.05; **p<0.01 vs. SD-Naïve control group) whereas OL (0.05%) and Per-OL (0.05%) experimental diets induced a significant Nrf2 and HO-1 overexpression in comparison with SD-CIA control group (#p<0.05; ##p<0.01 vs. SD-CIA control group) (Fig. 7).

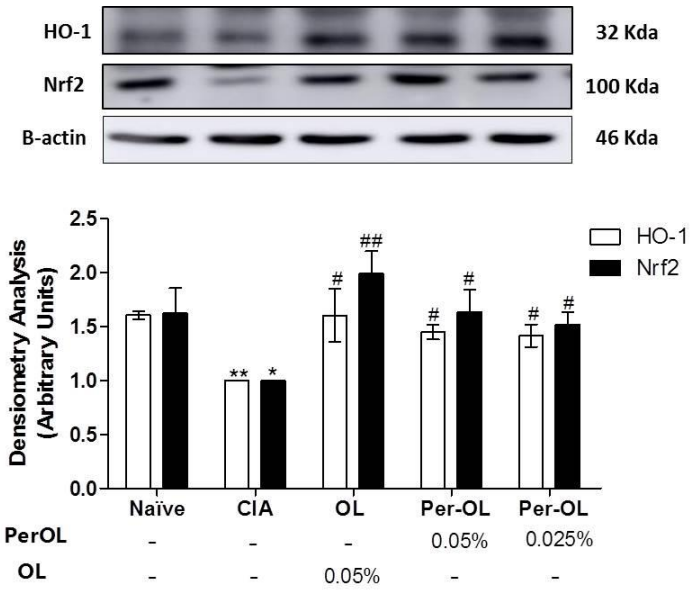


Figure 7. Effects of dietary OL and Per-OL on Nrf2/HO-1 antioxidant signaling pathway in hind paws. The expression was determined by Western Blot, quantified by densitometry analysis and normalized with respect to a house-keeping β-actin. Data represent mean ± S.E.M., n=5. $p<0.05$ and $^{**}p<0.01$ vs. SD-Naïve control group, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. SD-CIA control group.

4. DISCUSSION

In this study, dietary OL and Per-OL treatments effectively reduced the clinical arthritis severity and the histologic score in mice with CIA, a model of RA which is commonly used to study and investigate mechanisms involved in RA development as well as a new strategy of RA therapy (Ferrandiz et al., 2007). In addition, OL and Per-OL treatments markedly inhibited oxidative damage and reduced proinflammatory cytokine expression in inflamed joints. The main mechanisms by which OL and Per-OL exerted their antiarthritic efficacy was mediated by a dramatically increase on antioxidant Nrf2/HO-1 signaling and an inhibition of NF-κB and MAPKs signal pathways.

Arthritis is a chronic systemic inflammatory disorder affecting synovial lining of joints, bursae and tendon sheaths (Guo *et al.*, 2018). The objective of conventional pharmacological therapy of RA is to finish or reverse cartilage destruction and diminish the pain devoid of untoward effects. However, current treatments are not efficient in all patients and possess a number of disadvantages, such as high cost, necessity for parenteral administration and potential adverse effects. Consequently, nutritional therapy as complementary and alternative medicine is under development as an innovative strategy in RA management (Gaforio *et al.*, 2019). In fact, it has

been demonstrated that nutritive and non-nutritive dietary factors can affect the clinical outcome of rheumatic diseases. In this line, dietary polyphenols have been extensively investigated with regards to their antioxidant, anti-inflammatory, and immunomodulatory properties in many inflammatory chronic conditions, including RA. Several studies have shown that among bioactive polyphenols, hydroxytyrosol (HTy), hydroxytyrosol acetate (HTy-Ac), genistein, kaempferol and resveratrol seem to be effective in CIA, an experimental model for RA that shares a number of pathological, histological and immunological features with human RA, by decreasing the production of proinflammatory cytokines, and activating the antioxidant defense system (Rosillo *et al.*, 2014b, 2015; Oliviero *et al.*, 2018).

CIA induction resulted in the development of a pronounced synovitis associated with an autoimmune response against cartilage with inflammatory cells infiltrate and production of many cytokines and matrix-degrading, accompanying cartilage degradation and bone erosions (Schurgers, Billiau and Matthys, 2011; Bao *et al.*, 2017). Our results revealed that OL and Per-OL supplemented diets exhibited preventive effects in the development of inflammation and joint damage in SD-CIA in comparison with animals that were fed with OL and Per-OL enriched-diets. These results were correlated to an improved arthritis score and with a reduction of inflammatory cells infiltration into articular tissue, synovial hyperplasia and cartilage destruction. These results are in accordance with data published by Impellizzeri et al., who reported an amelioration of arthritis development caused by injection of collagen type II in mice and in by intraperitoneal administration of OL-aglycone (Impellizzeri, Esposito, Mazzon, Paterniti, Di Paola, Bramanti, *et al.*, 2011; Impellizzeri, Esposito, Mazzon, Paterniti, Di Paola, Morittu, *et al.*, 2011).

The secretion of MMPs, cytokines and growth factors contribute to the loss of normal homeostasis in the synovial joint leading inflammation and joint damage on RA. Cytokines regulate the phenotype of effector and regulatory T-cells in the synovium. Thus, an imbalance of cytokine network contributes to the development and progression of this autoimmune disease. Particularly, TNF-α, IL-6, IL-1β and IFN-γ are considered as disease promoting cytokines in RA (Komatsu and Takayanagi, 2012; Pradhan *et al.*, 2019). In synovium, patients with RA had high frequencies of Th1 cells and also of Th17 cells, which are thought to play a prominent pathogenic role in autoimmune arthritis. More recently, in plasma from RA patients, levels of IL-17, IL-23 and IFN-γ were significantly increased and correlated with a redox imbalance and oxidative damage (Pradhan *et al.*, 2019). Besides, COMP, a specific serological marker which evaluates the articular cartilage degradation and its turnover, exerts an active role in inflammation. Increased plasma levels of COMP have been detected in the degenerating cartilage, synovial fluid and serum of patients with knee injuries and primary osteoarthritis (OA) and RA (Lai et al., 2012; Haikal *et al.*, 2019). Among MMPs, MMP-3 (stromelysin) has been reported to be the major enzymes produced by fibroblasts and macrophage cells in the synovium, and is responsible for the degradation of

proteoglycan, various type of collagens and denatured type I and type II collagens, among others, besides of its direct enzyme activity its activation is necessary for full activation of collagenases (M L Castejón *et al.*, 2017). Our results revealed that those arthritic mice fed with OL and Per-OL enriched-diets showed a significant reduction in serum MMP-3 and COMP levels as well as in IL-17, TNF-α, IL1-β, IFN-γ and IL-6 proinflammatory cytokines levels in paw tissues in comparison with SD-CIA control group which was correlated with the macroscopic and histological findings. These results are also in agreement with Castejón et at., who reported that OL controlled the production of inflammatory mediators decreasing IL-6 and TNF-α cytokines, MMP-1 and MMP-3 levels and mPGES-1 and COX-2 overexpression in human synovial fibroblast cell line SW982 (Maria Luisa Castejón *et al.*, 2017).

The overexpression and activation of proinflammatory enzymes, including iNOS and COX-2, are known to intensify the disease severity of RA (Altomonte *et al.*, 1992), which promoting inflammation and oxidative damage of the arthritic joint (Hemshekhar *et al.*, 2017). In fact, COX-2 and iNOS protein expressions are increased on rats CIA model and consequently, contributing to the progression of RA (Jing et al., 2019). In accordance with Ryu et al., (S.-J. Ryu *et al.*, 2015) who reported that OL suppressed the release of LPS-induced NO production and iNOS/COX-2 overexpression in RAW 264.7 murine macrophages and in LPS-stimulated murine peritoneal macrophages (Maria Luisa Castejon *et al.*, 2019), we have demonstrated that dietary OL and Per-OL treatments reduced COX-2 inmunohistochemical expression in knee joints from DBA/1 mice CIA-model and this was accompanied by a significant decrease on iNOS protein expression in paw homogenates reducing efficiently the joint damage. In addition, previous studies have shown that OL induced a downregulation of NF-κB transcriptional activity and dramatically suppression of the MAPKs activation in human osteoarthritis chondrocytes and in murine peritoneal macrophages stimulated with LPS (Feng *et al.*, 2017; Maria Luisa Castejon *et al.*, 2019).

Similarly, the main intracellular signal transduction pathways involved in RA include MAPKs, NF-κB and JAK/STAT pathways. Understanding of the signal transduction pathways implicated in RA has led to drug development programmers targeting MAPKs and NF-κB inhibitors (Smolen and Steiner, 2003; Malemud, 2018). In addition, a wide range of evidence indicates that Nrf2 transcription factor, may control different mechanisms involved in the physiopathology of rheumatic condition playing a central role in the protection of cells against oxidative stresses, up-regulating several genes encoding anti-inflammatory and antioxidant proteins, such as HO-1 (Mohan and Gupta, 2018). HO-1 gene expression activation is considered to be an adaptive cellular response to survive exposure to environmental stresses (Rosillo *et al.*, 2016; Fan *et al.*, 2018). Besides, some studies have revealed an interaction between members of the Nrf2 and NF-κB pathways, since that oxidative stress-induced NF-κB activation plays a pivotal role in the proinflammatory overproduction factors associated with the pathogenesis of RA and the

activation of the Nrf2/ Antioxidant responsive elements (ARE) system disrupts this cycle (Ahmed *et al.*, 2017). Particularly, Nrf2 inhibition aggravates cartilage destruction and accelerates the effector phase of RA in mice. NF-κB is considered a key signaling pathway in the control of synovial inflammation, hyperplasia and matrix generation (Zou *et al.*, 2017) and is able to modulate the expression of several proinflammatory genes such as IL-1β, TNF-α, IL-6 and IL-17 in CIA experimental model (Rosillo *et al.*, 2016). High levels of these proinflammatory cytokines induce the recruitment of co-stimulary molecules which leading to the activation of NF-κB and MAPKs signaling pathways (Rosillo *et al.*, 2014a) which is responsible for COX-2 up-regulation (Xie *et al.*, 2013). For this reason, intra-articular or systemic blockade of NF-κB signaling pathway is an effective target in the management of RA (Min *et al.*, 2013). Our data revealed that Nrf2 and HO-1 protein expressions were decreased in SD-CIA control group; however, dietary OL and Per-OL treatments could restore Nrf2 and HO-1 expressions conferring a remarkable role of Nrf2/HO-1 signaling pathway in the beneficial effects of OL and Per-OL enriched-diets in CIA model of RA. These results are in agreement with Castejón et al., showed that both dietary OL and Per-OL treatments induced an activation of Nrf2/HO pathway and prevented the MAPKs activation and nuclear NF-κB-p65 and NF-κB-p50 translocations by blocking IκB-α degradation in a pristane-induced SLE murine model. This was accompanied by a decrease on many proinflamatory markers production involved in this inmunoinflammatory disease (M.L. Castejon et al., 2019). According to our results, induction of HO-1 expression may protect against cartilage destruction and decrease the secretion of proinflammatory cytokines in CIA model (Li *et al.*, 2014). Besides, upregulating the expression of Nrf2 may exert anti-inflammatory effects in RA (Wu *et al.*, 2016). In this sense, our data suggest that Nrf2 overexpression observed in those arthritic animals fed with OL and Per-OL dietary treatments significantly could prevent the nuclear NF-κB transcription factor, resulting in an ameliorated proinflammatory markers production reducing the joint inflammatory injury.

At the same time, MAPKs can participate in the regulation of NF-κB transcriptional activity. Furthermore, we determined that dietary OL and Per-OL treatments influenced the activation of MAPKs. It is well-established that MAPKs play important roles in transducing synovial inflammation and joint destruction and they are considered critical molecular targets for therapeutic intervention in RA (Thalhamer, McGrath and Harnett, 2007). JNK and $ERK_{1/2}$ MAPKs are closely associated with collagenase production and inflammatory responses of fibroblast-like synoviocytes, whereas p38 MAPK isoforms is involved in regulating many of cellular biological processes, concretely synovial inflammatory cytokine production, which participate to the RA pathogenesis (Zou *et al.*, 2017). $ERK_{1/2}$ also participates in promoting pannus formation and bone destruction in arthritic joints (Goodridge et al., 2003). In the present study, the phosphorylation of all three kinases ($ERK_{1/2}$, JNK and p38 MAPKs) was upregulated by inflammation and was related

with the results obtained in proinflammatory cytokines profile determinations. On the contrary, OL and Per-OL experimental diets were able to prevent the phosphorylation of JNK, p38 and $ERK_{1/2}$, suggesting a role of MAPKs and NF-κB in the mechanism through which OL and Per-OL may have antiarthritic activity.

In summary, our study showed, for the first time, the beneficial effects of dietary OL and Per-OL supplementation in CIA model of RA by reducing arthritic damage, serum proinflammatory biomarkers (MMP-3 and COMP), cytokines production (IL-6, IL-1β, TNF-α, IFN-γ, IL-17), and inhibiting both iNOS and COX-2 overexpression. The possible action mechanisms implicated in these beneficial effects could be related to an activation of the antioxidant Nrf2/HO-1 pathway as well as a blockage of MAPKs and NF-κB signaling pathways. Our results provide evidence for the anti-arthritic properties of both OL and Per-OL and corroborate their potential as a new promising dietary strategy for the prevention and management of RA.

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CHAPTER IV

OLEUROPEIN AND ITS NEW ACETYL DERIVATIVE DIETARY TREATMENTS MODULATE INFLAMMATORY RESPONSE IN PERITONEAL MACROPHAGES FROM PRISTANE-INDUCED SYSTEMIC LUPUS ERYTHEMATOSUS MICE VIA REGULATION OF CANONICAL AND NON-CANONICAL NLRP3 INFLAMMASOME

DIETAS ENRIQUECIDAS CON OLEUROPEINA Y SU NUEVO ACETIL-DERIVADO MODULAN LA RESPUESTA INFLAMATORIA EN MACRÓFAGOS PERITONEALES DE RATONES CON LES INDUCIDO POR PRISTANO MEDIANTE LA REGULACIÓN DE LA VIA CANÓNICA Y NO CANONÓNICA DEL INFLAMASOMA NLRP3

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RESUMEN

El lupus eritematoso sistémico (LES) es una enfermedad autoinmune inflamatoria crónica que puede afectar a múltiples órganos. Recientes estudios indican que la inmunidad innata juega un papel fundamental en el desarrollo de LES. La oleuropeína (OL) es uno de los compuestos fenólicos mayoritarios presentes en las aceitunas verdes y en las hojas del olivo, y además posee numerosas propiedades beneficiosas.

El objetivo principal de este estudio fue investigar el efecto de dietas enriquecidas con OL y OL peracetilada (Per-OL) en macrófagos peritoneales extraídos de ratones con LES inducido por pristano en ratones BALB/c y estimulados con lipopolisacárido bacteriano (LPS).

Los ratones recibieron una inyección de pristano vía intraperitoneal y fueron alimentados con las dietas experimentales enriquecidas con ambos compuestos. Veinticuatro semanas más tarde, los riñones de los ratones se recogen junto a los macrófagos peritoneales para evaluar los diferentes marcadores pro-inflamatorios y vías de señalización intracelular que puedan estar involucradas en el proceso inflamatorio.

Los análisis macroscópicos del tejido renal se llevaron a cabo mediante la tinción con Hematoxilina y Eosina, mientras que la producción de citoquinas inflamatorias fue evaluado mediante la técnica de ELISA y los cambios de expresión de las vías de señalización celular fue evaluado mediante la técnica de Western Blotting.

Nuestros resultados demuestran que el tratamiento con dietas experimentales con OL and Per-OL reducen significativamente los niveles de infiltrado inflamatorio en el tejido renal y además de los niveles de las citoquinas pro-inflamatorias en los sobrenadantes de los macrófagos obtenidos de los ratones con LES. La suplementación dietética con OL o Per-OL disminuyó significativamente la expresión de marcadores pro-inflamatorios como las enzimas ciclo-oxigenasa 2 (COX-2) y la

enzima óxido nítrico sintasa inducible (iNOS). Nuestros resultados nos permiten concluir que las dietas elaboradas con OL o Per-OL disminuyen la expresión de proteínas implicadas en el daño renal a través de la regulación de diversas vías implicadas en el LES, como es el factor nuclear kappa B (NF-κB), Janus cinasas-transductor de señal y activador de la transcripción (JAK/STAT) y la vía canónica y no canónica del inflamasoma NLRP3.

Estos resultados refuerzan el interés del tratamiento con dietas enriquecidas con OL o Per-OL como alternativa beneficial en la prevención o el manejo del LES.

OLEUROPEIN AND ITS NEW ACETYL DERIVATIVE DIETARY TREATMENTS MODULATE INFLAMMATORY RESPONSE IN PERITONEAL MACROPHAGES FROM PRISTANE-INDUCED SLE MICE VIA REGULATION OF CANONICAL AND NON-CANONICAL NLRP3 INFLAMMASOME

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ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disease which can affect many organ systems. Recent research indicated that innate immunity plays vital roles in SLE. Oleuropein (OL) is one of mainly biophenols in green olives and leaves, and possesses many beneficial properties.

The aim of the study was investigated the effects of OL and its acetyl-derivate (Per-OL) dietary treatments on peritoneal macrophages from pristane-induced mice.

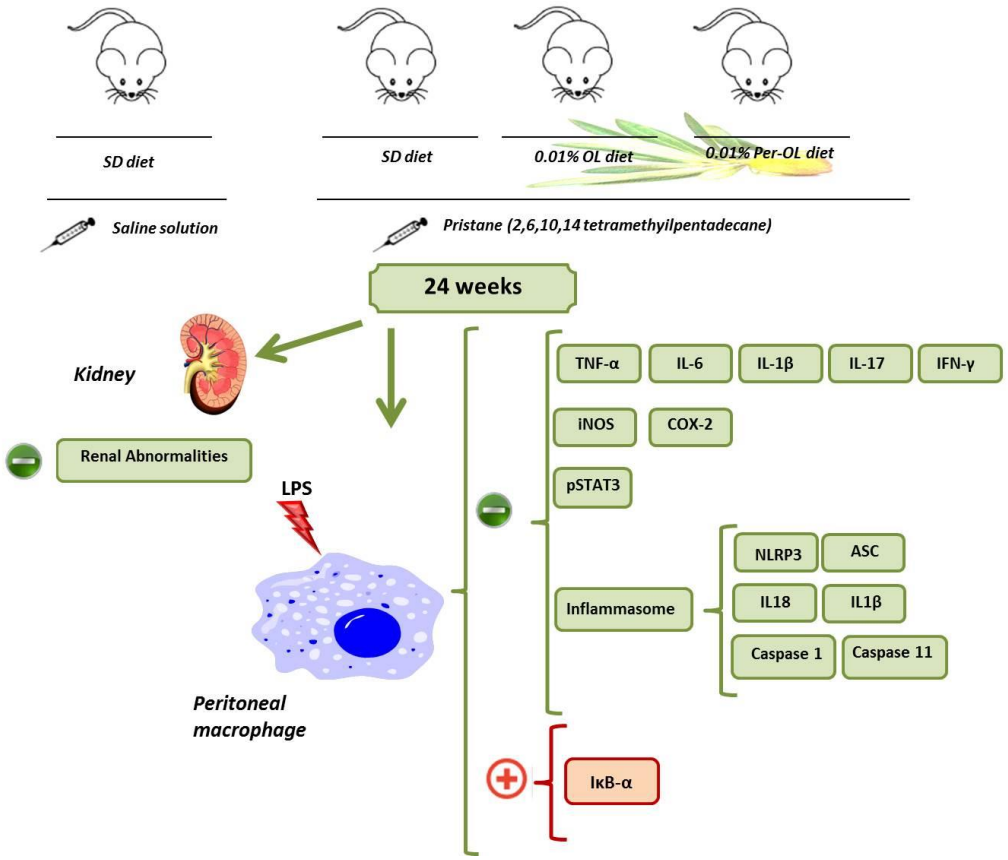
BALB/c mice received an injection of pristane or saline solution and were fed with experimental diets with OL and Per-OL. After 24 weeks, we recollected kidneys and peritoneal macrophages for experimental assays. In addition to macroscopic and histological analyses, pro-inflammatory cytokines production was evaluated by enzyme-linked immunoassay (ELISA). The protein expressions of pro-inflammatory markers and other signaling pathways activation were determined in macrophages by Western Blotting.

We have demonstrated that OL and Per-OL dietary treatments reduced significantly these levels in macrophages. Our data indicate that Janus kinase-signal transducer and activator of transcription (JAK/STAT), nuclear transcription factor-kappa B (NF-κB) and canonical and non-canonical NLR family pyrin domain-containing 3 (NLRP3) inflammasome-complex signaling pathways were drastically ameliorated.

These results support the interest of OL and Per-OL dietary treatments as a beneficial alternative that exerts a preventing/palliative role in the management of SLE.

Chapter IV

KEYWORDS: Inflammasome, lupus; macrophages; NLRP3; Oleuropein



1. INTRODUCTION

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disorder that courses with a combination of multiple genetic and environmental factors which break the threshold of immune tolerance (Perry *et al.*, 2011).

SLE damage has been characterized by disproportion of T-helper-cell (Th) subsets (Th1/Th2/Th17) and regulatory T-cells (Treg), autoantibodies and immune complexes. During the active step of the disease, there is an increase of the inflammatory response with higher serum and

plasma levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-6, IL-10, IL-12, IL-17 and IL-18 and lower levels of IL-2 (Aparicio-Soto, Sánchez-Hidalgo, Rosillo, *et al.*, 2016).

Although SLE pharmacological treatment has improved during the last decade and many potential new agents are in development, there is evidence, that diet therapy could be a promising approach in SLE due to its potential prophylactic effects without the side effects of classical pharmacology in addition to reduce comorbidities and improve quality of life in SLE patients (Aparicio-Soto, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2017).

Recent studies have confirmed that extra virgin olive oil (EVOO) consumption can contribute positive effects in rheumatic diseases. The beneficial properties of EVOO are linked to its multiple minor components, such as polyphenol compounds mainly flavonoids, lignans, secoiridoids and their hydrolysis products hydroxytyrosol (HTy) and tyrosol (Ty) among others. In this sense, previous studies from our research group have demonstrated that EVOO diet or olive biophenols-enriched diets improved inflammatory processes in experimental rheumatoid arthritis (RA) or SLE in mice (Aparicio-Soto, Sánchez-Hidalgo, Cárdeno, *et al.*, 2016). Particularly, dietary HTy and hydroxytyrosol acetate (HTy-Ac) supplementation prevented pristane-induced SLE in mice reducing pro-inflammatory cytokines and ameliorated renal damage with a considerably blockage of different inflammatory-related pathways (Aparicio-Soto *et al.*, 2017). In addition, phenolic fraction from EVOO treatment downregulated the gene expression of M1 macrophages markers and up regulated M2 molecules on M0 human macrophages. The phenolic fraction from EVOO also reduced both cytokine production and T cell activation, through blockage of nuclear transcription factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs)/ extracellular signal-regulated kinase (ERK) signaling pathways on peripheral blood mononuclear cells (PBMC) from patients with SLE in comparison with healthy donors (Aparicio-Soto *et al.*, 2018). Similar results were described by Rosillo et al., where phenolic fraction from EVOO administration prevented inflammatory response and joint damage in murine experimental arthritis (Rosillo *et al.*, 2014).

Among secoiridoids, specifically, oleuropein (OL) is an ester of HTy containing an oleosidic skeleton and a carbohydrate group which has been shown to possess antimicrobial, antiviral, anti-atherogenic, cardioprotective, anti-oxidative, anti-cancer and anti-inflammatory properties (Shamshoum, Vlavcheski and Tsiani, 2017; Şahin and Bilgin, 2018). Our previous studies have shown like OL was capable to prevent the inflammatory response and oxidative stress on IL-1β-induced SW982 human synovial fibroblasts cells and lipopolysaccharide (LPS)-induced murine peritoneal macrophages (Castejón *et al.*, 2017; Castejon *et al.*, 2019).

Some studies have shown the importance of acetyl derivatives of natural phenols since their lipophilic nature allows them to cross the cytoplasmic cell membranes and their uptake by cells, offering a possible protection of membrane components. In this sense, we confirmed better

anti-inflammatory effects of acetyl OL derivatives in comparison with OL in murine peritoneal macrophages which could be related to the modification of the chemical structure of the original compound (Castejon *et al.*, 2019).

Pristane-induced lupus model represents SLE from environmental activation with normal genetic background characterized by an autoimmune syndrome closely resembling SLE with lupus-specific autoantibodies, nephritis, arthritis, diffuse alveolar hemorrhage and hematological manifestations (Reeves *et al.*, 2009). Pristane induces chronic inflammation and enhances immune responses involving the continuous recruitment of leukocytes, including lymphocytes, neutrophils, dendritic cells and macrophages, to the peritoneal cavity and the spleen (Leiss *et al.*, 2013; Han *et al.*, 2017).

Taking into account, the present study was performed to investigate the anti-inflammatory effects of OL and its new acetyl derivative, peracetylated oleuropein (Per-OL) dietary treatments on peritoneal macrophages from pristane treated mice with the objective of characterize inflammatory mediators, mechanisms and signaling pathways involved.

2. MATERIALS AND METHODS

2.1. Reagents

OL was extracted according to reported literature (Stamatopoulos, Chatzilazarou and Katsoyannos, 2013), following the purification by column chromatography (CH_2Cl_2-MeOH 10:1→5:1) to give the product as a yellow solid.

The synthesis of Per-OL was carried out from OL. It was solved and stirred in a mixture of 1:1 (v/v) pyridine/acetic anhydride at 8ºC for 10 min. Then, the reaction was kept at room temperature overnight. After hydrolysing the acetic anhydride, the product was concentrated to dryness obtaining a residue that was purified by column chromatography (EtOAc-C. Hexane 1:1) to give the product (quant.) as a brown solid. Spectroscopy data was identical to reported literature (Procopio AS M.; Costa, N.; Nardi, M., 2008).

2.2. Animals and diets

To evaluate the beneficial effects of OL and Per-OL on pristane model, a total of 60 twelve-week-old BALB/c mice were used (Janvier Labs®, Saint-Berthevin Cedex, France). Mice were maintained under constant conditions of temperature (20-25 ºC) and humidity (40-60%) with a lighting regimen of 12L/12D. They were fed with standard rodent chow (Panlab A04®, Seville, Spain) and water ad libitum until SLE-like disease pristine induction.

Mice were randomized in four experimental groups: (i) Sham group fed with standard diet (SD) (n=10), (ii) pristane group fed with SD (n=20), (iii) pristane OL group fed with SD

supplement with OL (100mg/kg diet) (n=15) and (iv) pristane Per-OL group fed with SD supplement with Per-OL (100mg/kg diet) (n=15). Fresh diets were provided daily, and experimental diets were formulated on the basis of the American Instituted of Nutrition (AIN) standard reference diet, and were prepared by mixing the pertinent compounds and stored at -80ºC.

Mice were fed with the related diets along six months, and weight, water and food consumption and mortality were monitored weekly. All animal care and experimental procedures followed a protocol approved by the Animal Ethics Committee of the University of Seville, and all experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Counsel 2010/630/EU).

2.3. Pristane induction of SLE-like disease

At sixteen-weeks-old BALB/c mice received an intraperitoneal injection of 0.5 mL pristane (99% pure, Sigma Aldrich Co®, St Louis, MO, USA) to induce SLE according to previous described procedure by Satoh et al., 2009 (Reeves *et al.*, 2009). After 24 weeks (experimental period), animals were sacrificed and peritoneal macrophages, blood, thymus, lungs, spleen and kidneys were collected.

2.4. Histological evaluation

Kidneys were removed, fixed in neutral buffered 4% formaldehyde, and embedded in paraffin. Samples were sagittally sectioned at 5 μm and stained with hematoxylin and eosin (H&E); four samples per animal containing both cortex and medulla were selected for evaluation. Morphological changes were evaluated at the light microscopic level by two different observers who were unaware of the treatment group. Results are expressed as percentages of cases exhibiting histological changes.

2.5. Isolation and culture of murine peritoneal macrophages

Peritoneal macrophages were collected following the procedure described by Aparicio-Soto et al., 2015 (Aparicio-Soto *et al.*, 2015). Cells were washed with PBS and resuspended in RPMI 1640 medium (PAA®, Pasching, Austria) supplemented with 10% heat-inactivated foetal calf serum (FCS) (PAA®, Pasching, Austria), L-glutamine (2 mM), glucose (4.5 g/l), and HEPES buffer (10 mM), in the presence of 100 mg/ml streptomycin and 100 U/ml penicillin (PAA®, Pasching, Austria) and seeded in culture plates for 2 h at 37º C in a 5% CO_2 humidified atmosphere. After 2 h, cells were washed with phosphate buffered saline (PBS) and fresh RPMI 1640 medium without fetal bovine serum (FBS) containing different concentrations of assayed compounds (1-4). After 30

min, murine peritoneal macrophages were stimulated with 5 µg/ml of LPS from *Escherichia Coli* (Sigma-Aldrich®, St Louis, MO, US). In each experiment, viability was always ≥ 95% (data not shown). Cells supernatants were collected and stored at -80ºC for cytokine assays.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The cytokine production in peritoneal macrophages cell-culture supernatants was detected by enzyme-linked immunosorbent assay (ELISA) kits among manufacture's procedure to measure the levels of TNF-α, IL-1β (BD OptEIA®, San Jose, CA); IL-6, IFN-γ (Diaclone®, Besaçon Cedex, France) and IL-17 (Peprotech®, London, UK). The results were measured at 450 nm using an ELISA microplate reader (BioTek®, Bad Friedrichshall, Germany). All measurements were carried out in duplicate.

2.7. Immunoblotting

Cells (1×10^6 cells/mL) were stimulated with LPS for 18 h. After incubation, cells were rinsed, scraped off and collected in ice-cold PBS containing a cocktail of protease and phosphatase inhibitors and processed as described by Sánchez-Hidalgo et al., 2005 (Sanchez-Hidalgo *et al.*, 2005). Protein concentration was measured for each sample using a protein assay reagent (BioRad®, Múnchen, Germany) according to Bradford's method (Bradford, 1976). Aliquots of these supernatants which contains equal amount of protein (15 µg) were separated on 10-15% acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrophoretically transferred into a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-COX-2 and rabbit anti-iNOS (Cayman®, Ann Arbor, MI, USA), mouse anti-pSTAT3 (Cell Signalling Technology®, Danvers, MA, USA), mouse anti-NLRP3, mouse anti-ASC and rabbit anti-Caspase-1 (Cell Signalling Technology®, Danvers, MA, USA), rabbit anti-Caspase-11 (Novus Biologicals®, Littleton, CO, USA), rabbit anti-IL-18 (AbCam®, Cambridge, UK), rabbit anti-IκB-α (Cell Signalling Technology®, Danvers, MA, USA). To prove equal loading, the blots were analysed for β-actin expression using an anti-b-actin antibody (Sigma–Aldrich®, MO, USA). Immunodetection was performed using enhanced chemiluminescence light-detecting kit (Pierce®, Rockford, IL, USA). The immunosignals were captured using Amersham Imager 600 (GE Healthcare®, Buckinghamshire, UK), and densitometric data were studied following normalization to the housekeeping loading control. The signals were analysed and quantified by an Image Processing and Analysis in Java (Image J®, Softonic) and expressed in relation to pristane control group.

2.8. Statistical analysis

All values in the main text and figures are expressed as arithmetic means ± S.E.M. Data were evaluated with Graph Pad Prism® Version 5.04 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way

analysis of variance (ANOVA) followed by Tukey multiple comparisons test as post hoc test. *P* values of < 0.05 were considered statistically significant- In the experiments involving densitometry, the figures shown are representative of at least four different experiments performed on different days.

3. RESULTS

3.1. Effects of OL and Per-OL supplemented diets on pristine-induced model of SLE

SLE-pristane induced model is characterized by a multiorgan autoimmune disease with a wide array of clinical manifestations and multifactorial pathogenic pathways. In this case, comparison of different organ such as thymus, lungs and spleens weights between sham mice and pristane group revealed a significant increment in thymus and spleens weights (**p*<0.05 and ****p*<0.001 vs. SD-sham group) (Fig. 1). However, any significant difference were observed in which mice fed with OL and Per-OL diets in comparison with SD-SLE group mice.

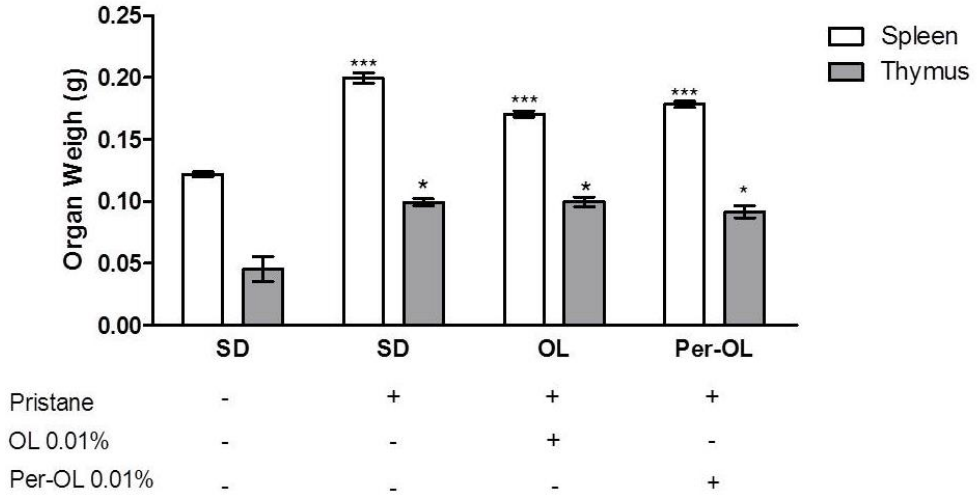


Figure 1. Thymus and spleen weight from different dietary BALB/c mice groups 6 months post-treatment. Each value represents the mean ± S.E.M. of every group (n=10). **p*<0.05; ****p*<0.001 vs. SD-sham group.

3.2. OL and Per-OL enriched-diets ameliorated pro-inflammatory infiltration in kidneys from SLE-pristane induced BALB/c mice

As shown in Fig. 2, most mice of pristane-treated group presented some degree of renal abnormalities in comparison with control mice, ranging from abundant inflammatory mononuclear cells in the renal interstitium to renal interstitial fibrosis. In pristane-treated mice, most kidneys (90%) displayed inflammatory infiltrate, preferably located around the intact vascular wall close to glomeruli (Fig. 2B). This inflammatory damage was considerably reduced after Per-OL diet (30%) and only lightly reduced after OL diet (70%) (Fig 2 A-D).

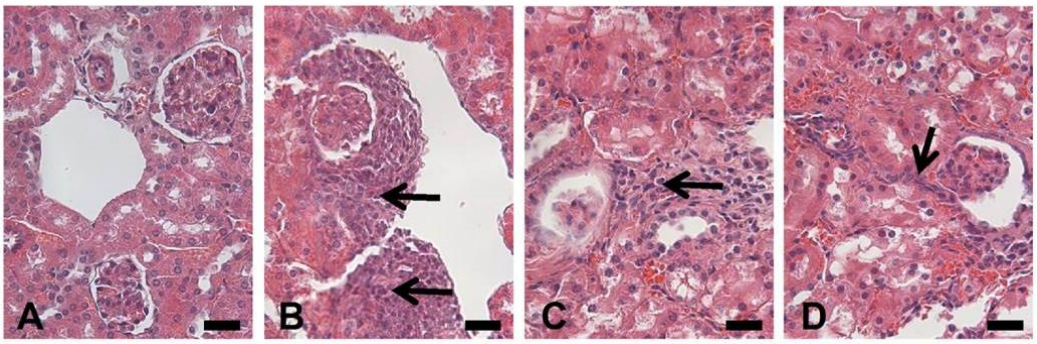


Figure 2. SLE model induced by pristane: effect of OL and Per-OL diets in renal histology. Representative histopathological appearance of mice kidneys after with H&E stain (A-D). Effects of OL (C) and Per-OL (D) diets in pristane-treated mice in comparison with normal (A) and pristane-treated controls (B). The presence of renal abnormalities and inflammatory mononuclear cells in the renal interstitium could be observed in kidneys. Bar: A-D: 20 μm.

3.3. Cell viability of peritoneal macrophages from SLE mice

Cells ($1x10^5$ cells/well) were incubated in 96 well-plates for 18 h, and their viability was analyzed by sulforhodamine B (SRB) assay (Skehan *et al.*, 1990). Cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells). In each experiment, viability was always ≥ 95% (data not shown).

3.4. Effects of OL and Per-OL supplemented diets in LPS-induced cytokine production in peritoneal macrophages from SLE mice

In order to know whether OL and Per-OL supplemented diets could modulate cytokine production by primary LPS-stimulated murine peritoneal macrophages from SLE-mice, we measured Th1, Th2 and Th17 cytokines production in supernatants of peritoneal macrophages from SLE-pristane mice after 24 h of LPS stimulation. The levels of TNF-α, IL-1β, IL-6, IFN-γ and IL-17 were found increased in SLE mice stimulated with LPS in comparison to sham mice

(***p<0.001 vs. SD-sham group). However, these Th1, Th2 and Th17 cytokines levels significantly decreased in LPS-stimulated macrophages from OL and Per-OL SLE mice (#p<0.05; ##p<0.01 and ###p<0.001 vs. SD-SLE group) (Figure 3). Low levels of cytokines were detected in non-stimulated macrophages (data not shown).

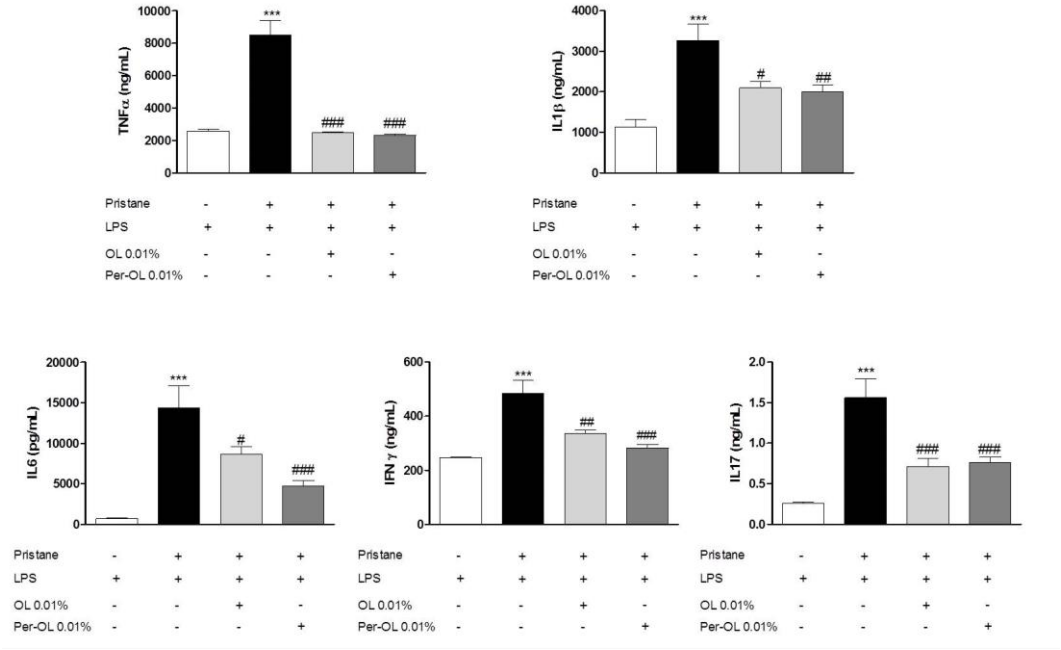


Figure 3: OL and Per-OL decreased the levels of pro-inflammatory cytokines on LPS-stimulated macrophages isolated from pristane-induced SLE mice. Levels of TNF-α, IL-1β, IL-6, IFN-γ and IL-17 were measured by ELISA. Data represent mean ± S.E.M. (n=10). ***p<0.001 vs. SD-sham group; #p<0.05, ##p<0.01, ###p<0.001 vs. SD-SLE group.

3.5. Effects of OL and Per-OL supplemented diets on over expression of ciclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) in peritoneal macrophages from SLE-mice

We evaluated the effect of OL and Per-OL enriched diets on COX-2 and iNOS protein expression. Whole cell lysates, from peritoneal macrophages stimulated with LPS from mice which were received experimental diets. COX-2 and iNOS proteins upregulation were markedly induced by LPS treatment (**p<0.01, ***p<0.001 vs. SD-sham group) (Fig. 4). However, a significant downregulation on these pro-inflammatory markers expressions were seen in SLE-OL and SLE-Per-OL macrophages (#p<0.05, ##p<0.01, ###p<0.001 vs. SD-SLE group).

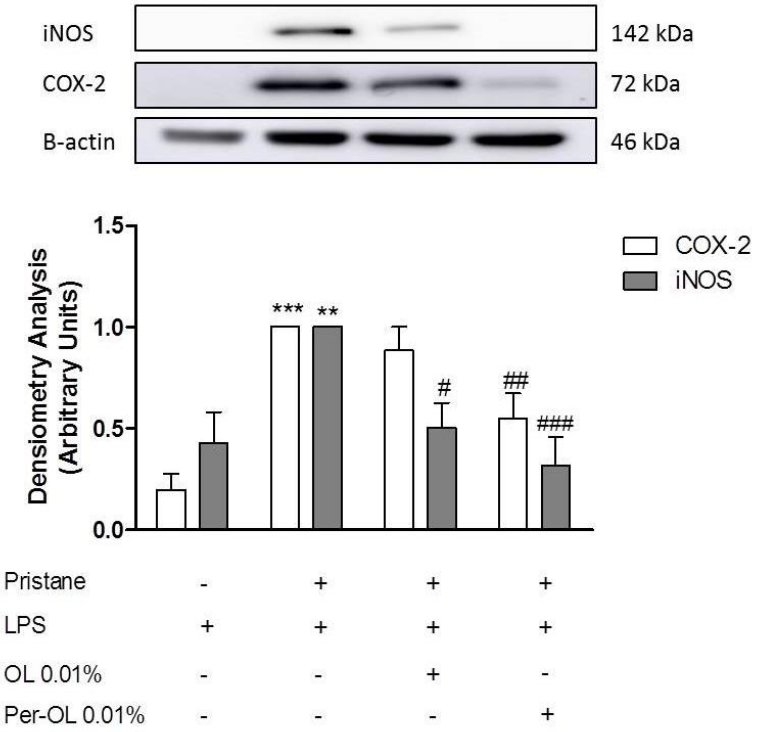


Figure 4: OL and Per-OL dietary induced the down-regulation of COX-2 and iNOS protein expressions in peritoneal macrophages stimulated with LPS from pristane-induced SLE mice. Densitometry analysis was performed following normalization to the control (β-actin housekeeping gene). Data are expressed as the mean ± S.E.M. (n=6). $^{**}p<0.01$, $^{***}p<0.001$ vs. SD-sham group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. SD-SLE group.

3.6. OL and Per-OL supplemented diets modulate NF-κB signaling pathway in murine peritoneal macrophages of pristane-induced SLE

To further explore the molecular mechanism underlying OL and Per-OL anti-inflammatory effects in murine peritoneal macrophages isolated from SLE-induced mice, we studied the role of NF-κB signaling pathway. NF-κB is normally complexed with inhibitor of NF-κB (IκB-α) in the cytoplasm (inactive state), but after cellular stimulation, for example with LPS, IκB-α is phosphorylated, ubiquinated and subsequently degraded by the proteasome (Sánchez-Fidalgo *et al.*, 2013). For this reason, we measured the expression levels of IκB-α in cytosolic fractions of peritoneal macrophages from pristane-induced mice treated with 5 μg/mL LPS and incubated for

18 h. As shown in Fig. 5, treatment with OL and Per-OL significantly prevented IκB-α translocation (*p<0.05 vs. SD-sham group; #p< 0.05 vs. SD-SLE group).

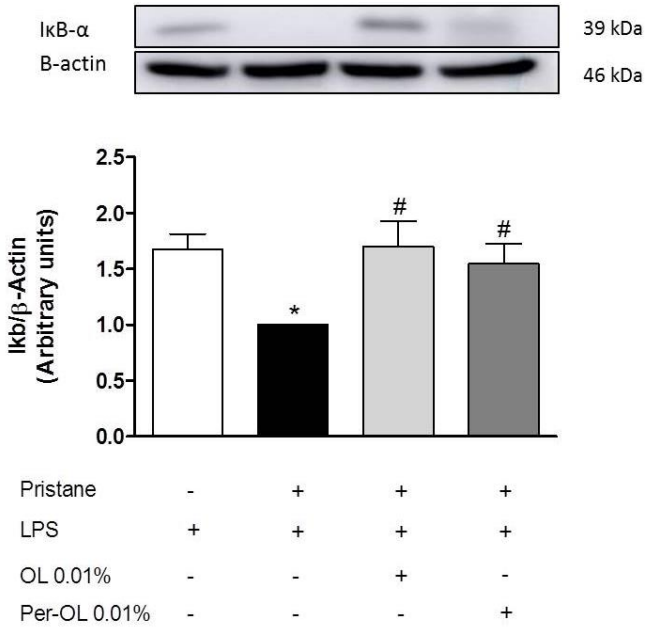


Figure 5: OL and Per-OL dietary treatment prevented IκB-α degradation in macrophages isolated from pristane-induced SLE mice stimulated with LPS. Densitometry analysis was performed following normalization to the control (β-actin housekeeping gene). Data are expressed as the mean ±S.E.M. (n=6). *p<0.05 vs. SD-sham group; #p<0.05 vs. SD-SLE group.

3.7. Effects of OL and Per-OL supplemented diets in expression of signal transducer and activator of transcription (STAT)-3 signaling pathways in peritoneal macrophages from SLE-mice

As Fig. 6 shows a higher induced STAT3 phosphorylation in peritoneal macrophages was obtained from SLE-induced mice in comparison with control group. However, OL and Per-OL treatments could down-regulated the expression of p-STAT3 mice peritoneal macrophages (**p<0.01 vs SD-sham group, ##p<0.01 vs SD-SLE group).

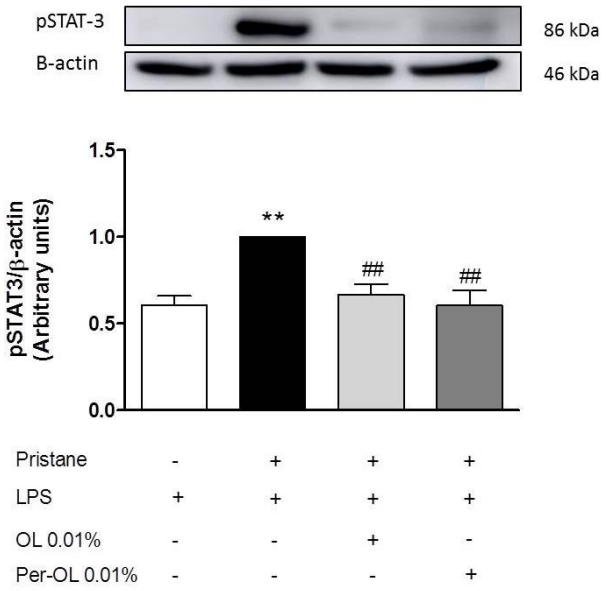


Figure 6: OL and Per-OL treatment modulated STAT-3 signaling pathways in LPS-stimulated murine peritoneal macrophages isolated from pristane-induced SLE mice. Densitometry analysis was perfomed following normalization to the control (β-actin housekeeping gene). Data are expressed as the mean ± S.E.M. (n=6). $^{**}p<0.01$ vs. SD-sham group; $^{\#\#}p<0.01$ vs. SD-SLE group.

3.8. Effects of OL and Per-OL supplemented diets on inflammasome: canonical and non-canonical signaling pathways in peritoneal macrophages from SLE-mice

NLRP family pyrin domain-containing 3 inflammasome (NLRP3) is considered to oligomerize and recruit apoptosis-associated speck-like protein containing (ASC) and pro-caspase-1, thereby triggering the maturation of IL-1β and IL-18 (Zhang, Liu and Li, 2018). For this reason, we decided to evaluate the effects of OL and Per-OL on this multiprotein complex modulation deeping in the canonical and non-canonical activation. We observed a significant increment of the expression of ASC and NLRP3 in murine peritoneal macrophages obtain from SLE-induced group, but OL and Per-OL supplemented diet could able to reduce them ($^{*}p<0.05$ and $^{***}p<0.001$ vs. SD-sham group; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. SD-SLE group). Indeed, we found that OL and Per-OL reduced the maturation and secretion of pro-inflammatory cytokines like IL-1β and IL-18, in comparison with pristane group animals ($^{***}p<0.001$ vs. SD-sham group; $^{\#\#\#}p<0.001$ vs. SD-SLE group) (Fig. 7A). Therefore, we evaluated the activation of caspase-1 in peritoneal macrophages obtain from SLE-induced stimulated with LPS, and we could observe a significantly reduction of

expression of cleaved-caspase-1 with OL and Per-OL supplemented diet (**p*<0.05 vs. SD-sham group; #*p*<0.05, ##*p*<0.01 vs. SD-SLE group) (Fig. 7B).

To study the possible implication of non-canonical inflammasome NLRP3 activation, we evaluated the effect of OL and Per-OL in peritoneal macrophages in SLE-induced mice stimulated with LPS, and in this case we only could observed a significantly decrease of caspase-11 activation in Per-OL group in comparison with pristane control group (**p*<0.05 vs. SD-sham group; #*p*<0.05 vs. SD-SLE group) (Fig. 7B).

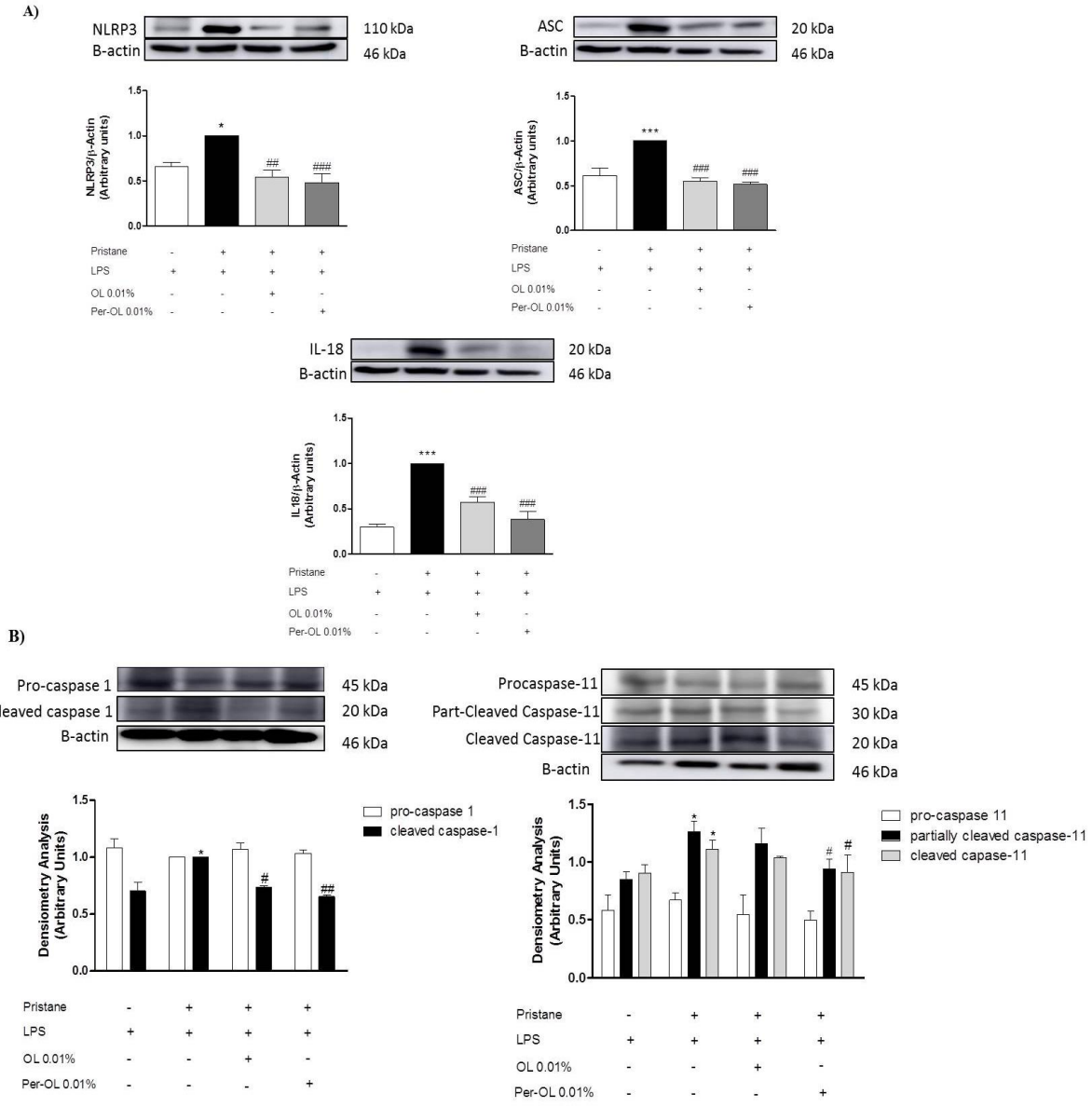


Figure 7: Effect of OL and Per-OL dietary treatment on NLRP3 inflammasome complex in LPS-stimulated macrophages from pristane-induced SLE mice. (A) OL and Per-OL diet modulated NLRP3, ASC and IL-18 protein expression levels in LPS-stimulated peritoneal macrophages from pristane-induced SLE mice. **(B)** OL and Per-OL dietary treatments modulated canonical and non-canonical inflammasome pathway in peritoneal macrophages from pristane-induced SLE mice stimulated with LPS. Densitometry analysis was performed following normalization to the control (β-actin housekeeping gene). Data are expressed as the mean ± S.E.M. (n=6). $^{*}p<0.05$, $^{***}p<0.001$ vs. SD-sham group; $^{\#}p<0.05$; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. SD-SLE group.

4. DISCUSSION

In the present study, we have depth insight into the effects of dietary treatments OL and its new lipophilic acetyl derivative Per-OL on murine peritoneal macrophages from pristane-SLE mice, evaluating the clinical, pathological, and mechanistic contribution. Patients with SLE show an impaired oxidative status and increased levels of IL-1β, TNF, IL-17 and IL-18, which are closely linked to inflammation and correlated with disease activity (Wu *et al.*, 2016). It has been reported that IL-1β can promote the proliferation, migration, and invasion of other cells via activating STAT3. Besides, STAT3 signaling plays a crucial role in the Th17 generation and an upregulated STAT3/IL-17 expression had been described in lupus patients (Chen *et al.*, 2019). In the present study, we found that OL and Per-OL dietary treatments reduced the inflammatory infiltrated in kidneys. Anti-inflammatory response was accompanied by a significant reduction of Th1, Th2 and Th17 cytokines levels as well as p-STAT3 downregulation in LPS-stimulated macrophages from OL and Per-OL treated SLE mice.

Our results are in agreement with Campolo et al., (Campolo *et al.*, 2013) who observed that OL was capable of reducing the secondary injury associated to intestinal damage attenuating TNF-α and IL-1β production in the ileum from splanchnic arterial occlusion shocked mice. Similarly, administration of OL caused a significant reduction of TNF-α and IL-1β, in a mouse model of carrageenan-induced pleurisy (Impellizzeri *et al.*, 2011). In addition, Ryu et al. showed that OL reduced LPS-induced nitric oxide (NO) production in murine macrophages by suppressing iNOS and COX-2 as well as the release of pro-inflammatory IL-1β and IL-6 (Ryu *et al.*, 2015). In this line, several authors have reported that STAT3 signaling regulates the inhibition of myocardial isquemia/reperfusion in rats treated with OL (Jin *et al.*, 2018) and the inflammatory response in OL- and Per-OL- treated LPS-induced murine peritoneal macrophages (Castejon *et al.*, 2019).

Recently, it has been described that acute consumption of olive oil decreased the activation of NF-κB system on mononuclear cells from healthy donors and that OL inhibits LPS-triggered NF-κB and activator protein (AP)-1 activation. NF-κB induces the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines such as TNF-α, IL-1β, IL-

6, iNOS and COX-2, among others, and also participates in the regulation and assembly of inflammasome complex contributing to the initiation and development of inflammatory diseases (Liu *et al.*, 2017).

By inhibiting the activation of NF-κB, the production of inflammatory mediators under its control may be reduced. In this regard, Miles et al. demonstrated that OL significantly decreased the concentration of IL-1β in LPS-stimulated human whole blood culture (Carluccio *et al.*, 2003; Miles, Zoubouli and Calder, 2005; Perez-Martinez *et al.*, 2007). Similar results were described in human synovial fibroblast cell line SW982 where OL as well as the phenolic fraction from EVOO exerted anti-inflammatory and anti-oxidant effects via down-regulation of MAPKs and NF-κB signaling pathways and induction of nuclear factor E2-related factor 2 (Nrf2)-linked heme oxygenase-1 (HO-1) controlling the production of inflammatory mediators such as IL-6 and TNF-α cytokines, metalloproteinase (MMP)-1 and MMP-3 levels and microsomal prostaglandin E synthase- 1 (mPGEs-1) and COX-2 overexpression (Castejon *et al.*, 2017; Rosillo *et al.*, 2019).

The inflammasome was described as a large intracellular signaling complex that contains a cytosolic pattern recognition receptor, especially a NLRP. Among NLR inflammasome complex, the most characterized is NLRP3 inflammasome (Jo *et al.*, 2016). Inflammasome machinery is dysregulated in SLE and plays an important role in promotion of organ damage. Maturation and secretion of IL-1β and IL-18 translation are mediated by inflammasome activated caspase-1 (Shirato *et al.*, 2017). Canonical inflammasome induce inflammatory response by processing inactive procaspase-1 into cleaved caspase-1. Caspase 1 is able to regulate the maturation of IL-1β and IL-18 into active cytokines which are involved in the lupus injury(Jo *et al.*, 2016). Besides, non-canonical inflammasome there is an alternative via which has been described to activate caspase-11, and also serve as an additional pathway of maturation and secretion of IL-1β and IL-18 in macrophage-mediate immune response (Kayagaki *et al.*, 2011).

We found, for the first time that OL and Per-OL dietary treatments inhibited canonical and non-canonical activation of NLRP3 inflammasome/IL-1β in murine peritoneal macrophages isolated from pristane-SLE mice. This important finding indicates that OL and Per-OL inhibited disease progression which was at least partly dependent on inhibition of NLRP3 inflammasome. Similar data were found after HTy and peracetylated-hydroxytyrosol (Per-HTy) treatments on LPS-induced inflammatory response in murine peritoneal macrophages (Montoya *et al.*, 2018).

Taken together, OL and Per-OL exerted a protective effect against inflammatory lupic response in murine peritoneal macrophages from pristane-SLE mice via STAT3 and NF-κB signaling expression coupled with inhibition of canonical and non-canonical NLRP3 inflammasome.

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CHAPTER V

DIETARY OLEUROPEIN AND ITS NEW ACETYL-DERIVATIVE, ATTENUATE MURINE LUPUS NEPHRITIS THROUGH HO-1/NRF2 ACTIVATION AND SUPPRESSING JAK/STAT, NF-KB, MAPK AND NLRP3 INFLAMMASOME SIGNALING PATHWAYS

Capítulo II

DIETAS ENRIQUECIDAS CON OLEUROPEINA Y SU NUEVO ACETIL-DERIVADO MODULAN LA NEFRITIS LÚPICA MURINA A TRAVÉS DE LA ACTIVACIÓN DE LA VÍA DE SEÑALIZACIÓN NRF2/HO-1 JUNTO A LA SUPRESIÓN DE LAS VÍAS JAK/STAT, NF-KB, MAPKS Y DEL INFLAMASOMA NLRP3

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RESUMEN

El lupus eritematoso sistémico (LES) es una enfermedad autoinmune inflamatoria crónica que puede afectar a múltiples órganos y para la que actualmente no se dispone de un tratamiento efectivo y seguro. Por lo tanto, la terapia nutricional puede constituir una interesante alternativa para el tratamiento del LES, debido a sus potenciales beneficios sin los efectos adversos asociados a la farmacoterapia clásica. Los extractos de la hoja del olivo tienen un alto interés debido a sus múltiples efectos terapéuticos. La oleuropeína (OL) es el componente mayoritario de los extractos de las hojas del olivo y posee muchas propiedades beneficiosas.

En este estudio, evaluamos los efectos de dos dietas suplementadas con OL y un derivado semisintético, oleuropeína peracetilada (Per-OL) en un modelo de LES inducido por pristano en ratones BALB/c.

Los ratones recibieron una inyección intraperitoneal de pristano o solución salina según los grupos experimentales y fueron alimentados con dietas experimentales enriquecidas con OL y con Per-OL. Los cambios en los niveles de citoquinas y marcadores pro-inflamatorios fueron evaluados por la técnica de ELISA. Los cambios en la expresión proteica de las enzimas óxido nítrico sintasa inducible (iNOS), prostaglandina E sintasa microsomal 1 (mPGEs-1) así como la hemo-oxigenasa (HO-1), factor nuclear eritroide-2 (Nrf2), las proteínas cinasas p38, C-jun NH_2-terminal cinasa (JNK) y cinasa regulada por señal extracelular (ERK), el factor de transducción de la señal y activador de la transcripción (STAT)-3, el factor nuclear-kappa B (NF-κB) junto a la activación del inflamasoma NLRP3 fueron determinados mediante la técnica de Western Blotting.

OL y Per-OL redujeron significativamente el daño renal y redujeron los niveles séricos de la metaloproteinasa (MMP)-3 así como los niveles de prostaglandina E_2 (PGE_2) renales. Nuestros estudios indicaron que las dietas enriquecidas con OL y Per-OL producen un incremento en la

expresión de la vía antioxidante de Nrf2/HO-1, junto a una disminución de la activación de las vías de señalización janus quinasas-transductor de señal y activador de la transcripción (JAK/STAT), de las proteínas cinasas activadas por mitógenos (MAPKs), NF-κB e inflamasoma NLRP3.

Estos resultados sugieren que la suplementación dietética con OL y Per-OL podrían constituir una nueva alternativa de abordaje terapéutico como preventivo o paliativo de la nefritis en el manejo del LES.

Chapter V

DIETARY OLEUROPEIN AND ITS NEW ACETYL-DERIVATE, ATTENUATE MURINE LUPUS NEPHRITIS THROUGH HO-1/NRF2 ACTIVATION AND SUPPRESING JAK/STAT, NF-KB, MAPK AND NLRP3 INFLAMMASOME SIGNALING PATHWAYS

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ABSTRACT

Systemic Lupus Erythemathosus (SLE) is a chronic inflammatory and autoimmune disease which can affect multiple organ system, without an effective and safe treatment. Olive leaf extracts are of special interest for their therapeutic effects. Oleuropein (OL) is the most abundant constituents of olive leaf extract and possess many beneficial properties.

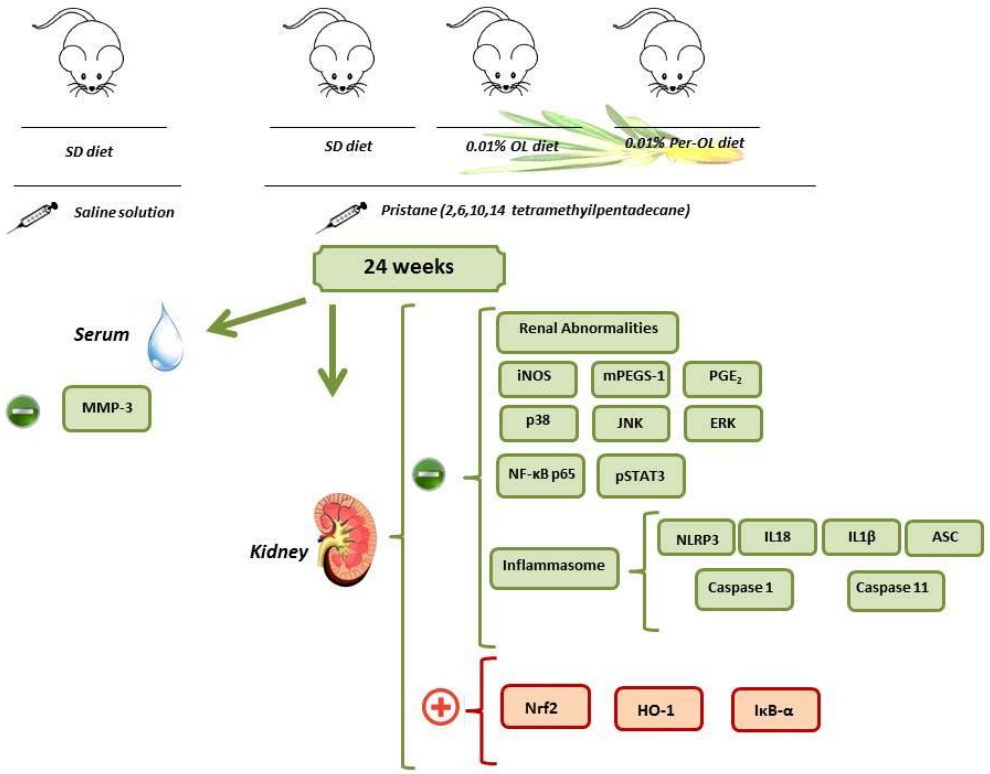
In this study, we evaluated the effects of dietary OL and its new derivate, Peracetylated oleuropein (Per-OL), in a pristane-induced SLE model.

Mice received an injection of pristane or saline solution and were fed with experimental diets: enriched with OL and Per-OL. The levels of proinflammatory cytokines and markers were evaluated by enzyme linked immunosorbent assay (ELISA). The protein expressions of nitric oxide synthase inducible (iNOS), microsomal prostaglandin E synthase 1 (mPGEs-1), heme oxygenase-1 (HO-1), nuclear factor E2-related factor 2 (Nrf2), mitogen-activated protein kinases (MAPKs), Janus kinase/signal transducer and activator of transcription (JAK/STAT), nuclear transcription factor-kappa B (NF-κB) and inflammasome nucleotide-binding domain, leucine-rich repeats-containing family, pyrin domain-containing-3 (NLRP3) pathways activation were determined in kidneys by Western Blot.

OL and Per-OL significantly reduced renal damage and decreased serum matrix metalloproteinase 3 (MMP-3) and prostaglandin E_2 (PGE_2) kidneys levels. Our findings indicate that Nrf2 and HO-1 antioxidant protein expressions were up-regulated in mice fed with OL and Per-OL diets, whereas the activation of JAK/STAT, MAPK, NF-κB and NLRP3 inflammasome pathways were significantly ameliorated.

These results suggest that OL and Per-OL supplementation might provide a new alternative approach as a preventive/palliative treatment of nephritis in SLE management.

KEYWORDS: HO-1/Nrf2; inflammasome; lupus nephritis; MAPK; NF-kB; oleuropein.



1. INTRODUCTION

Systemic lupus erythematosus (SLE) is a highly heterogeneous disorder, characterized by differences in autoantibody profile, serum cytokines, and a multi-system involvement commonly affecting the skin, musculoskeletal, and hematopoietic systems clinical manifestations involving with a special importance of the renal damage (Goulielmos *et al.*, 2018). Pathogenic drivers of SLE are multifactorial and are not fully known. Interaction of genetic susceptibility with environmental and potential stochastic factors leads to an early break of immune tolerance with preclinical autoimmunity (Rose and Dörner, 2017). Immunological abnormalities includes imbalance of T-helper-cell (Th) subsets (Th1/Th2/Th17) and regulatory T-cells (Treg), resulting in a loss of self-tolerance, activation of auto reactive T and B cells and production of large amounts of pro-

inflammatory cytokines which contribute actively to tissue damage and local inflammation (Miyake, Akahoshi and Nakashima, 2011).

Previous studies have demonstrated that the molecular signaling pathways involved in SLE include nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPKs), Janus kinase and signal transducer and activator of transcription proteins (JAK/STAT) , nuclear factor E2-related factor 2 (Nrf2)/ heme oxygenase-1 (HO-1) pathways. More recently, the nucleotide-binding domain, leucine-rich repeats-containing family, pyrin domain-containing-3 (NLRP3) inflammasome which integrates caspase-1 activation resulting in the cleavage of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-18 precursors to their mature forms and their eventual secretion induces pyroptotic cell death, which contributing to both, host defense and sterile inflammation (Schroder and Tschopp, no date; Kuryłowicz and Nauman, 2008; Kawasaki *et al.*, 2011; Lorenz, Darisipudi and Anders, 2014; Nie *et al.*, 2015; Zhao *et al.*, 2015; Aparicio-Soto, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2017; Liu *et al.*, 2018).

The treatments of SLE include a numerous options like as corticosteroids, immunosuppressive drugs and cell-based therapies. Despite of the improvements in care, patients usually suffer a loss of quality of life due to the long-used of these medications (Aparicio-Soto *et al.*, 2016). Therefore, more effective and less toxic therapies for SLE are required. For this purpose, the interest on nutritional therapy and dietary supplements is currently increasing. Phenolic compounds, which are typically included in Mediterranean diet through extra virgin olive oil (EVOO), olives and wine, have shown a wide range of bioactive properties. In this context, our research group have previously demonstrated the anti-inflammatory and immunomodulatory properties of hydroxytyrosol (HTy), tyrosol (Ty), hydroxytyrosyl acetate (HTy-Ac), oleocanthal and oleuropein (OL) on lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages (Aparicio-Soto *et al.*, 2014) on IL-1β-stimulated human synovial fibroblasts cell line SW982 (Castejón *et al.*, 2017; Rosillo *et al.*, 2017) and on experimental models of chronic autoimmune diseases such as SLE, rheumatoid arthritis (RA) and ulcerative colitis (UC) (Cardeno *et al.*, 2014; Rosillo *et al.*, 2014; Aparicio-Soto *et al.*, 2015).

OL is the main secoiridoid component of olive leaves, roots and unprocessed olive drupes, which is hydrolysed by endogenous β-glucosidases during fruit maturation and forms different products, including HTy. It contributes to the bitter taste and stability of olive oil and functions as a hydrophilic phenolic antioxidant (Cornwell and Ma, 2008; Barbaro *et al.*, 2014; Imran *et al.*, 2018). Previous studies have reported its antioxidant, anti-inflammatory, anti-allergic, anti-atherogenic, anti-thrombotic, and anti-mutagenic effects (Barbaro *et al.*, 2014; Gorzynik-Debicka *et al.*, 2018; Nassir *et al.*, 2019; Zhang and Zhang, 2019). In fact, OL was able to suppress inflammatory response on LPS-stimulated RAW 264 (Ryu *et al.*, 2015; Mao *et al.*, 2019). Also it has been shown to exert a significantly protective effects in a mouse model of carrageenan-induced pleurisy (Impellizzeri *et*

al., 2011) , on cartilage slowing down the progression of osteoarthritic lesions in Guinea pigs (Horcajada *et al.*, 2015), and in several models of neuroinflammation (Hornedo-Ortega *et al.*, 2018).

Besides, some studies have shown the importance of acetyl derivatives of natural phenols due to their lipophilic nature allows them to cross the cytoplasmic cell membranes and their uptake by cells, providing a potential protection of membrane constituents. Thus, this increased lipophilicity means that peracetylated-oleuropein (Per-OL) could be better absorbed across intestinal epithelial cell monolayers than OL. Also a deacetylation step by lipases of the human digestive system may delay the excretion process (Stamatopoulos, Chatzilazarou and Katsoyannos, 2013). Besides, acetyl-derivatives have shown anti-inflammatory activities greater than parent compounds putting in evidence a strict correlation between lipophilicity and bioavailability (de Araújo *et al.*, 2017; Rizzo *et al.*, 2017). Consequently, the acetylation may improve pharmacodynamics and pharmacokinetics profiles compared to the natural compound and it could be an appealing strategy in the management of different inflammatory process (Montoya *et al.*, 2018).

Taking this into account, the aim of the present study was to investigate the potential effects of OL and its new acetyl-derivative, Per-OL, enriched-diets in murine pristane-induced nephritis on the production of inflammatory mediators focusing into the action mechanisms and signaling pathways involved after dietary treatments.

2. MATERIALS AND METHODS

2.1. Reagents

OL was extracted according to reported literature (Stamatopoulos, Chatzilazarou and Katsoyannos, 2013), following the purification by column chromatography (CH_2Cl_2-MeOH 10:1→5:1) to give the product as a yellow solid. The synthesis of Per-OL was carried out from OL. It was solved and stirred in a mixture of 1:1 (v/v) pyridine/acetic anhydride at 8ºC for 10 min. Then, the reaction was kept at room temperature overnight. After hydrolysing the acetic anhydride, the solution was concentrated to dryness obtaining a residue that was purified by column chromatography (EtOAc-C. Hexane 1:1) to give the product (quant.) as a brown solid. Spectroscopy data was identical to reported literature (Procopio AS M.; Costa, N.; Nardi, M., 2008).

2.2. Pristane induction of SLE-like disease

Sixteen-weeks-old BALB/c mice received an intraperitoneal injection of 0.5 mL pristane (99% pure, Sigma Aldrich Co®, St Louis, MO, USA) to induce SLE according to previous described procedure by Satoh and Reeves, 1994 (Satoh and Reeves, 1994). After 24 weeks (experimental period), animals were sacrificed and blood and kidneys were collected.

2.3. Animals and diets

To evaluate the beneficial effects of OL and Per-OL on pristine-SLE induced model, 60 twelve-week-old BALB/c mice (27.32 ± 0.37 g) were used (Janvier Labs®, Saint-Berthevin Cedex, France). Mice were maintained under constant conditions of temperature (20-25 ºC) and humidity (40-60%) with a lighting regimen of 12L/12D. They were fed with standard rodent diet (Panlab A04®, Seville, Spain) and water ad libitum until SLE-like disease pristane induction.

Mice were randomized in four experimental groups: (i) Sham group were fed with standard diet (SD) (SD-sham group) (n=10), (ii) pristane group received SD (SD-SLE group) (n=20), (iii) pristane OL group (OL) were fed with SD supplement with OL (100 mg/kg diet) (n=15) and (iv) pristane Per-OL group (Per-OL) were fed with SD supplement with Per-OL (100 mg/kg diet) (n=15). Fresh diets were provided every day, and experimental diets were formulated on the basis of the American Instituted of Nutrition (AIN) standard reference diet, and were prepared by mixing the pertinent compounds and stored at -80ºC.

Mice were fed with the related diets along six months. Weight, water and food consumption were monitored weekly. The food consumption was on average 4 g/day, with an estimated intake of 4 mg of OL or Per-OL per mice, in accordance with previous data (Aparicio-Soto *et al.*, 2017). All animal care and experimental procedures complied with the Guidelines of the European Union regarding animal experimentation (Directive of the European Counsel 2010/630/EU) and followed a protocol approved by the Animal Ethics Committee of the University of Seville.

2.4. Histological studies of renal tissue

Kidneys were collected, fixed in 4% formaldehyde, and embedded in paraffin. Samples were sagittally sectioned at 5 μm and stained with Masson trichrome and periodic acid-Schiff (PAS); four samples per animal containing both cortex and medulla were selected for evaluation. Morphological changes were evaluated by two different observers who were unaware of the treatment group. Results are expressed as percentages of cases exhibiting histological changes.

2.5. Measurement of inflammatory markers

Bloods were harvested directly from the heart of mice, allowed to clot for 2 h at room temperature and centrifuged for 20 min at 4000 rpm, 20 ºC and stored at -80ºC. Enzyme-linked immunoassay (ELISA) kits were used to determine serum levels of matrix metalloproteinase 3 (MMP-3) (R&D Systems®, Abingdon, UK). Frozen kidney tissues were weighed and homogenized in ice-cold protease-inhibitor buffer. Homogenates were centrifuged (4000 *g*, 10 min, 4°C), and the supernatants were used for the determination of prostaglandin E_2 (PGE_2) (Cayman Chemical Company®, Ann Arbor, MI, USA) and IL-1β (BD OptEIA®, San Jose, CA) levels using the ELISA kit

according to the manufacturers' instructions. All measurements were carried out in duplicate. The results were measured in picograms per milliliter at 450 nm using an ELISA microplate reader (BioTek®, Bad Friedrichshall, Germany).

2.6. Immunoblotting

Protein concentration was measured of kidney homogenates using a protein assay reagent (BioRad®, Múnchen, Germany) according to Bradford's method (Bradford, 1976). Aliquots of these supernatants which contains equal amount of protein (50µg) were separated on 10-15% acrylamide gel by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, electrophoretically transferred into a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-iNOS (Cayman®, Ann Arbor, MI, USA) (1:1000); mouse anti-pSTAT3, mouse anti-pJNK, rabbit anti-JNK, mouse anti-pp38, rabbit anti-p38, (Cell Signaling Technology®, Danvers, MA, USA) (1:1000); rabbit polyclonal anti p-ERK and mouse anti-ERK½, rabbit anti-HO1 and rabbit anti-Nrf2 (Cell Signaling Technology®, Danvers, MA, USA) (1:500), mouse anti-NLRP3, mouse anti-ASC and rabbit anti-Caspase-1 (Cell Signaling Technology®, Danvers, MA, USA) (1:500, 1:100, 1:400, respectively), rabbit anti-Caspase-11 (novus Biologicals®, Littleton, CO, USA), rabbit anti-IL-18 (AbCam®, Cambridge, UK) (1:500) ,rabbit anti- mPGEs-1 (Cayman®, Chemical Company, Ann Arbor, MI, USA) (1:500), rabbit anti-IκB-α and rabbit anti-NFκB-p65 (Cell Signaling Technology®, Danvers, MA, USA) (1:1000) overnight at 4ºC. Then, after rising, membranes were incubated with a horseradish peroxidise (HRP)-labelled secondary antibody anti-rabbit (Cayman®, Chemical Company, Ann Arbor, MI, USA) (1:50000) or anti-mouse (Dako® Cytomation, USA) (1:50000) containing blocking solution for 2 h at room temperature. To prove equal loading, the blots were analysed for β-actin expression using an anti-b-actin antibody (Sigma–Aldrich®, MO, USA). Immunodetection was performed using enhanced chemiluminescence light-detecting kit (Pierce®, Rockford, IL, USA). The immunosignals were captured using Amersham Imager 600 (GE Healthcare®, Buckinghamshire, UK), and densitometric data were studied following normalization to the housekeeping loading control. The signals were analysed and quantified by an Image Processing and Analysis in Java (Image J®, Softonic) and expressed in relation to SD-SLE control group.

2.7. Statistical evaluation

All values in the figures and text are expressed as arithmetic means ± standard error of the mean (S.E.M.). Data were evaluated with Graph Pad Prism® Version 5.04 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance followed by Tukey's multiple-comparisons test as *post hoc* test. *P* values of < 0.05 were considered statistically significant. In the experiments involving

densitometry, the figures shown are representative of at least four different experiments performed on different days.

3. RESULTS

3.1. OL and Per-OL diets ameliorated renal abnormalities in SLE-pristane induced BALB/c mice

As shown in Figure 1, in control group or treated with OL or Per-OL diets there were no obvious kidney alterations showing a significant improvement of the cytoarchitecture. However, most mice of SD-SLE group presented some degree of renal abnormalities in comparison with SD-sham group, ranging from abundant inflammatory mononuclear cells in the renal interstitium to renal interstitial fibrosis. In relation to renal interstitial fibrosis, it also increased in pristane-treated mice and persisted randomly after OL and Per-OL diets but generally at less magnitude (Fig 1 E-F). The presence of scattered renal tubules filled with protein aggregates ("thyroidization") was also observed in around of 20% of the pristane treated-mice, however, it was rather maintained after different diets.

Oil granulomas were frequently observed in peritoneum of pristane-treated mice which were not modified after OL and Per-OL diets (Fig 1 G-H). Finally, an outstanding finding was the presence of large lightly stained areas of renal tubules in pristane-treated mice (10%), that considerably increased after OL (80%) and Per-OL (70%) diets (Fig 1 I-L). These hyaline-like renal tubule areas were not positive for the PAS technique, discarding their mucous nature.

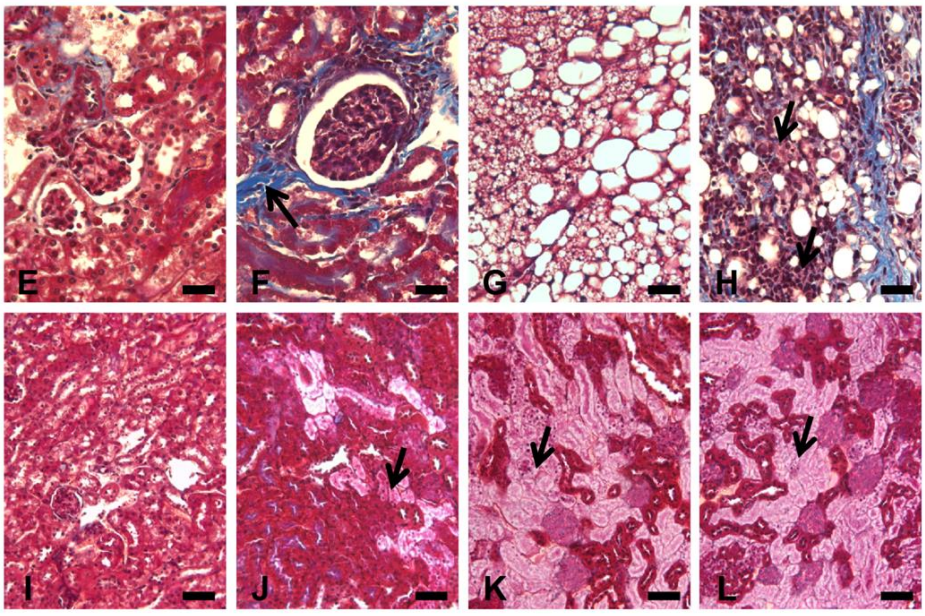


Figure 1. SLE model induced by pristane: effect of OL and Per-OL diets in renal histology. Representative histopathological appearance of mice kidneys stained and trichrome stain (E-L). Effects of OL (K) and Per-OL (L) diets in pristane-treated mice in comparison with normal (E, G, I) and pristane-treated controls (F, H, J). The presence of fibrosis (E-F), peritoneum oil granulomas (G-H) and hyaline-like renal tubule areas (I-L) can be observed in kidneys. Bar: A-H = 20 µm; I-L= 10µm.

3.2. OL and Per-OL supplemented diets decreased serum levels of MMP-3 in pristane-induced SLE

It has been reported that lupus nephritis and its progression might depend on the disintegration of the membrane due to the effects of MMP activities. To evaluate the effects of OL and Per-OL supplemented diets in MMP-3 levels in SLE, serum levels of MMP-3 were measured after sacrifice. In SD-SLE group, MMP-3 serum levels were considerably increased compared with sham group (**p<0.01 vs. SD-sham group). On the contrary, serum MMP-3 levels were significantly lower in animals which were fed with OL and Per-OL diet in comparison with SD-SLE group ($^{\#\#}p$<0.01 vs. SD-SLE group). Nevertheless, no significant statistical differences between OL or Per-OL treatments were observed (Figure 2).

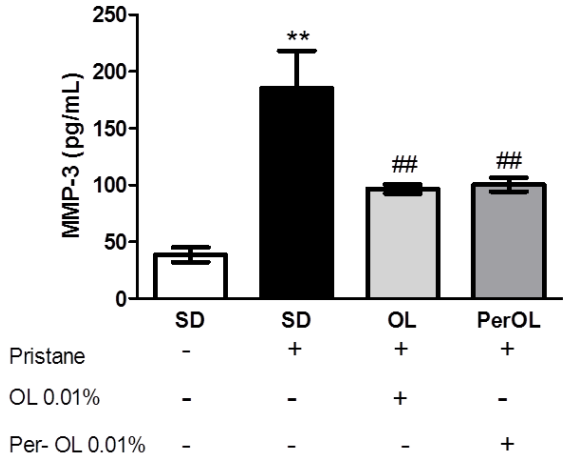
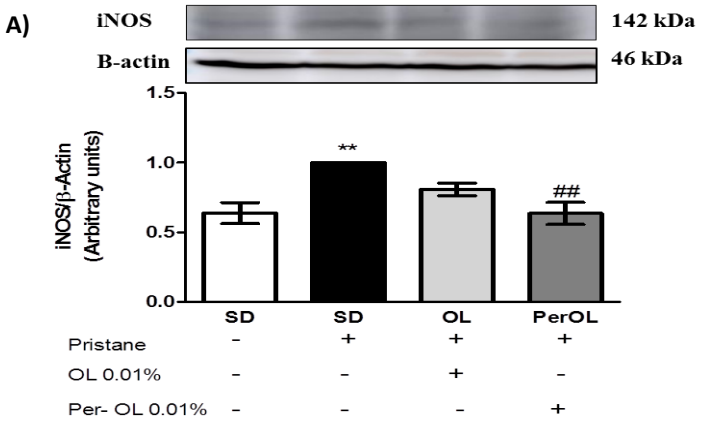


Figure 2. OL and Per-OL diets reduced the serum levels of MMP-3 from pristane-induced SLE mice. Serum samples from mice in each group were collected at the end of the experiment and the MMP-3 levels were detected by ELISA. Data are represented as mean ± S.E.M. (n=10). **p<0.01 vs. SD-sham group; $^{\#\#}p$<0.01 vs. SD-SLE group.

3.3. Effects of OL and Per-OL supplemented diets on inducible nitric oxide synthase (iNOS) and microsomal prostaglandin E synthase -1 (mPGEs-1) protein expression and PGE_2 production in kidney in pristane-induced SLE

Pristane-induced SLE is characterized by an increasing of iNOS expression in kidney (Botte *et al.*, 2014). Accordingly, iNOS expression was increasing in SD-SLE group mice in comparison with SD-sham group (***p*<0.01 vs. SD-sham group). Furthermore, the expression of this parameter was significantly decreased in animals fed with Per-OL supplemented diet, in comparison with pristane group (##*p*<0.01 vs. SD-SLE group) whereas OL-SLE group showed similar levels to SD-Sham group (Figure 3A).

In other hand, previous studies have demonstrated that mPGEs-1 and PGE_2, play an important role in inflammatory kidney injury (Lazarus *et al.*, 2001; Suganami *et al.*, 2003). To determine whether OL and Per-OL diet could modulate PGs, we analyzed mPGEs-1 protein expression and kidney PGE_2 levels production. Both parameters were considerably augmented in pristane group in comparison with sham group (***p*<0.01; ****p*<0.001 vs. SD-sham group), however, a significant reduction was observed in animals which were fed with experimental diets (OL and Per-OL) in comparison with SD-SLE group (#*p*<0.05; ##*p*<0.01; ###*p*<0.001 vs. SD-SLE group) showing similar levels to SD-Sham group (Figure 3B).



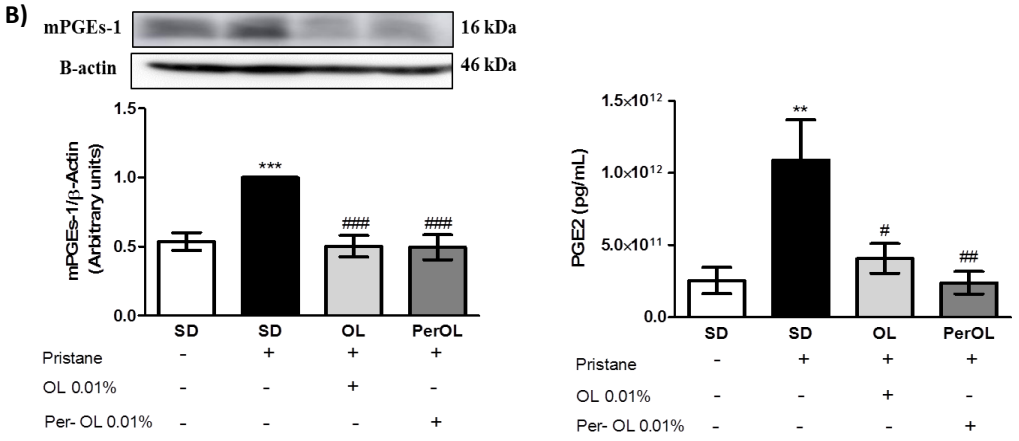


Figure 3. (A) OL and Per-OL diet modulated iNOS protein expression in kidneys from pristane-induced SLE mice. (B) OL and Per-OL diet down-regulated the mPGEs-1 protein expression and PGE_2 production in kidneys from pristane-induced SLE mice. PGE_2 levels were determined by ELISA. Data are represented as mean ± S.E.M. (n=10). Proteins obtained from kidney tissues of OL and Per-OL treated mice were analyzed by western blot using specific antibody against iNOS and mPGEs-1. Densitometry analysis was performing following normalization to the control (β-actin housekeeping gene). Data are expressed as the mean ± S.E.M. (n=6). $^{**}p<0.01$,$^{***}p<0.001$ vs. SD-sham group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. SD-SLE group.

3.4. OL and Per-OL supplemented diets down-regulated HO-1 protein expression via Nrf2 activation in kidney from of SLE-pristane induced BALB/c mice

To recognize whether up-regulation of Nrf-2-dependent signaling by OL and Per-OL supplemented diets could diminish renal inflammation in pristane-induced kidney damage, we measured Nrf-2 and HO-1 protein expressions by western blotting. As shown in Fig. 4, protein expressions of HO-1 and Nfr2 were significantly down-regulated in pristane group compared with sham group ($^{*}p<0.05$ vs. SD-sham group). Nonetheless, OL supplemented diet produced an increment of the expression of Nrf2 in comparison with pristane group ($^{\#\#}p<0.01$ vs. SD-SLE group), while a significant up-regulation of both proteins were detected in Per-OL supplemented diet ($^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ vs. SD-SLE group).

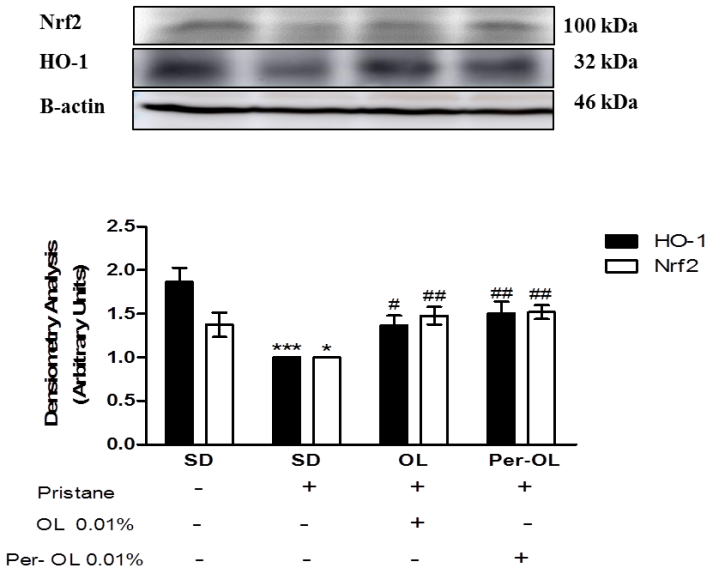


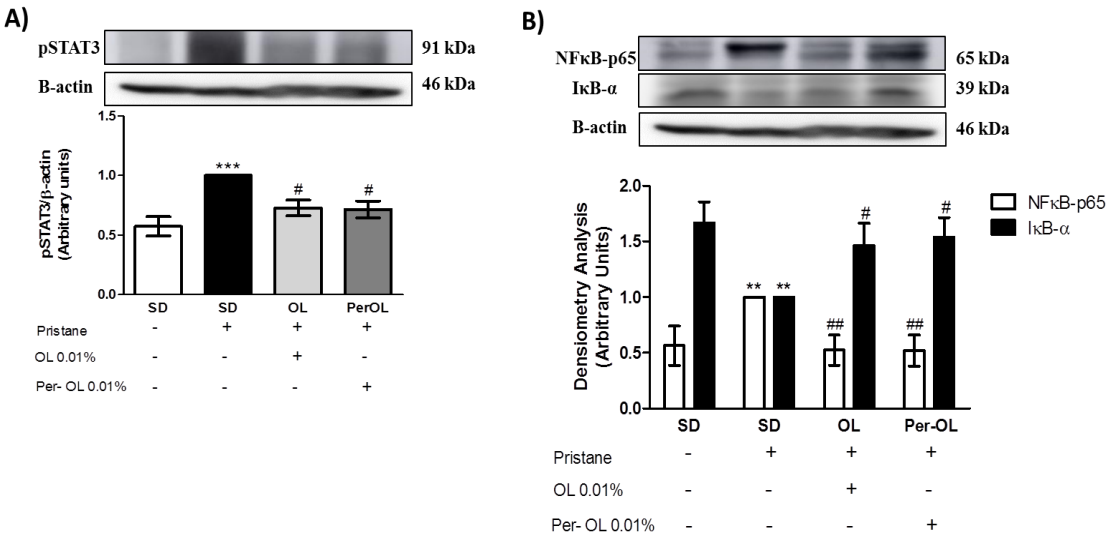
Figure 4. OL and Per-OL dietary induced the up-regulation of HO-1 and Nrf2 protein expressions in kidneys from pristane-induced SLE mice. Densitometry analysis was performed following normalization to the control (β-actin housekeeping gene). Data are expressed as the mean ± S.E.M. (n=6). *p<0.05, ***p<0.001 vs. SD-sham group; #p<0.05,##p<0.01 vs. SD-SLE group.

3.5. OL and Per-OL diets modulates signal transducer and activator of transcription (STAT)-3, NF-κB and MAPKs signaling pathways in pristane-induced SLE

To have to delve in the study of mechanisms underlying in the effects of OL and Per-OL diet anti-inflammatory effects in SLE model, we also determined the role of STAT-3, NF-κB and MAPK signaling pathways. According to our results, we observed a markedly increment of p-STAT-3 expression in SD-SLE group compared with SD-sham group (***p<0.001 vs. SD-sham group), but this parameter were significantly down-regulated with OL and Per-OL diet (#p<0.05 vs. SD-SLE group) (Fig. 5A).

Also, as shown in Fig. 5B, we observed an important degradation of the inhibitor of NF-κB (IκB)-α in kidney in pristane group mice (**p<0.01 vs. SD-sham group); nonetheless, Per-OL diet was able to prevent its degradation after pristane injection (#p<0.05 vs. SD-SLE group). For other hand, p65 translocation into the nucleus was remarkably increased after pristane injection (**p<0.01 vs. SD-sham group), whereas OL and Per-OL supplemented diet were able to prevent the nuclear migration (##p<0.01 vs. SD-SLE group). Similarly, we observed an increase of p38, C-jun NH_2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) phosphorylation after pristane injection (**p<0.01; ***p<0.001 vs. SD-sham group). On the contrary, there was a significant

prevention of MAPKs phosphorylation in OL and Per-OL groups ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ vs. SD-SLE group) (Fig.5C).



C)

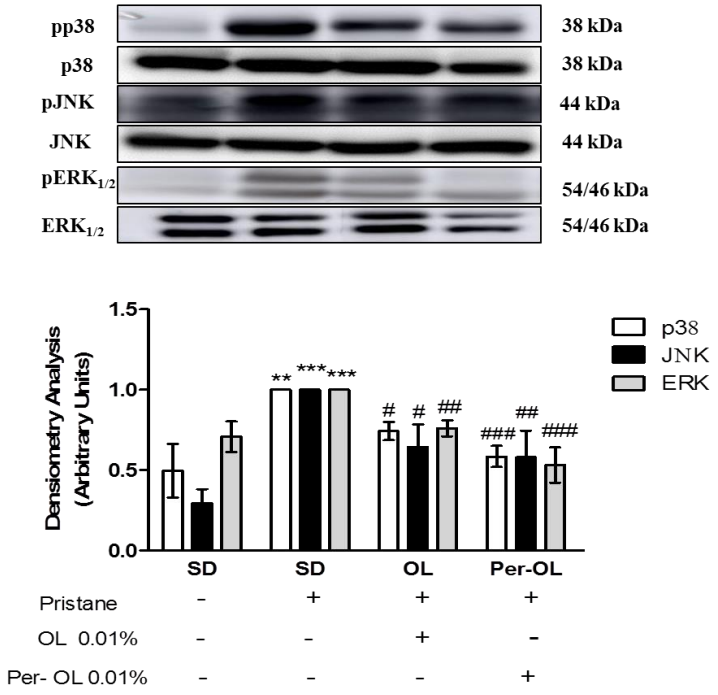


Figure 5. (A) OL and Per-OL dietary treatment modulated STAT3 and prevented inhibitor of NF-κB (IκB-α) degradation and nuclear p65 migration (B) in kidneys from pristane-induced SLE mice. Densitometry analysis was performed following normalization to the control (β-actin housekeeping gene). **(C) Dietary treatment of OL and Per-OL down-regulated the renal expression levels of pp38, pJNK and pERK$_{1/2}$ MAP kinases in pristane-induced SLE mice.** Densitometry analysis was performed following normalization to the control (p38, JNK and ERK$_{1/2}$ housekeeping genes, respectively). Data are expressed as the mean ± S.E.M. (n=6). **p<0.01, ***p<0.001 vs.SD-sham group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 vs. SD-SLE group.

3.6. Effects of OL and Per-OL diet on inflammasome: canonical and non-canonical signaling pathways in pristane-induced SLE

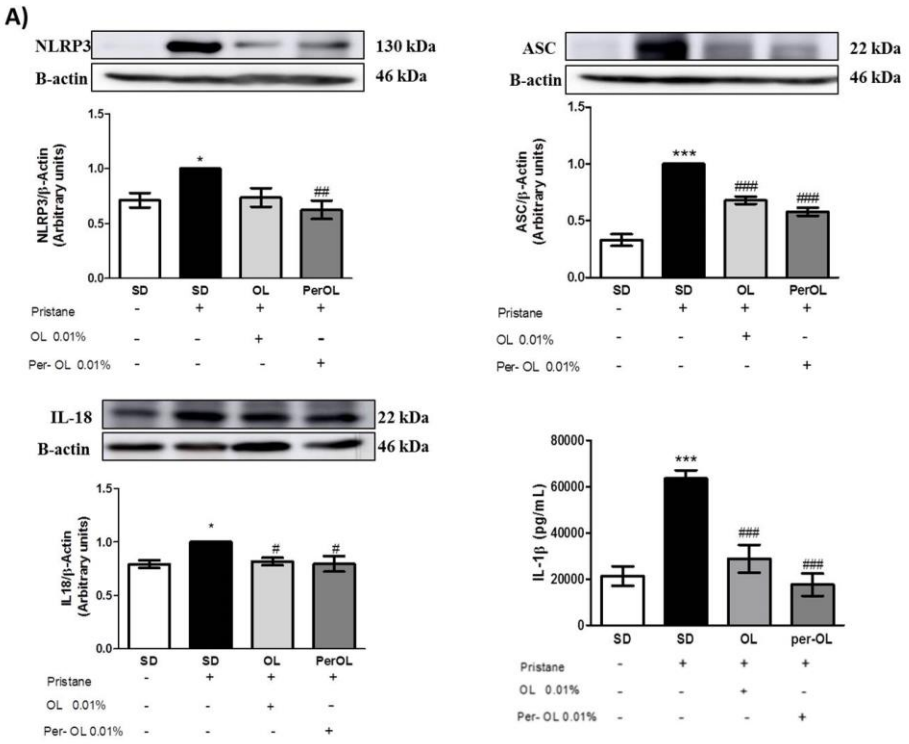
Recent studies suggest that inflammasome complex is dysregulated in SLE, playing an important role in promotion of organ damage and development of lupus pathologies (Kahlenberg and Kaplan, 2014). For this reason, we decided to study the effects of OL and Per-OL on this multiprotein complex modulation. The NLRP3 inflammasome complex, comprising NLRP3, apoptotic speck protein containing a caspase recruitment domain (ASC), and cysteine aspartic acid protease 1 (Caspase 1) (Li *et al*., 2018).

In our studies, we observed how pristane injection produced an important increased of IL-18 (*p<0.05 vs. SD-sham group), IL-1β levels (***p<0.001 vs. SD-sham group) and NLRP3 expression (*p<0.05 vs. SD-sham group); nevertheless, these parameters were down-regulated with OL and

Per-OL supplemented diet ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ vs. SD-SLE group). Furthermore, ASC expression were notably augmented in pristane group compared with sham group ($^{***}p<0.001$ vs. SD-sham group) whereas OL and Per-OL supplemented diet group had a significant down-regulation of its expression ($^{\#\#\#}p<0.001$ vs. SD-SLE group) (Fig. 6A).

Also, we have studied the canonical inflammasome activation through the caspase-1 protein expression. We have observed a remarkably increment of cleaved caspase-1 in SD-SLE mice in comparison with SD-sham group ($^{*}p<0.05$ vs. SD-sham group), but it has been modulated with enriched-diets ($^{\#}p<0.05$ vs. SD-SLE group). We could not observe any significant changes in pro-caspase-1 expression in any experimental groups (Fig. 6B).

According to Kayagaki et al., (Kayagaki *et al.*, 2011), there is an alternative mechanism for caspase-1 activation: the non-canonical inflammasome pathway, which involves caspase-11 to activate procaspase-11 and the subsequent release IL-1β and IL-18 (Montoya *et al.*, 2018). Non-canonical effects of NLRP3 and ASC also could contribute to lupus nephritis (Lorenz, Darisipudi and Anders, 2014). Taking this into account, we decided to determine the OL and Per-OL diet effects on non-canonical inflammasome pathway through the caspase-11 activation. In Fig. 6C, we represented the procaspase-11 partially cleaves and cleaved caspase-11 after pristane injection ($^{*}p<0.05$; $^{***}p<0.001$ vs. SD-sham group). We could demonstrated that Per-OL supplemented diet was a significant down-regulation of caspase-11 protein expression (procaspase and cleaved forms) in comparison with SD-SLE group ($^{\#}p<0.05$; $^{\#\#}p<0.01$ vs. SD-SLE group).



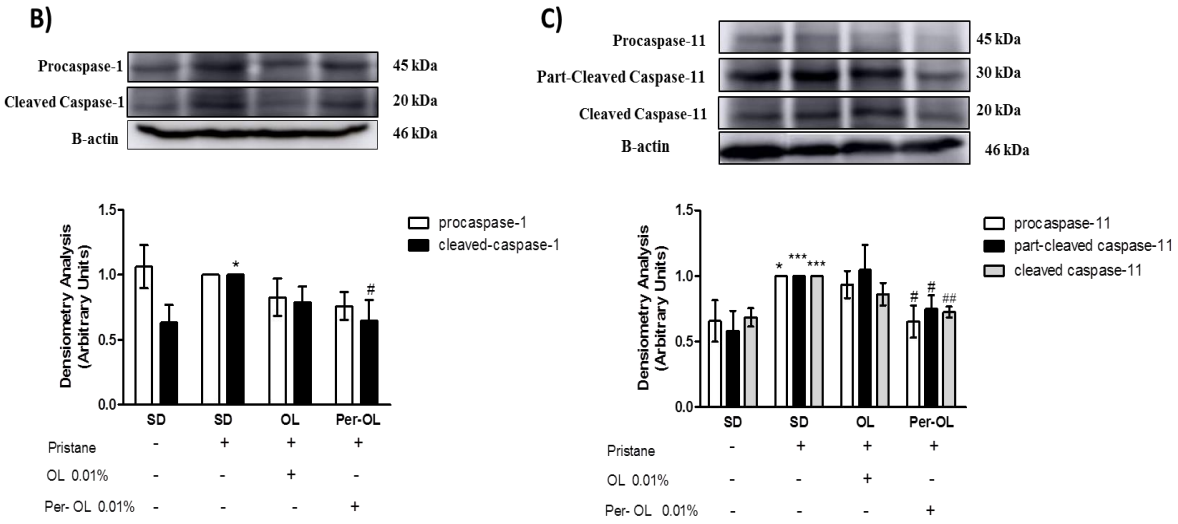


Figure 6: Effect of OL and Per-OL dietary treatment on NLRP3 inflammasome complex in kidneys from pristane-induced SLE mice. **(A)** OL and Per-OL diet modulated NLRP3, ASC and IL-18 protein expression levels, and down-regulation IL-1β levels in kidneys from pristane-induced SLE mice. IL-1β levels were determined by ELISA. Data are represented as mean ± S.E.M. (n=16) and densitometry analysis was performed following normalization to the control (β-actin housekeeping gene). Data are expressed as the mean ± S.E.M. (n=6). **(B)** OL and Per-OL dietary treatment modulated canonical and non-canonical inflammasome pathway **(C)** in kidneys from pristane-induced SLE mice. Densitometry analysis was performed following normalization to the control (β-actin housekeeping gene). Data are expressed as the mean ± S.E.M. (n=6).*p<0.05, ***p<0.001 vs. SD-sham group; #p<0.05; ##p<0.01 and ###p<0.001 vs. SD-SLE group.

4. DISCUSSION

SLE is as autoimmune disease, characterized by autoantibody generation, immune complexes, autoreactive or inflammatory T cells and inflammatory cytokines that may initiate and amplify inflammation and damage to various organs such as kidney contributing to the clinical manifestations of SLE (Aparicio-Soto, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2017; Xu and Li, 2018).

We have demonstrated, for the first time, that OL and Per-OL supplemented diets could modulate SLE on pristane-induced model in mice, specifically preventing renal injury through modulating NF-κB, STAT3 and MAPKs signaling pathways, and inhibiting the NLRP3 inflammasome complex.

Pristane-treated mice presented some degree of renal abnormalities in comparison with control mice, ranging from abundant inflammatory mononuclear cells in the renal interstitium to renal interstitial fibrosis. On the contrary, kidneys from pristane -treated mice fed with OL and Per-OL showed a significant improvement of the renal cytoarchitecture, The appearance of

hyaline-like renal tubule areas after pristane treatment and, preferably, after administration of oil diets, has not been previously referenced in the literature, excepting by Ruan et al. (Ruan *et al.*, 2016) that described the presence of a large lightly stained mucous-like substance. The histological aspects of renal tissue in our studies were very similar to those described by Ruan et al. but being them PAS negative, their mucous nature is questionable (Ruan *et al.*, 2016).

The MMPs are a family of zinc-dependent enzymes that degrade different components of extracellular matrix acting on pro-inflammatory cytokines, chemokines and other proteins regulating varied aspects of inflammation and immunity (Aparicio-Soto *et al.*, 2017). Particularly, MMP-3 (stromelysin) levels were markedly elevated in SLE. with a possible role in the pathogenesis of lupus nephritis (Gheita *et al.*, 2015). Accordingly with our previous findings (Aparicio-Soto *et al.*, 2016, 2017). MMP3 serum levels were increased in SD-SLE mice which, however serum MMP-3 levels were significantly reduced in those pristane-treated mice that received OL and Per-OL enriched-diets contributing to minimize pristane-induced renal damage.

PGE_2 is a bioactive lipid mediator biosynthetised by mPGEs-1 (Schneider, Boeglin and Brash, 2004), which plays a crucial role in the development of inflammatory response, acting as a mediator of Th1/Th2/Th17 cytokines and it has been implicated in the glomerular filtration regulation (Imig, Breyer and Breyer, 2002; Adamik *et al.*, 2013). Besides, PGE_2 high urinary levels of this prostanoid have been reported in lupus nephritis (Herrera-Marcos *et al.*, 2017). In previous studies by our research group (Aparicio-Soto *et al.*, 2016, 2017) high levels of PGE_2 and mPGEs-1 protein expression were found in pristane-induced SLE mice. However, both parameters were significant reduced in renal tissue of pristane-induced animals that were received OL and Per-OL supplemented diet in comparison with those animals which were fed with SD The generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) has a wide impact in acute and chronic kidney injuries in patients with lupus nephritis. In fact, nitric oxide (NO) is overproduced in the setting of lupus activity (Oates and Gilkeson, 2006). NO works as an intracellular messenger which regulates the formation of ROS and RNS synchronizing the inflammatory response (Montoya *et al.*, 2018). Our results showed, accordingly with previous studies (Jiang *et al.*, 2014) that iNOS expression was remarkably increased in kidneys from SD-SLE mice, however experimental diets enriched with OL and Per-OL could restore reduced iNOS expression.

Nrf2 signaling pathway is a crucial regulator of the antioxidant response in mammalian cells in response to endogenous and exogenous stress (Yu *et al.*, 2014) modulating the transcription of antioxidant genes, including HO-1. The induction of antioxidant enzymes has been considered to be adaptive cellular responses to oxidative stress (Lv *et al.*, 2018). Previous studies have demonstrated that Nrf2 improves lupus nephritis in a pristane- induced SLE model by neutralizing reactive oxygen species and by negatively regulating the NF-κB in patients with SLE. In addition, a significant decrease in HO-1 expression (Jiang *et al.*, 2014), specifically in monocytes (Herrada *et al.*,

2012) has been found, thus HO-1 induction could ameliorate lupus nephritis, probably by multiple mechanisms including NO synthesis suppression, inhibition of antibody production and modulation of cytokine production (Takeda *et al.*, 2004). In our studies, OL and Per-OL dietary treatments strongly could reestablish Nrf2 and HO-1 expressions conferring a role of Nrf2/HO-1 signaling in the beneficial effects of dietary OL and Per-OL in this SLE model. Furthermore, these data are in agreement with our previous results in pristine- induced SLE model (Aparicio-Soto *et al.*, 2016, 2017; Rosillo *et al.*, 2016). According to the results, Jiang et al., (Jiang *et al.*, 2014) had established that Nrf2 inhibits SLE development of lupus by suppressing NF-κB-mediated inflammatory response as well.

NF-κB is a redox sensitive transcription factor which controls the expression of several genes involved in inflammatory responses such as pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, IL-1, 6 and IL-17 and is crucial in self-reactive T- and B-lymphocyte, survival and proliferation in lupus development (Okamoto, 2006). In this sense, lower IκB-α protein and high p65 expressions have been observed after pristane injection. However, kidneys from pristane mice fed with either OL or Per-OL showed similar IκB-α than in SD-sham group. In accordance with these results, p65 translocation was also reduced in OL and Per-OL groups compared to pristane group mice. These results are in agreement with our previous findings where other olive oil polyphenols supplemented diets increased IκB-α expression and reduced p65 nuclear translocation in pristane models of SLE. (Sanchez-Fidalgo *et al.*, 2010; Rosillo *et al.*, 2015; Aparicio-Soto *et al.*, 2016). Thus, our data suggest that dietary OL and Per-OL may protect against lupus nephritis by activating Nrf-2/HO-1 inhibiting the activation of the NF-κB pathway and deposition of extracellular matrix.

MAPKs family members (p38 kinases, $ERK_{1/2}$ and JNK) are implicated in several cell processes such as regulation of the synthesis of cytokines, chemokines, adhesion molecules and PGs involved in the regulation of autoimmune response (Thalhamer, McGrath and Harnett, 2007). Importantly, MAPKs phosphorylate the STAT3, important in pro-inflammatory cytokine-mediated signaling pathways leading to STAT3 activation by phosphorylation on tyrosine residues, resulting in the formation of STAT dimers that translocate into the nucleus to bind specific deoxyribonucleic acid (DNA) sequences (Wang, Cherukuri and Luo, 2005). Our findings show that STAT3 and MAPKs phosphorylation were increased in kidneys from SLE pristane mice; however, dietary OL and Per-OL supplementation significantly reduce both STAT3 and MAPKs activation at transcriptional level. Altogether, our results suggest that dietary OL and Per-OL may ameliorate inflammatory biomarkers production interfering negatively with JNK, p38, ERK MAPKs and STAT-3 signaling pathways.

On the other hand, inflammasomes are multi-protein complexes associated with several immune, inflammatory and auto-inflammatory diseases which use a central support and adaptor

molecules to recruit and activate caspase-1 and caspase-11. Canonical inflammasomes induce inflammatory response by processing inactive procaspase-1 into cleaved active caspase-1 (Montoya *et al.*, 2018). These canonical inflammasomes contain the nucleotide-binding oligomerization domain (NOD)-like receptor, the adapter molecule ASC and caspase-1 (Liu, Berthier and Kahlenberg, 2017). Then, active caspase-1 cleaves the pro-inflammatory cytokines IL-1β and IL-18 to their actives forms which are known to contribute directly to the inflammatory injury in lupus.

Different types of NLR family members, such as NLRP4, NLRP1, NLRP3 and AIM2 have been identified (Lamkanfi and Dixit, 2014). Increased expression of NLRP3 and caspase-1, among others inflammasome components has been reported in lupus nephritis biopsies (Kahlenberg *et al.*, 2011), suggesting that this tissue may be aware for inflammasome activation. The way in which the inflammsome is triggered in LES is an important concept to understand its role in this disease. It has been shown that inmmune complexed formed secondary to antibody recognition of DNA or ribonucleic acid (RNA) antigens stimulate inflammasome activation through upregulation of Toll-like receptor (TLR)-dependent activation of NF-κB and subsequent activation of the NLRP3 inflammasome (Kahlenberg and Kaplan, 2014). In this line, in previous studies, it has been reported an increment of inflammasome components expression, including NLRP3 and caspase-1 in lupus nephritis biopsies, suggesting that this tissue may be primed for inflammasome activation (Kahlenberg and Kaplan, 2014). Similarly, maturation and secretion of IL-1β and IL-18 translation are mediated by inflammsome activated caspase-1 (Shirato *et al.*, 2017).

In our study, we observed that pristane-induced SLE significantly increased NLRP3, ASC and caspase 1 protein expression, leading to the release of IL-1β and IL- 18, whereas only Per-OL supplemented diet could reduce NLRP3-ASC-inflammasome-activated caspase-1 cleavage inhibiting the canonical inflammasome pathway. Changes on immunosignals of active forms of caspase-1 (pro-caspase-1 and caspase-1) were not observed after OL dietary treatment. For other hand, the non-canonical inflammasome, an alternative mechanism, has been described to activate caspase-11 enzyme and in turn induce not only pyroptosis but also serves as an additional pathway for maturation and secretion of IL-1β and IL-18 in macrophage-mediated innate immune responses (Kayagaki *et al.*, 2011). Our data shown like pro-caspase-11 and caspase-11 activation was induced by pristane. Nevertheless, Per-OL treatment could inhibit NLRP3-inflammasome-activated caspase-11 cleavage. Our results have demonstrated a decreasing of IL-1β and IL-18 levels, probably as a consequence of NLRP3 down-regulation, highlighting the importance of the NLRPs regulation of avoiding inflammasome activation and therefore, the cleaving of the pro-forms to active cytokines forms. This finding above indicated that Per-OL might exert the anti-inflammatory effects in pristane-induced SLE via inhibiting canonical and non-canonical inflammasome pathways.

In conclusion, this study showed, for the first time, the immunomodulatory effects of dietary OL and Per-OL supplementation in pristane-induced SLE in mice by inhibiting pro-inflammatory biomarkers such as cytokines production IL-1β, and IL-18, MMP-3, PGE_2, as well as iNOS and mPGEs-1 over expression. The mechanisms underlying these protective effects could be related to the activation of the Nrf2/HO-1 antioxidant pathway as well as and the inhibition of relevant signaling pathways including JAK/STAT, MAPKs and NF-κB.

In addition, the new synthetic acetyl-derivatives exhibited a better anti-inflammatory profile that the original compound OL inhibits the canonical and non-canonical NLRP3 inflammasome signaling pathways. Consequently, the acetylation may improve pharmacodynamics and pharmacokinetics profiles compared to the natural compound. Both OL and Per-OL may offer a new promising dietary strategy for the prevention and management of SLE, which needs to be further explored.

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General Discussion

In the present Thesis we tried to elucidate the potential benefits of Oleuropein (OL), a secoiridoid which is the majority component of olive tree leaves, roots and unprocessed drupes of *Olea europaea* L., and the beneficial effects of its semi-synthetic acetyl-derivatives in different models of immunoinflammation such as, an *in vitro* model of rheumatoid arthritis (RA) using human synovial fibroblasts stimulated with interleukin (IL)-1β, an *ex vivo* model of inflammation using murine peritoneal macrophages stimulated with LPS, and in experimental *in vivo* RA and systemic lupus erythematosus (SLE), and clarify the specific mechanisms by which them may exert their anti-inflammatory and immunomodulatory effects.

The focus of the **chapter I** has been to demonstrate the potential protective role of OL in the modulation of inflammatory response in murine peritoneal macrophages. Macrophages are major inflammatory and immune effector cells, with a crucial role in the pathogenesis and development of inflammatory chronic diseases, including SLE and RA. The exposition to bacterial lipopolysaccharide (LPS) drives the macrophages to an activated state that produces and balance disruption of the intracellular reduction-oxidation state accompanied by reactive oxygen species (ROS)-mediated damage and evokes the production of different inflammatory mediators. For this reason, the stimulation of macrophages with LPS constitutes a validate model for the screening and subsequent evaluation of the effects of candidate drugs on the inflammatory pathway (Brüne *et al*., 2013; Sánchez-Miranda *et al.*, 2013; Cardeno, Sanchez-Hidalgo, Aparicio-Soto, Sanchez-Fidalgo, *et al.*, 2014).

The results of the present study showed, for the first time, that three new acetylated OL derivatives exhibited significant anti-inflammatory activities and attenuated the oxidative events induced by LPS in murine peritoneal macrophages, exhibiting better results than the observed with original compound OL. Among them, Peracetylated OL (Per-OL) displayed the most effective anti-inflammatory behavior.

Balance disruption of the intracellular reduction-oxidation state has been observed in stimulated macrophages, which leads to oxidative stress characterized by a major shift in the cellular redox balance and usually accompanied by ROS-mediated damage. In fact, the new secoiridoid acetylated derivatives were able to reduce ROS levels acting as effective anti-oxidants. Thus, modulators of ROS production and ROS-induced signalling pathways, especially in macrophages, could represent potential strategies for anti-inflammatory intervention. Our findings are in concordance with other studies in which OL showed a strong antioxidant effects in a similar range tested in our study (50-100 μM). Also, other olive polyphenols like hydroxytyrosol (HTy) showed important antioxidant effects acting as free radicals scavengers (Granados-Principal *et al*., 2011) **[Chapter I. Figure 2].**

A good balance of cell permeability and aqueous solubility, for optimal gastrointestinal absorption of a drug, has been suggested to be in the range of $0 < \log P < 3$. Therefore, OL and its three acetylated derivatives fulfil this requirement, although for Per-OL with the acetylated glucose moiety should have better cell permeability, in accordance with the excellent results observed in *in vitro* assays (Kerns and Di, 2008).

Particularly, stimulation of macrophages by LPS induces transcription of cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) genes and generation of large amounts of nitric oxide

(NO) that acts as an intracellular messenger, modulating the formation of endogenous ROS that orchestrate the inflammatory response (Li *et al.*, 2012). COX-2 , the inducible isoform of COX is essential for the inflammatory response and is responsible for the overproduction of prostaglandin E_2 (PGE_2) in inflammation (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, Sanchez-Fidalgo, *et al.*, 2014). Likewise, PGE_2 modulates a variety of immune processes at sites of inflammation, including production of pro-inflammatory cytokines (Imig, Breyer and Breyer, 2002). In the present study nitric oxide (NO) levels and iNOS expression were decreased by OL treatment. Similar data were reported by Ryu et al., 2015 in RAW 264.7 cells (Ryu *et al.*, 2015). Moreover, compounds (2), (3) and (4) at 25 and 50 μM produced a stronger reduction of both NO levels and iNOS protein expression when compared to OL. Besides, LPS stimulus upregulated COX-2 protein expression and increased PGE_2 levels in murine peritoneal macrophages in concordance with previous studies by Aparicio-Soto et al., 2014 (Aparicio-Soto *et al.*, 2014) **[Chapter I. Figure 3].**

LPS-stimulated macrophages are closely related to an imbalance of cytokine network. It is well known that this kind of inflammatory process is characterized by an increase of helper T cells (Th)-1 and Th17 proinflammatory cytokines mainly, tumor necrosis factor (TNF)-α, IL-1β, IL-6, IL-17 and interferon (IFN)-γ. Our results showed that OL and its derivatives inhibited the overproduction of these proinflammtory cytokines. In fact, the treatment with OL significantly reduced both IL-6 and IL-1β cytokines in culture medium in agreement with a previous study (Ryu *et al.*, 2015). However, TNF-α, IFN-γ and IL-17 levels were not decreased after OL treatment. On the contrary, IL-6, IL-1β , IFN-γ and IL-17 levels were diminished by OL derivative (3), compound (4) could decrease IL-6, IL-1β , TNF-α and IFN-γ levels and finally, the derivative (2) significantly reduced the production of all tested Th1 and Th17 pro-inflammatory cytokines showing the best anti-inflammatory profile at the assayed concentrations (25 and 50 μM) in a dose-dependent manner **[Chapter I. Figure 4].**

In attempt to elucidate the action mechanisms of these phenolic compounds, we next studied the involvement of mitogen-activated protein kinases (MAPKs), Janus kinase signal-transducer and activator of transcription (JAK/STAT) and nuclear factor E2-related factor 2 (Nrf2)/ heme oxygenase-1 (HO-1) signaling pathways. MAPKs pathway is a critical axis essential for both induction and propagation of the inflammatory LPS-activated macrophage response (Radnai *et al.*, 2009). MAPKs include extracellular signal-regulated ($ERK_{1/2}$), c-Jun NH_2-terminal (JNK) and p38 MAPKs. The increase activity of MAPKs as well as their involvement in the regulation of the inflammation mediator synthesis at the transcription level and translation make them potential targets for anti-inflammatory therapy (Cardeno, Sanchez-Hidalgo, Aparicio-Soto and Alarcon-de-la-Lastra, 2014). In effect, MAPKs have been shown to play crucial roles in iNOS and COX-2 upregulation induced by various stimuli in mammalian cells (Guha and Mackman, 2001). Our study showed that LPS evoked a significant increment of the p38, JNK and $ERK_{1/2}$ MAPKs phosphorylation in peritoneal macrophages after 18 h of stimulation. In contrast, OL treatment and its acetylated derivatives (25 and 50 μM) reduced MAPKs phosphorylation, which was consistent with previous studies reporting that OL inhibited MAPKs activation in RAW 264.7 cells (Ryu *et al.*, 2015) and in human osteoarthritis chondrocytes (Feng *et al.*, 2017) **[Chapter I. Figure 5]**.

Moreover, MAPKs are also involved in the activation of JAK/STAT, an important signalling transduction pathway for the biological function of many cytokines. Our results are in agreement with previous studies reporting that OL treatment attenuated the induced activation of signal transducer and activator of transcription (STAT)-3 in a murine colitis-associated colorectal cancer model (Giner *et al.*, 2016). Besides, (2) and (3) OL derivatives also exhibited significant effects in the prevention of the induced phosphorylation of STAT-3, showing better results that the described after the treatment with the parent. Altogether, our data suggest that all tested compounds present a potential anti-inflammatory effect mediated in part by STAT-3 inactivation **[Chapter I. Figure 6A]**. Nrf2, is a redox-sensitive transcription factor and binds to antioxidant response elements (ARE) located in the promoter regions of many detoxifying/antioxidant genes, including HO-1 (Bang *et al.*, 2012). In the presence of oxidative stress, Nrf2 could migrate to the nucleus, bind to the oxidant response element sequence, and induce phase II gene transcription resulting in a cytoprotective response characterized by up regulation of HO-1 among others, and decreased sensitivity to oxidative stress damage. In inflammatory conditions, HO-1 expression protein could be part of an adaptive mechanism to limit cytotoxicity via several mechanisms including scavenging of ROS and nitrogen species, regulation of cell proliferation and prevention of apoptosis. Our results in concordance with Aparicio-Soto et al., 2015 (Aparicio-Soto *et al.*, 2015), showed that LPS stimulation decreased Nrf2 and HO-1 expression. On the contrary, OL derivatives treatments (25 and 50 μM) were able to increase these reduced expressions of Nrf2 and HO-1 induced by LPS, but we did not observed any significant changes in the expression of HO-1 and Nrf2 after the treatment with the original compound. Altogether, our data suggest that acetylated OL derivatives present also a potential antioxidant effect which is mediated by Nrf2/HO-1 antioxidant signaling pathway **[Chapter I. Figure 6B]**.

In conclusion, this study showed, for the first time, the new acetylated OL derivatives: Per-OL, 2'', 3'', 4'', 6''-Tetra-*O*-acetyloleuropein and 6''-*O*-Acetyloleuropein have an important role in the balance of the inflammatory microenvironment induced by LPS in murine peritoneal macrophages by inhibiting pro-inflammatory cytokines production, such as IL-1β, TNF-α, IL-6, IL-17 and IFN-γ, as well as iNOS and COX-2 overexpression. The mechanisms underlying these protective effects could be related via the Nrf2/HO-1 antioxidant pathway activation and inhibition of both JAK/STAT and MAPKS signalling pathways. Furthermore, these new synthetic OL derivatives exhibit a better anti-inflammatory profile that the natural compound OL, that could be due to their better pharmacokinetic/pharmacodynamics profiles related to the modification of their chemical structure.

Thereby, these new OL derivatives may offer a new promising therapeutic strategy in the management of inflammatory related pathologies, which needs to be further investigated. It could be a useful option to increase the quality of life of patients with these types of diseases, probably as a dietary supplement.

In the **chapter II**, we investigated the potential effects of OL in IL-1β-stimulated SW982 cells (human RA- synovial fibroblasts (SFs) cell line). RA pathology is characterized by chronic synovitis in poly-articular joints. In RA joints, there are hyperplasia and hypertrophy of the synovial lining cells, specifically, SFs. In fact, SFs act as a major cell population in the invasive pannus to participate in the chronic inflammatory responses (Firestein, 1996). SFs contribute significantly to matrix degradation in

RA through the production of inflammatory mediators such as cytokines mainly TNF-α and IL-6 sustaining regulatory feedback loops that induce the production of enzymes, such as, metalloproteinases (MMPs) through the activation of cellular signaling pathways involving MAPKs (McInnes and Schett, 2007; Su *et al.*, 2016). TNF-α is reportedly involved in early joint swelling, chronic joint inflammation and the concomitant erosive changes in cartilage and bone (Sommerfelt *et al.*, 2013). On the other hand, IL-6 is a key cytokine inducing a decrease in type II collagen (CII) production, increase in MMPs synthesis, and changes in the subchondral bone layer (Chenoufi *et al.*, 2001). High levels of IL-6 have been detected in both sera and synovial fluids from the affected joints of RA patients. In this study, we have demonstrated that treatment with OL caused a significant inhibition of IL-1β-induced TNF-α and IL-6 release in human synovial SW982 cells **[Chapter II. Figure 3].**

Among MMPs, MMP-1 (colagenase 1) and MMP-3 (stromelysin) have been reported to be the major enzymes produced by fibroblasts and macrophage-like cells in the synovium, and their levels are significantly higher in synovial fluids from patients with RA (Rosillo *et al.*, 2016). MMP-1 is one of the critical neutral proteinases which degrade native fibrillary collagens in the extracellular matrix (Yamanishi and Firestein, 2001). On the other hand, MMP-3 is responsible to degrade proteoglycan, type IV and type IX collagens and denatured type I and type II collagens, fibronectin, gelatin and laminin and is believed to be especially important because, besides of its direct enzyme activity, its activation is necessary for full activation of collagenases (Chen and Mattey, 2012). Our data showed that the production of both MMP-1 and MMP-3 was significantly induced by IL-1β stimulation in human SW982 cells, whereas pre-treatment with OL induced a significant down-regulation of both MMPs levels in IL-1β-stimulated SW982 cells **[Chapter II. Figure 4]**.

COX-2, the inducible isoform of COX, and microsomal prostaglandin E synthase- 1 (mPGEs-1), enzymes responsible for the overproduction of PGE_2 in inflammation, are up-regulated contributing to the progression of RA through prostaglandin E_2 receptor 4 (EP_4) activation (McCoy, Wicks and Audoly, 2002). We have shown that OL decreased expression of both COX-2 and mPGEs-1 in IL-1β-stimulated SW982 cells **[Chapter II. Figure 5].**

Nuclear transcription factor kappa-B (NF-κB) has been implicated in cytokine release, activation, autoantibody production, cellular proliferation, inhibition of apoptosis, and numerous other processes associated with RA. Also plays a pivotal role in the development and activation of Th-1 responses and is responsible in addition to MAPKs for COX-2 up-regulation (Makarov, 2001). NF-κB, as a dimeric transcription factor exists in the cytoplasm as an inactive complex with the inhibitory protein IkB-α. An inflammatory signal such as IL-1β induced the activation of IκB-α kinase complex to phosphorylate members of the IkB family. Phosphorylated IkB becomes ubiquitinated and is then targeted for degradation by the proteasome. The NF-κB dimmers can then translocate to the nucleus and activate the transcription and repression of genes (Hayden and Ghosh, 2008; Scheinman, 2013).

On other hand, MAPKs have previously been shown to play a critical role in the regulation of the synthesis of chemokines, cytokines, adhesion molecules and prostaglandin (PG)s involved in RA and are considered as the major tyrosine phosphorylation proteins in human synovial stimulated with IL-

1β (Barchowsky, Frleta and Vincenti, 2000). Besides JNK MAPK modulates MMPs production by synovial fibroblasts and drives osteoclast differentiation in RA (Han *et al.*, 2001). In particular, p38 MAPK regulates MMP-3 induction in fibroblasts and osteoclast differentiation (Rosillo *et al.*, 2015). Accumulating studies have demonstrated that inhibitors of MAPKs and NF-κB alleviated synovial inflammation, bone destruction, and cartilage damage in animal models of arthritis, including adjuvant arthritis in rats and CIA in mice (Han *et al.*, 2001; Nishikawa *et al.*, 2003).

As expected, the exposure of IL-1β significantly increased the degradation of IκBα and phosphorylation of $ERK_{1/2}$, JNK and p38, indicating NF-κB and MAPKs activation in SW982 cells which was consistent with previous studies reported by Schett et al., and Inoue et al., (Schett *et al.*, 2000; Inoue *et al.*, 2001). However, pre-treatment with OL, at concentration of 50 and 100 µM strongly inhibited the IκB-α degradation and prevented the activation of MAPKs. These results indicate that OL inhibit inflammation in IL-1β-treated SW982 cells by regulating the NF-κB and MAPKs pathways **[Chapter II. Figure 6 and 7].**

In basal conditions, Nrf2 in sequestered in the cytoplasma by Kelch-likeECH-associated protein 1 (Keap1) and degraded by the ubiquitin-dependent 26S proteasome system. In inflammatory and immune conditions, the expression of this protein could be part of an adaptive mechanism to limit cytotoxicity via several mechanisms including scavenging of reactive oxygen or nitrogen species, regulation of cell proliferation and prevention of apoptosis (Rosillo *et al.*, 2016). Furthermore, the deficiency of HO-1 in both mice and humans induces the characteristic phenotype of an increased inflammatory state, whereas the induction of HO-1 in animals leads to the protection of the progression of arthritis with decreased levels of matrix MMPs and the prevention of cartilage degradation suggesting the HO-1 plays the key role in RA therapeutic strategy (Su *et al.*, 2016).

Our data showed that OL treatment strongly augmented Nrf2 and HO-1 expression conferring a role of HO-1 in the beneficial effects of OL in IL-1β-stimulated SW982 cells. Thus HO-1 could represent a potential molecular target susceptible to modulation with treatment with OL, which has not been demonstrated previously **[Chapter II. Figure 8].**

In conclusion, our study has demonstrated, for the first time, that OL prevented the inflammatory response and oxidative stress of IL-1β -induced SW982 human synovial cells. The mechanisms underlying these protective effects could be related via down-regulation of MAPKs and NF-κB and induction of Nrf2-linked HO-1 signaling pathways controlling the production of pro-inflammatory cytokines, MMP-1 and MMP-3 levels as well as mPGES-1 and COX-2 overexpression. Thus, OL might provide a basis for developing a new dietary strategy for the prevention and management of RA.

The aim of the **chapter III** has been to demonstrate the potential protective role of a diet elaborated with OL and Per-OL in the prevention and development of inflammatory arthritis and joint damage in a collagen induce arthritis (CIA) murine model. This model is commonly used to investigate mechanisms relevant to RA as well as new antiarthritic treatments (Ferrandiz *et al.*, 2007). CIA induction resulted in the development of a remarkable synovitis associated with an autoimmune response against cartilage and production of matrix degrading enzymes accompanying cartilage degradation and bone erosion (Schurgers, Billiau and Matthys, 2011).

Our results indicate that OL and Per-OL dietary treatments exhibited preventive and therapeutic effects in the development of inflammatory arthritis and joint damage in CIA arthritic mice (SD-CIA) in comparison with CIA mice fed with standard diet (SD) **[Chapter III. Figure 1A and 1B].** These effects were correlated to an improved arthritis score, a minor inflammatory cells infiltration into articular tissues, reduced exudation into the synovial space, synovial hyperplasia and cartilage erosion **[Chapter III. Figure 1C].**

The pathogenesis of RA is related to an imbalance of cytokine network contributing to the development and progression of both CIA and RA (Komatsu and Takayanagi, 2012). In RA synovium, elevated levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-17 and IFN-γ are produced by macrophages and synovial fibroblasts. Our results indicate that mice fed with OL and Per-OL enriched-diets showed a significant reduction in all-pro-inflammatory cytokines levels in comparison with SD-CIA control group **[Chapter III. Figure 3].**

Also, COX-2 and iNOS mRNA expression levels are up-regulated in LPS-stimulated RAW 264.7 cells and consequently, contributing to the progression of RA (Zhao *et al.*, 2019). We have shown that OL and Per-OL dietary treatments reduce COX-2 expression in the knee joint, and also these results showed a significant decreased of iNOS protein expression in paw homogenates in mice which were fed with Per-OL enriched-diet at both doses assayed **[Chapter III. Figure 4].**

MMP3 (stromelysin) have been reported to be the major enzymes produced by fibroblasts and macrophage cells in the synovium, and is responsible for the degradation of proteoglycan, various type of collagens and denatured type I and type II collagens, among others, besides of its direct enzyme activity its activation is necessary for full activation of collagenases (Castejón *et al.*, 2017). Collagen oligomeric matrix protein (COMP), a prominent non-collagenous component of cartilage shows great potential as a biological marker of cartilage metabolism in arthritis (Rosillo *et al.*, 2015). It has been reported to increase in patients with knee osteoarthritis (OA) and early RA. It has been proposed that COMP molecules are important for maintaining the properties and integrity of the collagen network and contribute to the material properties of biological tissue (Haikal *et al.*, 2019). Our data showed that the production of both MMP-3 and COMP was significantly increased in SD-CIA control group; whereas OL and Per-OL dietary treatment induced a significant down-regulation of both serum parameters levels **[Chapter III. Figure 2].**

MAPKs also play important roles in transducing synovial inflammation and joint destruction and they are considered critical molecular targets for therapeutic intervention in RA (Thalhamer, McGrath and Harnett, 2007). MAPK JNK and $ERK_{1/2}$ play a major role in modulating collagenase production and invasion by RA fibroblasts, while p38 MAPK isoforms are involved in regulating many of cellular biological processes, concretely synovial inflammatory cytokine production, which participate to the pathogenesis of RA (Zou *et al.*, 2017). $ERK_{1/2}$ also participates in promoting pannus formation (Goodridge *et al.*, 2003). Our data indicate that OL and Per-OL dietary treatments were able to reduce the phosphorylation of JNK, p38 and $ERK_{1/2,}$ suggesting that these experimental diets could control the activation of these signaling pathways during arthritic inflammatory process **[Chapter III. Figure 6].**

NF-κB is considered a key signaling molecule in the control of synovial inflammation, hyperplasia and matrix generation (Zou *et al.*, 2017). It is a mechanism involucrate in RA because at inflammatory cytokines are produced by immune-activated cells and exert various actions on inflammation through the activation of the NF-κB signaling pathway (Aupperle *et al.*, 2001). Our data are in agreement with Castejón et al., that showed that OL and Per-OL dietary treatments increased the inhibitory protein IκB-α and reduced NF-κB-p65 and p50 translocation, indicating that these dietary treatments inhibited the NF-κB activation by blocking IκB-α degradation in pristane induced systemic lupus erythematosus in mice (Castejon *et al.*, 2019) **[Chapter III. Figure 5].**

It was reported that activation of Nrf2/HO-1 signaling pathway plays a critical role in the prevention and relief of RA (Fan *et al.*, 2018). Induction of HO-1 expression protects against cartilage destruction and decreases the secretion of proinflammatory cytokines in CIA model (Li *et al.*, 2014). Nrf2 plays an important role for expression of HO-1. Nrf2 inhibition aggravates cartilage destruction and accelerates the effector phase of RA in mice. However, upregulating the expression of Nrf2 exerts anti-inflammatory effects in RA (W.-J. Wu *et al.*, 2016). In the present study, we have demonstrated that Nrf2 and HO-1 protein expression were decreased in SD-CIA control group; however dietary Per-OL treatments at both doses assayed could restore Nrf2 and HO-1 expressions conferring a role of Nrf2/HO-1 signaling pathway in the beneficial effects of Per-OL enriched-diets in CIA model of RA **[Chapter III. Figure 7].**

In conclusion, this study showed, for the first time, the beneficial effects of dietary OL and Per-OL supplementation in CIA model of RA by inhibiting pro-inflammatory biomarkers such as cytokines production (IL-6, IL-1β, TNF-α, IFN-γ, IL-17) in paw homogenates and MMP3 and COMP serum levels, as well as, iNOS and COX-2 over expression. The mechanisms underlying these protective effects could be related to the activation of Nrf2/HO-1 antioxidant pathway as well as the inhibition of important signalling pathways including MAPKs and NF-κB. Both OL and Per-OL may offer a new promising dietary strategy for the prevention and management of RA, which needs to be further explored.

In **chapter IV**, we have dept insight into the effects of OL and its new lipophilic acetyl-derivative Per-OL dietary treatments on murine peritoneal macrophages from pristane-SLE mice, evaluating the clinical, pathological, and mechanistic contribution.

SLE is as autoimmune disease, characterized by autoantibody generation, immune complexes, autoreactive or inflammatory T cells and inflammatory cytokines that may initiate and amplify inflammation and damage to various organs such as kidney contributing to the clinical manifestations of SLE (Aparicio-Soto, Sánchez-Hidalgo and Alarcón-de-la-Lastra, 2017; Xu and Li, 2018).

Patients with SLE show an impaired oxidative status and increased levels of IL-1β, TNF, IL-17 and IL-18, which are closely linked to inflammation and correlated with disease activity (C.-Y. Wu *et al.*, 2016). It has been reported that IL-1β can promote the proliferation, migration, and invasion of other cells via activating STAT3. Besides, STAT3 signaling plays a crucial role in the Th17 generation and an upregulated STAT3/IL-17 expression had been described in lupus patients (Chen *et al.*, 2019).

In the present study, we found that OL and Per-OL dietary treatments reduced the inflammatory infiltrated in kidneys **[Chapter IV. Figure 2].** Anti-inflammatory response was accompanied by a significant reduction of Th1, Th2 and Th17 cytokines levels as well as p-STAT3 downregulation in LPS-stimulated macrophages from OL and Per-OL treated SLE mice **[Chapter IV. Figure 3 and 6].**

Recently, it has been described that acute consumption of olive oil decreased the activation of NF-κB system on mononuclear cells from healthy donors and that OL inhibits LPS-triggered NF-κB and activator protein (AP)-1 activation. NF-κB induces the expression of various proinflammatory genes, including those encoding cytokines and chemokines such as TNF-α, IL-1β, IL-6, iNOS and COX-2, among others, and also participates in the regulation and assembly of inflammasome complex contributing to the initiation and development of inflammatory diseases (Liu *et al.*, 2017).

By inhibiting the activation of NF-κB, the production of inflammatory mediators under its control may be reduced. Our results have demonstrated that experimental diets with OL and Per-OL were able to reduce the IκB-α translocation into nucleus and controlling the NF-κB activation **[Chapter IV. Figure 5]**. In this regard, Miles et al. demonstrated that OL significantly decreased the concentration of IL-1β in LPS-stimulated human whole blood culture (Carluccio *et al.*, 2003; Miles, Zoubouli and Calder, 2005; Perez-Martinez *et al.*, 2007). Similar results were described in human synovial fibroblast cell line SW982 where OL as well as the phenolic fraction from EVOO exerted anti-inflammatory and anti-oxidant effects via down-regulation of MAPK and NF-κB signaling pathways and induction of Nrf2-linked HO-1 controlling the production of inflammatory mediators such as IL-6 and TNF-α cytokines, MMP-1 and MMP-3 levels and mPGEs-1 and COX-2 overexpression (Castejon *et al.*, 2017; Rosillo *et al.*, 2019).

The inflammasome was described as a large intracellular signaling complex that contains a cytosolic pattern recognition receptor, especially a NLRP. Among NLR inflammasome complex, the most characterized is NLR family pyrin domain-containing 3 inflammasome (NLRP3) (Jo *et al.*, 2016). Inflammasome machinery is dysregulated in SLE and plays an important role in promotion of organ damage. Maturation and secretion of IL-1β and IL-18 translation are mediated by inflammasome activated caspase-1 (Shirato *et al.*, 2017). Canonical inflammasome induce inflammatory response by processing inactive procaspase-1 into cleaved caspase-1. Caspase 1 is able to regulate the maturation of IL-1β and IL-18 into active cytokines which are involved in the lupus injury (Jo *et al.*, 2016). Besides, non-canonical inflammasome, there is an alternative via which has been described to activate caspase-11, and also serve as an additional pathway of maturation and secretion of IL-1β and IL-18 in macrophage-mediate immune response (Kayagaki *et al.*, 2011).

We found, for the first time that OL and Per-OL dietary treatments inhibited canonical and non-canonical activation of NLRP3 inflammasome/IL-1β in murine peritoneal macrophages isolated from pristane-SLE mice. This important finding indicates that OL and Per-OL inhibited disease progression which was at least partly dependent on inhibition of NLRP3 inflammasomes **[Chapter IV. Figure 7].** Similar data were found after HTy and Peracetylated-HTy (Per-HTy) treatments on LPS-induced inflammatory response in murine peritoneal macrophages (Montoya *et al.*, 2018).

Taken together, OL and Per-OL exerted a protective effect against inflammatory lupic response in murine peritoneal macrophages from pristane-SLE mice via STAT3 and NF-κB signaling expression coupled with inhibition of canonical and non-canonical NLRP3 inflammasome.

Finally, in the chapter V, we have demonstrated that OL and Per-OL supplemented diets could modulate SLE on pristane-induced model in mice, specifically preventing renal injury through modulating NF-κB, STAT3 and MAPKs signaling pathways, and inhibiting the NLRP3 inflammasome complex.

Pristane-treated mice presented some degree of renal abnormalities in comparison with control mice, ranging from abundant inflammatory mononuclear cells in the renal interstitium to renal interstitial fibrosis. On the contrary, kidneys from pristane-treated mice fed with OL and PER-OL showed a significant improvement of the renal cytoarchitecture, The appearance of hyaline-like renal tubule areas after pristane treatment and, preferably, after administration of oil diets, has not been previously referenced in the literature, excepting by Ruan et al. (Ruan *et al.*, 2016) that described the presence of a large lightly stained mucous-like substance. The histological aspects of renal tissue in our studies were very similar to those described by Ruan et al. but being them periodic acid Shiff (PAS) negative, their mucous nature is questionable (Ruan *et al.*, 2016) **[Chapter V. Figure 1].**

MMP-3 (stromelysin-1) levels were markedly elevated in SLE. with a possible role in the pathogenesis of lupus nephritis (Gheita *et al.*, 2015). Accordingly with our previous findings (Aparicio-Soto *et al.*, 2016, 2017). MMP-3 serum levels were increased in SD-SLE mice which, however serum MMP-3 levels were significantly reduced in those pristane-treated mice that received OL and Per-OL enriched-diets contributing to minimize pristane-induced renal damage **[Chapter V. Figure 2].**

PGE_2 is a bioactive lipid mediator biosynthesized by mPGEs-1 (Schneider, Boeglin and Brash, 2004), which plays a crucial role in the development of inflammatory response, acting as a mediator of Th1/Th2/Th17 cytokines and it has been implicated in the glomerular filtration regulation (Imig, Breyer and Breyer, 2002; Adamik *et al.*, 2013). Besides, PGE_2 high urinary levels of this prostanoid have been reported in lupus nephritis (Herrera-Marcos *et al.*, 2017). In previous studies by our research group (Aparicio-Soto *et al.*, 2016, 2017) high levels of PGE_2 and mPGEs-1 protein expression were found in pristane-induced SLE mice. However, both parameters were significant reduced in renal tissue of pristane-induced animals that were received OL and Per-OL supplemented diet in comparison with those animals which were fed with SD. NO is overproduced in the setting of lupus activity (Oates and Gilkeson, 2006). NO works as an intracellular messenger which regulates the formation of ROS and RNS synchronizing the inflammatory response (Montoya *et al.*, 2018). Our results showed, accordingly with previous studies (Jiang *et al.*, 2014) that iNOS expression was remarkably increased in kidneys from SD-SLE mice, however experimental diets enriched with OL and Per-OL could restore reduced iNOs expression **[Chapter V. Figure 3].**

Nrf2 signaling pathway is a crucial regulator of the antioxidant response in mammalian cells in response to endogenous and exogenous stress (Yu *et al.*, 2014) modulating the transcription of antioxidant genes, including HO-1. Previous studies have demonstrated that Nrf2 improves lupus nephritis in a pristane- induced SLE model by neutralizing reactive oxygen species and by negatively

regulating the NF-κB in patients with SLE. In addition, a significant decrease in HO-1 expression (Jiang *et al.*, 2014), specifically in monocytes (Herrada *et al.*, 2012) has been found, thus HO-1 induction could ameliorate lupus nephritis, probably by multiple mechanisms including NO synthesis suppression, inhibition of antibody production and modulation of cytokine production (Takeda *et al.*, 2004). In our studies, OL and Per-OL dietary treatments strongly could reestablish Nrf2 and HO-1 expressions conferring a role of Nrf2/HO-1 signaling in the beneficial effects of dietary OL and Per-OL in this SLE model **[Chapter V. Figure 4].**

NF-κB is a redox sensitive transcription factor which controls the expression of several genes involved in inflammatory responses such as pro-inflammatory cytokines including TNF-α, IL-1, 6 and IL-17 and is crucial in self-reactive T- and B-lymphocyte, survival and proliferation in lupus development (Okamoto, 2006) In this sense, lower IκB-α protein and high p65 expressions has been observed after pristane injection. However, kidneys from pristane mice fed with either OL or Per-OL showed similar IκB-α than in SD-sham group. In accordance with these results, p65 translocation was also reduced in OL and Per-OL groups compared to pristane group mice **[Chapter V. Figure 5B].** These results are in agreement with our previous findings where other olive oil polyphenols supplemented diets increased IκB-α expression and reduced p65 nuclear translocation in pristane models of SLE.

MAPK family members (p38 kinases, $ERK_{1/2}$ and JNK) are implicated in several cell processes such as regulation of the synthesis of cytokines, chemokines, adhesion molecules and PGs involved in the regulation of autoimmune response (Thalhamer, McGrath and Harnett, 2007). Importantly, MAPKs phosphorylate the STAT3, important in pro-inflammatory cytokine-mediated signaling pathways leading to STAT3 activation by phosphorylation on tyrosine residues, resulting in the formation of STAT dimers that translocate into the nucleus to bind specific DNA sequences (Wang, Cherukuri and Luo, 2005). Our findings show that STAT3 and MAPKs phosphorylation were increased in kidneys from SLE pristane mice; however, dietary OL and Per-OL supplementation significantly reduce both STAT3 and MAPKs activation at transcriptional level. Altogether, our results suggest that dietary OL and Per-OL may ameliorate inflammatory biomarkers production interfering negatively with JNK, p38, ERK MAPKs and STAT-3 signaling pathways **[Chapter V. Figure 5A and 5C].**

In other hand, inflammasomes are multi-protein complexes associated with several immune, inflammatory and auto-inflammatory diseases which use a central support and adaptor molecules to recruit and activate caspase-1 and caspase-11. Canonical inflammasomes induce inflammatory response by processing inactive procaspase-1 into cleaved active caspase-1 (Montoya *et al.*, 2018). These canonical inflammasomes contain the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR), the adapter molecule apoptosis-associated speck-like protein that contains a CARD (ASC) and caspase-1 (Liu, Berthier and Kahlenberg, 2017). Then, active caspase-1 cleaves the pro-inflammatory cytokines IL-1β and IL-18 to their actives forms which are known to contribute directly to the inflammatory injury in lupus. Increased expression of NLRP3 and caspase-1, among others inflammasome components has been reported in lupus nephritis biopsies (Kahlenberg *et al.*, 2011), suggesting that this tissue may be aware for inflammasome activation. In our study, we observed that

pristane-induced SLE significantly increased NLRP3, ASC and caspase 1 protein expression, leading to the release of IL-1β and IL- 18, whereas only Per-OL supplemented diet could reduce NLRP3-ASC-inflammasome-activated caspase-1 cleavage inhibiting the canonical inflammasome pathway. Changes on immunosignals of active forms of caspase-1 (pro-caspase-1 and caspase-1) were not observed after OL dietary treatment **[Chapter V. Figure 6A and 6B].**

For other hand, the non-canonical inflammasome, an alternative mechanism, has been described to activate caspase-11 enzyme and in turn induce not only pyroptosis but also serves as an additional pathway for maturation and secretion of IL-1β and IL-18 in macrophage-mediated innate immune responses (Kayagaki *et al.*, 2011). Our data shown like pro-caspase-11 and caspase-11 activation was induced by pristane. Nevertheless, Per-OL treatment could inhibit NLRP3-inflammasome-activated caspase-11 cleavage. Our results have demonstrated a decreasing of IL-1β and IL-18 levels, probably as a consequence of NLRP3 down-regulation, highlighting the importance of the NLRPs regulation of avoiding inflammasome activation and therefore, the cleaving of the pro-forms to active cytokines forms. This finding above indicated that Per-OL might exert the anti-inflammatory effects in pristane-induced SLE via inhibiting canonical and non-canonical inflammasome pathways **[Chapter V. Figure 6C].**

In conclusion, this study showed, for the first time, the immunomodulatory effects of dietary OL and Per-OL supplementation in pristane-induced SLE in mice by inhibiting pro-inflammatory biomarkers such as cytokines production IL-1β, and IL-18, MMP-3, PGE_2, as well as iNOS and mPGEs-1 over expression. The mechanisms underlying these protective effects could be related to the activation of the Nrf2/HO-1 antioxidant pathway as well as and the inhibition of relevant signalling pathways including JAK/STAT, MAPKs and NF-κB.

In addition, the new synthetic acetyl-derivatives exhibited a better anti-inflammatory profile that the original compound OL inhibits the canonical and non-canonical NLRP3 inflammasome signaling pathways. Consequently, the acetylation may improve pharmacodynamics and pharmacokinetics profiles compared to the natural compound. Both OL and Per-OL may offer a new promising dietary strategy for the prevention and management of SLE, which needs to be further explored.

Altogether, this Thesis provides preliminary evidence that OL exerts preventive/palliative effects in the development of experimental RA and SLE, and also its new acetyl derivative, Per-OL which demonstrate its beneficial effects in these pathologies. However, the current challenge is to confirm that all these in vitro and in vivo observed phenomena described in this Thesis, also occur in human after OL and Per-OL consumption, and that results in a decreased risk of developing RA and SLE, or suffering outbreaks of these diseases and its related symptoms, which requires future and further studies.

Nevertheless, OL and Per-OL may be considered as supportive nutritional therapy for patients of RA and SLE or OL and Per-OL supplement might provide an attractive nutraceutical complement in the management of RA and SLE, owing to their notable effects without the side effects of classical pharmacology, and their potential contribution to reducing comorbidities and improving quality of life in patients with RA and SLE, thereby offering a new promising strategy in the nutritional therapy

of this immune-inflammatory disease, as well as a new fields of research which need to be pursued further.

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Conclusions

Los resultados obtenidos en la presenta **Tesis Doctoral** nos permiten concluir:

1. La OL así como sus derivados acetilados obtenidos semisintéticamente demostraron marcadas actividades antioxidantes previniendo la formación de especies reactivas de oxígeno y antiinflamatoria, disminuyendo la producción de nitritos, de citocinas inflamatorias, la expresión de las proteínas COX-2 e iNOS, así como los niveles de PGE_2 en macrófagos peritoneales murinos estimulados con LPS. Además, tanto OL como sus derivados, redujeron la fosforilación de las MAP cinasas y la vía JAK/STAT y además incrementaron la expresión de la vía antioxidante Nrf2/HO-1. Estos resultados ponen de manifiesto la capacidad moduladora de la respuesta inmune e inflamatoria de este compuesto obtenido de las hojas del olivo, así como de los derivados obtenidos a partir del compuesto natural, lo que podría ser de interés para el abordaje terapéutico de enfermedades de carácter inmunoinflamatoria.

2. Tras el tratamiento con OL en FS humanos (SW982) estimulados con IL-1β, pusimos de manifiesto una disminución significativa en la producción de niveles de las citoquinas pro-inflamatorias IL-6 y TNF-α, de las proteasas MMP-1 y MMP-3, y una inhibición de la expresión proteica de la enzima pro-inflamatoria COX-2, posiblemente asociados a una disminución en la activación de la vía de las MAP quinasas y una prevención en la degradación de la proteína inhibitoria IκB-α, así como un incremento en la expresión de la vía antioxidante regulada por Nrf2 y HO-1. Estos resultados preliminares sugieren que la OL podría participar en el desarrollo de una nueva estrategia terapéutica dirigida a los elementos del sistema inmunitario cuyas limitaciones en eficacia y/o seguridad son bien conocidas y constituir un complemento nutricional de gran valor en el tratamiento y/o prevención de la AR al actuar sobre procesos FS-dependientes.

3. Las dietas enriquecidas con OL y su derivado peracetilado, Per-OL, redujeron significativamente la incidencia y gravedad del daño articular en el modelo de artritis inducida por colágeno II en ratones DBA 1/J en comparación con aquellos alimentados con una dieta estándar. Se apreció una reducción del número y severidad de articulaciones afectadas y un menos índice clínico de la enfermedad desde los días 30 hasta 42.

4. Tras el tratamiento con las dietas enriquecidas con OL y Per-OL en ratones con artritis, se observó una atenuación de las alteraciones histomorfológicas asociadas a la lesión articular. La respuesta inflamatoria articular con las dietas experimentales se relación con una disminución significativa en los niveles de citoquinas pro-inflamatorias y de los marcadores serólogicos de inflamación sinovial y degradación del cartílago MMP-3 y COMP, respectivamente. Dichos efectos estuvieron asociados a la inhibición de las vías de señalización molecular de las MAP cinasas y al factor de transcripción NF-κB modulando la expresión de marcadores pro-inflamatorios COX2 e iNOS, así como a la activación de la vía antioxidante Nrf2/HO-1, rutas que parecen estar funcionalmente interconectadas. Por todo ello, este tipo de suplementación dietética podría ser considerado como una alternativa en la terapia nutricional de pacientes con AR.

5. En macrófagos peritoneales procedentes de ratones con LES inducido por pristano, dietas experimentales enriquecidas con OL y Per-OL al 0.01 % produjeron una disminución de los niveles de citoquinas pro-inflamatorias, acompañada de una disminución en la expresión de mediadores pro-inflamatorios como la COX.2 e iNOS, regulados por la inactivación del factor de transcripción JAK/STAT y de un incremento en la expresión de la proteína inhibitoria IκB-α. Además, se produjo una regulación por la inactivación tanto de la vía canónica regulada por la caspasa 1, como de la no-canónica regulada por la caspasa 11 del complejo proteico del inflamasoma NLRP3, contribuyendo a los efectos beneficiosos que aporta el enriquecimiento de la dieta con OL y con Per-OL en el manejo del LES experimental.

6. Las dietas elaboradas con los compuestos fenólicos OL y Per-OL redujeron significativamente las alteraciones histopatológicas renales y mejoró la expresión de diversas proteínas implicadas en el daño renal en un modelo murino de LES inducido por pristano, en comparación con aquellos animales alimentados con una dieta estándar. En este modelo se puso de manifiesto una reducción de marcadores serólogicos de inflamación como las MMP-3, además de la reducción de mediadores pro-inflamatorios como iNOS, PGE_2 y mPGEs-1. Todo ello a través de la modulación de las vías de señalización de las MAP cinasas, NF-κB, JAK/STAT y Nrf2/HO-1, así como de la modulación del complejo proteico compuesto por el inflamasoma NLRP3, tanto en su activación canónica como no-canónica.

En conclusión, esta Tesis Doctoral proporciona resultados preliminares sobre el papel preventivo y paliativo de la OL y de sus derivados acetilados en el desarrollo de enfermedades inmunoinflamatorias como la AR y el LES experimentales. Estos secoiridoides mejoran el proceso inmunoinflamatorio inducido por lo que podrían constituir nuevas estrategias sobre las que es necesario profundizar para afianzar el papel de estos compuestos como nutracéuticos útiles para prevenir, cambiar el curso e incluso tratar este tipo de enfermedades.

Sin embargo, el desafío actual es confirmar que los resultados in vitro e in vivo mostrados en la presente Tesis Doctoral también ocurren tras el consumo de estos compuestos en humanos, traduciéndose en un menos riesgo de desarrollar estas patologías de carácter inmunoinflamatorias, previniendo la aparición de brotes y disminuyendo los síntomas asociados a estas enfermedades; lo cual requiere de futuros estudios multicéntricos de intervención nutricional, que avalen a la OL y a sus derivados como nuevas estrategias nutricionales en el manejo de estas enfermedades.