# MEDICINAL PLANTS FROM ANDALUSIA:

## Potential use as intestinal anti-inflammatory agents

Tesis Doctoral

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Departamento de Farmacología
Universidad de Granada

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## FACULTAD DE FARMACIA

### Departamento de Farmacología

# MEDICINAL PLANTS FROM ANDALUSIA:
# Potential use as intestinal anti-inflammatory agents

Tesis doctoral para aspirar al grado de doctor presentada por


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Bajo la dirección de los Doctores

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*Alla mia famiglia.*

*I have no special talent. I am only passionately curious.*

*Albert Einstein*

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# RESUMEN

# RESUMEN

## INTRODUCCIÓN

El término Enfermedad Inflamatoria Intestinal (EII) comprende dos patologías: la colitis ulcerosa (CU) y la enfermedad de Crohn (EC). Ambas se caracterizan por una inflamación crónica del intestino derivada de una respuesta inmune intestinal exacerbada ante un determinante antigénico desconocido. En ambas se alternan periodos de remisión de la enfermedad con intervalos de exacerbación de los síntomas [1, 2].

Aunque hasta el momento se desconocen los mecanismos responsables de la iniciación y perpetuación del proceso inflamatorio intestinal, es aceptado que en su fisiopatología están implicados factores genéticos, ambientales e inmunológicos. Así, numerosos estudios han establecido que, en personas genéticamente predispuestas, una activación exagerada y descontrolada del sistema inmune intestinal frente a un determinante antigénico puede desencadenar la aparición de la respuesta inflamatoria intestinal exagerada [3]. Esta respuesta inmunológica genera numerosos mediadores de carácter proinflamatorio (citocinas, eicosanoides y especies reactivas derivadas del oxígeno y/o del nitrógeno) que actúan de forma sinérgica y sincronizada promoviendo la amplificación y cronificación del proceso inflamatorio del intestino [4-7].

Ya que no se conoce la etiología de la EII, actualmente no existe un tratamiento curativo para esta patología y los objetivos principales de la terapia farmacológica consisten en promover la remisión del ataque agudo de la enfermedad y reducir la incidencia de recurrencias. Esto se consigue inhibiendo la respuesta inmunológica exacerbada que tiene lugar tanto en EC como en CU, bien actuando sobre el mayor número posible de mediadores proinflamatorios involucrados en la patogénesis de estas enfermedades, o bien actuando en concreto sobre alguno de ellos que posea un papel relevante.

Algunos de los tratamientos disponibles en la actualidad son los fármacos antiinflamatorios, los inmunosupresores, o las terapias biológicas. El 5-ASA (5-aminosalicílico) es el antiinflamatorio de elección en las fases iniciales de la enfermedad,

cuando la inflamación afecta solo a una pequeña porción del colon. Cuando los pacientes presentan una patología de intensidad moderada deben pasar a tratarse con corticoides orales o en enemas como budesonida, y cuando la afectación colónica es más extensa, los pacientes no responden a budesonida o 5-ASA o presentan una enfermedad muy activa, deben recibir prednisona o esteroides parenterales [8, 9].

Si no se consigue controlar la actividad de la enfermedad con estos fármacos, está indicada el uso de inmunosupresores como azatioprina, metotrexato, infliximab y finalmente, en caso de fracaso fármaco-terapéutico se puede recurrir a la cirugía [10-12]. Todos estos tratamientos tienen una eficacia relativa, ademas de manifestar importantes efectos adversos que limitan su utilización prolongada en el tiempo. Por ello es importante la investigación de nuevas estrategias terapéuticas que aúnen eficacia y seguridad.

Distintos estudios han puesto de manifiesto la tendencia actual por parte de los pacientes con EII de emplear medicinas alternativas y/o complementarias en el tratamiento de su enfermedad, probablemente como consecuencia de la falta de eficacia que el tratamiento convencional tiene en muchas ocasiones, o por la elevada incidencia de reacciones adversas asociadas que les caracteriza [13].

Entre las distintas terapias utilizadas destaca el empleo de plantas medicinales con propiedades anti-inflamatorias: se trata de tratamientos seguros, ya que se han venido empleando durante muchos años, y que contienen una mezcla de principios activos que podrían promover la actuación simultánea sobre distintas dianas terapéuticas del proceso inflamatorio intestinal.

En la Comunidad Autónoma Andaluza existe un gran número de especies vegetales autóctonas utilizadas por la Medicina Tradicional como anti-inflamatorios y en dolencias digestivas, que requieren de su validación científica para poder ser utilizadas en el futuro para el tratamiento de pacientes con EII, bien como medicina alternativa o complementaria.

**OBJETIVO**

El presente trabajo de Tesis doctoral pretende evaluar la actividad anti-inflamatoria intestinal de distintas especies vegetales utilizadas en la medicina tradicional como anti-

inflamatorias y comparar sus efectos con aquellos obtenidos con un extracto vegetal estandarizado de *Thymus serpyllum* (*Serpylli herba*) que se encuentra comercializado en la actualidad.

Así, se propusieron dos objetivos principales:

1. Evaluar el efecto anti-inflamatorio intestinal de distintas dosis de *Serpylli herba*, *Phlomis lychnitis*, *Phlomis purpurea*, *Lavandula dentata* y *Lavandula stoechas*. Para ello, los diferentes extractos vegetales fueron administrados por vía oral en un modelo de colitis experimental inducida por ácido trinitrobencenosulfónico (TNBS) en ratas y en el modelo del sulfato de dextrano sódico (DSS) en ratones siguiendo un protocolo curativo.

2. Establecer los mecanismos de acción involucrados en el efecto beneficioso de los extractos vegetales a través de experimentos in vitro en diferentes células implicadas en la respuesta del sistema inmune. Además se evaluó la actividad antioxidante, el efecto sobre la respuesta inmune y sobre la integridad del epitelio intestinal de los diferentes extractos.

**METODOLOGÍA**

Inicialmente, para llevar a cabo una primera aproximación a la naturaleza de los extractos, se estableció el contenido de polifenoles y la actividad antioxidante mediante del método del DPPH [14].

Para llevar a cabo los experimentos sobre la actividad biológica, en el modelo de TNBS se siguió un protocolo de tratamiento curativo: una vez establecida la colitis tras la administración intra-rectal del TNBS [15], se administraron diariamente las diferentes dosis orales de los extractos durante una semana, con el objeto de controlar el proceso inflamatorio. Al final de las experiencias, los animales fueron sacrificados y el colon fue extraído en su totalidad. El daño macroscópico fue valorado en función de la relación peso/longitud del colon y una evaluación visual a la que se asignó un índice de daño macroscópico (IDM) de acuerdo con el criterio descrito por Bell y col. (1995) [16]. Para la evaluación microscópica, secciones de las muestras de colon fueron teñidas con

hematoxilina eosina y los cambios histológicos fueron evaluados según el criterio establecido por Camuesco y col. (2004) [17].

Las determinaciones bioquímicas incluyeron la valoración del estado oxidativo colónico, mediante la determinación de la actividad de la enzima mieloperoxidasa (MPO) [18], el contenido de glutation (GSH) total [19] y la expresión proteica de la enzima óxido nítrico sintasa inducible (iNOS) intestinal mediante Western blot. Asimismo se valoró la producción de citocinas pro-inflamatorias como el factor de necrosis tumoral α (TNF-α) o las interleucinas (IL) -1β e IL-6 mediante técnicas de ELISA. Finalmente, se procedió al análisis de la expresión génica, mediante PCR cuantitava y semi-cuantitativa, de distintos marcadores del proceso inflamatorio. Esto incluye citoquinas como TNF-α, IL-1β, IL-6, IL-17; mediadores quimiotácticos como la proteína quimio atrayente de monocitos MCP-1 (*monocyte chemoatractant protein*), la molécula quimio atrayente de neutrófilos inducidos por citoquina CINC-1 (*cytokine-induced neutrophil chemoattractant*) y la proteína inflamatoria de macrófagos MIP (*macrophage inflammatory protein*)-2; la molécula de adhesión intercelular ICAM (*inter-cellular adhesion molecule*)-1, y marcadores de la función de barrera intestinal como mucinas (MUC-2 y MUC-3), *trefoil factor* (TFF)-3, y proteínas de las uniones estrechas del epitelio como *Zonula occludens* (ZO)-1.

Cuando se realizaron los ensayos en el modelo del DSS se siguió también un protocolo de tratamiento curativo. Una vez desarrollada la colitis por la presencia de DSS al 2,5% en el agua de bebida durante 5 días, se administraron los distintos extractos por vía oral. Durante el desarrollo de las experiencias se controlaron diariamente una serie de parámetros generales, que incluyen el consumo diario de comida de los animales, la evolución del peso corporal, y la aparición de heces diarreicas y sanguinolentas por visualización de restos perianales [20]. Tras el sacrificio de los animales, el colon fue extraído en su totalidad, y las alteraciones intestinales fueron caracterizadas en base a parámetros macroscópicos, microscópicos y bioquímicos, de manera similar a lo que se realizó en el modelo experimental anterior, evaluando el efecto anti-inflamatorio intestinal de los diferentes tratamientos administrados.

Además en el caso de *L. dentata* y *L. stoechas* se profundizó más el estudio evaluando los efectos beneficiosos de estos extractos sobre la maduración de los macrófagos intestinales, lo cuales desempeñan un papel clave en el mantenimiento de la tolerancia de la *lamina propria* del colon.

Se llevaron a cabo diferentes experimentos in vitro en distintas células involucrada en la respuesta inmune: línea celular de células epiteliales de carcinoma rectal de ratón (CMT-93) y de células de adenocarcinoma rectal humanas (Caco-2), macrófagos derivados de medula ósea de ratón (BMDM), células mononucleares de sangre periférica (PBMCs) y células dendríticas derivadas de monocitos (moDCs).

Además, la EII se asocia también a la aparición de síntomas extra-intestinales y, por este motivo, podría ser considerada una patología sistémica. Estos síntomas pueden aparecen antes, simultàneamente o a posteriori del diagnóstico de la EII, y determinan un empeoramiento de la calidad de vida de los pacientes la cual ya se ve comprometida por las manifestaciones intestinales de la patología [21]. Entre todas las manifestaciones extra-intestinales, las más comunes son las afecciones articulares, con síntomas similares a un proceso reumático [22, 23]. Por esto, de manera adicional, se evaluó el efecto anti-inflamatorio de los extractos de *L. dentata* y *L. stoechas* sobre edema plantar inducido por carragenina en ratones, confirmando la capacidad de estos extractos para modular la respuesta inmune también en este modelo experimental.

**RESULTADOS**

***Thymus serpyllum***. El extracto *Serpylli herba* manifestó efecto anti-inflamatorio intestinal en los dos modelos de colitis experimental utilizados: modelo experimental inducido por TNBS en ratas y el inducido por DSS en ratones. Mediante de los estudios histológicos (Figuras 14 y 17) se observó una recuperación del daño intestinal y, a través de los análisis bioquímicos, se puso de manifiesto una mejora de los diferentes marcadores del proceso inflamatorio, entre los cuales se encuentran la actividad de la MPO (Tabla 6), el contenido de glutatión total, los niveles de leucotrieno B4 (Figura 16) y la expresión enzimática de iNOS y COX-2 (Figura 15). Este efecto beneficioso estuvo asociado a una reducción de la expresión de distintas citoquinas como TNF-α, IL-6, IL-

1β, IFN-γ e IL-17, la quimocina MCP-1 y la molécula de adhesión ICAM-1 (Figuras 16 y 18), mejorando así la respuesta inmune alterada asociada a la inflamación del colon. Por todo ello, podemos concluir que *Serpylli herba* posee un efecto anti-inflamatorio en diferentes modelos de colitis experimental que podría ser atribuido a sus propiedades moduladoras de la respuesta inmune.

***Phlomis lychnitis* y *Phlomis purpurea*.** Los análisis histológicos (macroscópicos y microscópicos) (Figura 20) de las muestras de colon evidenciaron que los extractos de *Phlomis lychnitis* y *Phlomis purpurea* ejercen un efecto anti-inflamatorio que ha sido confirmado bioquímicamente a través de una disminución de la actividad de la MPO (Tabla 7), que es un marcador de la infiltración de neutrófilos, un aumento del contenido de glutation total, que contrarresta el estrés oxidativo asociado al proceso inflamatorio, y una reducción de la expresión de la iNOS (Figura 21). Sin embargo, solo el extracto de *Phlomis purpurea* redujo la expresión de las citocinas pro-inflamatorias IL-1β e IL-17 (Figura 22), las quimocinas CINC-1 y MCP-1, así como también la molécula de adhesión ICAM-1 (Figura 23), mejorando la respuesta inmune alterada asociada a la inflamación colónica. Además, tanto *Phlomis lychnitis* como *Phlomis purpurea* fueron capaces de incrementar significativamente la expresión de marcadores de la integridad del epitelio intestinal como MUC-2, MUC-3 y villin (Figura 24), poniendo de manifiesto una mejora de la permeabilidad del colon que se encuentra alterada en la inflamación intestinal. Ambos extractos manifiestan actividad en el modelo de colitis experimental inducido por TNBS, estableciendo una base experimental que sustenta sus usos tradicionales en desordenes digestivos. Además de sus propiedades anti-oxidantes, otros mecanismos pueden contribuir al efecto beneficioso, como la mejora de la función barrera del epitelio intestinal y una reducción de la respuesta inmune.

***Lavandula dentata* y *Lavandula stoechas*.** Los extractos de *L. dentata* y *L. stoechas* revelaron propiedades inmunomoduladoras en experimentos in vitro, llevados a cabo en distintos tipos celulares involucrados en la respuesta inmune. Ambos extractos redujeron la producción de diferentes mediadores de la inflamación como citocinas y óxido nítrico. Además, manifestaron efecto anti-inflamatorio intestinal en el modelo de colitis experimental inducido por TNBS en ratas, reduciendo los niveles de MPO e

incrementando el contendido total de glutatión (Tabla 9), indicando una disminución de la infiltración de neutrófilos y una mejora del estado oxidativo. Asimismo, ambos extractos modularon la expresión de citocinas pro-inflamatorias y quimiocinas mejorando la alteración de la función barrera del epitelio intestinal (Figuras 34 y 35). Los extractos de *L. dentata* y *L. stoechas* mostraron efecto anti-inflamatorio en otro modelo experimental, el edema plantar inducido por carragenina en ratones, donde se pudo observar una disminución significativa del grosor de las patas. Este efecto se acompañó con una reducción de la expresión de diferentes enzimas inducibles como MMP-9, iNOS y COX-2 , asi como citoquinas pro-inflamatorias (Figura 40). Es importante destacar que ambos extractos fueron capaces de influir en el proceso de diferenciación de los macrófagos intestinales, que se encontraba alterado en la inflamación inducida por DSS en ratones. Los macrófagos intestinales, gracias a sus propiedades anti-inflamatorias, contribuyen al mantenimiento de la tolerancia intestinal [24], asi, los tratamientos con los extractos de *L. dentata* y *L. stoechas* fueron capaces de favorecer la diferenciación de dichos macrófagos, manifestando entonces su actividad anti-inflamatoria (Figura 39). En conclusión, ambos extractos presentan efecto anti-inflamatorio intestinal, confirmando, nuevamente, sus potenciales usos como remedios naturales en patologías digestivas. Asimismo, sus efectos beneficiosos se manifiestan también en la inflamación articular sugiriendo una posible utilización para el tratamiento de los síntomas extra-intestinales de la EII.

**CONCLUSIONES**

1. *Serpylli herba* manifestó efecto anti-inflamatorio intestinal en dos modelos experimenales de colitis; su efecto podría ser atribuido a sus propiedades inmunomoduladoras.

2. Los extractos de *Phlomis lychnitis*, *Phlomis purpurea*, *Lavandula dentata* y *Lavandula stoechas* presentaron actividad anti-inflamatoria intestinal en el modelo de colitis experimental inducida por TNBS en ratas. Los resultados obtenidos fueron similares a los logrados con el extracto estandarizado de *Serpylli herba*. Esto confirma su

uso tradicional como anti-infamatorios en desordenes del trato gastrointestinal y demuestra su potencial desarrollo como agentes anti-inflamatorios para el tratamiento de la EII.

3. Además de sus propiedades antioxidantes, otros mecanismos pueden contribuir a este efecto beneficioso, como una mejora de la barrera epitelial intestinal y una reducción de la respuesta inmune. Todos los extractos han demostrado poseer un efecto inmunomodulador directo en diferentes tipos celulares. En particular, *Lavandula dentata* y *Lavandula stoechas* fueron capaces de mejorar la maduración de los macrófagos residentes del intestino y podrían interferir en la presentación del antígeno de las DCs a las células T. Esta inmunomodulación podría contribuir a una mejora del proceso inflamatorio que caracteriza la EII

# INTRODUCTION

## INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract characterized by chronic and spontaneously relapsing inflammation of the intestine. IBD comprises two major conditions: Crohn’s disease (CD) and ulcerative colitis (UC). They differ in terms of their distributions in the gastrointestinal tract and in their macroscopic and histological features. CD is characterized by a transmural, segmental, and typically granulomatous inflammation, and the inflammatory process may involve any part of the gastrointestinal tract, from mouth to anus. Unlike CD, UC is a non-transmural inflammatory disease that is restricted to the colon, being the inflammatory changes limited to the mucosa and submucosa, with cryptitis and crypt abscesses [1, 2]. It is not always possible to distinguish between CD and UC, thus the definitive differentiation often evolves during the course of disease.

The appearance of the symptoms depends on the severity and the site of inflammation. Most of them are common between CD and UC patients, and include diarrhoea, fever, abdominal pain and cramping, blood and/or mucus in the stool, and even clinical signs of bowel obstruction [1, 25]. Moreover, IBD is often associated with extraintestinal symptoms, and for this reason IBD could be also considered as a systemic disease. These symptoms can appear before, simultaneously and after the diagnosis of IBD, and are also associated with a worsening of the quality of life of the patients, which is already clearly compromised with the intestinal manifestation of the disease [21]. In fact, it has been reported that the severity of some of these extraintestinal manifestations parallels the activity of IBD, and respond after treatment of the underlying disease; however, others seem to follow an independent course and require targeted treatment [26]. Although joint involvement is the most common extraintestinal manifestation in human IBD, skin, eyes, lungs, heart and vascular system can be also affected [22, 23].

## 1. Etiology

The etiology of IBD has not been fully elucidated but it is probably related to dysregulation of the mucosal immune response toward the resident bacterial flora together with genetic and environmental factors [3] (Figure 1).

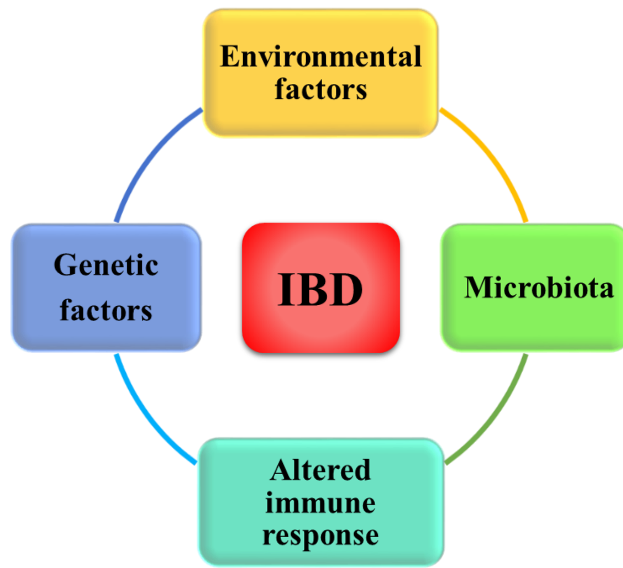


**Figure 1.** Factors involved in Inflammatory Bowel Disease (IBD).

**Genetic factors:** Genetic factors play an important role, as shown by epidemiologic studies on monozyotic twins. These studies suggested a key heritable component for both CD and UC. Concordance rates in monozygotic twins is 36% for CD whereas that for UC is 16%. Concordance rates for dizygotic twin pairs are 4% for both diseases [27].

Genetic loci associated with IBD were initially identified using linkage studies, which first demonstrated an association with a locus on chromosome 16 [28]. Many of these genes can act influencing autophagy, maintenance of the integrity of the epithelial barrier, innate and adaptive immune responses, injury repair, response to oxidative stress and microbial defence and antimicrobial activity [2]. Other interesting facts are that immunological functions can be influenced by several risk loci through a single pathway.

The *intracellular nucleotide oligomeration domain 2* (NOD2) [29, 30], CARD9 [31] and ITLN1 [32] in the innate immune response; Interleukin (IL)-23R [32], STAT3 [32] and TNFSF15 [33] in the IL-23-Th17 pathway, ATG16L1 [34, 35] and IRGM [36] in autophagy, XBP-1 [37] and ORMDL3 [38] in endoplasmic reticulum (ER) stress, and protein–tyrosine phosphatase, non-receptor type (PTPN2) [39, 40] in T cell response, have been identified. Moreover, genetic polymorphisms can act synergically to modify the Paneth cell functions [41]. Although the vast majority of loci are shared between both diseases with similar directions of effect, some loci (such as NOD2 and ATG16L1) are specifically associated with CD alone and others only with UC [42]. The NOD2*/caspase recruitment domain 15 gene* (CARD15) variant was the first IBD gene discovered, associated with ileal CD [29]. This gene is expressed in different cellular types like dendritic cells (DCs), Paneth cells and mucosal epithelial cells. This intracellular molecule recognizes bacterial components, like peptidoglycans, which leads to a cascade activating nuclear factor κB (NF-κB) [43]. Therefore, NOD2/CARD15 may represent an antibacterial factor and its mutation might results in decreased of immune defence. Furthermore, this gene modulates the secretion of defensins that are endogenous molecules produced by Paneth cells: NOD2/CARD15 mutations have also a negative impact on the release of defensins [44, 45]. However, it is important to note that only ≈20% of CD patients are homozygous for NOD2 variants [46, 47]; mutations in this gene alone does not spontaneously result in IBD. Hence, genetics highlight the key part played by the interaction between the internal microenvironment in the form of gut microbial dysbiosis and associated immunological response, both processes influenced by the external environment.

**Environmental factors:** Incidence and prevalence of IBD are increasing with time. The annual incidences of CD and UC are very similar: 0–20.2 per 100,000 in North America and 0.3–12.7 per 100,000 in Europe for CD; and 0–19.2 per 100,000 in North America and 0.6–24.3 per 100,000 in Europe for UC [48]. Moreover, it has been shown that the frequency of IBD is higher in developed countries than in developing ones [49]. This is confirmed by different studies in which the incidence of UC in first-generation

and second-generation Indian migrants to the UK became similar to the native UK population, and higher than the incidence in the countries of origin, whilst the incidence of CD was lower [50-52]. There is large number of risk factors for the development of IBD, as shown by different epidemiologic studies like: smoking, dietary habits, appendectomy, excessive use of drugs as nonsteroidal anti-inflammatory drugs (NSAID) [53] and oral contraceptive [54, 55], lifestyle [56-58] and hygiene. The hygiene hypothesis was proposed in 1989 [59] to explain the higher incidence of autoimmune diseases in the developed world. The lack of exposure to different antigens, typically of common infections, has been inversely associated with risk of IBD. All these factors can influence the gut microbiota and, in turn, the development and maturation of immune responses. This hypothesis has been supported by the gradual increase of IBD prevalence reported in those developing countries that have modified their habits by acquiring the western ones [60, 61].

**Microbiota:** The gastrointestinal tract is exposed to a wide variety of antigens: dietary components, and, particularly, microorganisms, such as bacteria, viruses and fungi [62]. While the small bowel is normally not exposed to extensive colonization, in the colon, up to 60% of the stool mass consists of bacteria, which means that each gram of stool contains $10^{10}$-$10^{12}$ bacteria [63]. It is interesting to note that before birth, the gastrointestinal tract is sterile, the colonization starts when the first microbial exposure occurs and different subsequent events like diet, assisted or vaginal childbirth, level of sanitation and exposure to antibiotics will determine its composition [64, 65]. After birth, the gut of newborns, being an aerobic environment, can afford only facultative anaerobes growth, such as members of the *Enterobacteriaceae* family. However, the intestinal lumen turns anaerobic after a few days and changes also the flora composition: the gut becomes a favourable site for the colonization of strict anaerobes, such as *Bifidobacterium*, *Clostridium*, and *Bacteroides* [66]. After that, the colonization continues with *Enterococcaceae*, *Streptococcaceae*, *Lactobacillaceae*, *Clostridiaceae*, and *Bifidobacteriaceae*, being the predominant bacterial taxa, resembling the maternal skin and vaginal microbiome. Moreover, since the diet of an infant until weaning consists of milk, oligosaccharide fermenters like bifidobacteria are the most relevant bacterial

group in this stage [67-69]. Another important change occurs when the introduction of solid foods takes place: there is an increase in abundance of *Bacteroides*, *Clostridium*, *Ruminococcus*, and a decrease in *Bifidobacterium* and *Enterobacteriaceae* [70, 71]. After weaning, the composition of microbiota gradually alters to adult-like gut microbiota, which is abundant in *Ruminococcaceae*, *Lachnospiraceae*, *Bacteroidaceae* and *Prevotellaceae* [67].

The composition of microbiota is not static, but it changes with age, geography and is influenced by other external factors, like diet and medications [67, 72, 73]. High-throughput 16S ribosomal ribonucleic acid (RNA) gene sequencing techniques have accelerated the knowledge of gut microbiome diversity [74]. Studies based on this technique have highlighted that only 7–9 of the 55 known divisions or phyla of the Bacteria domain are detected in faecal or mucosal samples from the human gut [75-77]. Among these, *Bacteroides*, *Faecalibacterium* and *Bifidobacterium* are the most abundant genera in human faecal samples, but their relative abundance is highly variable between individuals [78].

Several studies have demonstrated an association between microbiota composition and various aspects of host health, including physiological development, metabolism, and immunological response [79-82]. This important role attributed to gut microbiota has been confirmed by experiments performed with germ-free animals; the results showed that these animals are more susceptible to infections, showing reduced vascularity, digestive enzyme activity, muscle wall thickness, cytokine production and serum immunoglobulin levels [83].

Appropriate development of the immune response is dependent on gastrointestinal colonization. The immune system of the mucosa interacts continuously with the bowel contents, nutrients and bacterial species specific for a given location are tolerated. The development of mucosal immune system is very complex, mainly because is very important to distinguish commensal microbiota from pathogens. The epithelium provides the first line of defence and tolerates commensal organisms through numerous mechanisms. These include the masking or modification of microbial-associated

molecular patterns that are usually recognized by pattern recognition receptors (PRR)s, such as Toll-Like Receptors (TLRs) [84] and NOD/CARD [85], and the inhibition of the NF-κB inflammatory pathway [86]. The commensal microbiota plays an important role in human health by acting as a barrier against pathogens and their invasion with a highly dynamic modality, exerting metabolic functions and stimulating the development of the immune system.

The failure to obtain or maintain this complex homeostasis has a negative impact on the intestinal and systemic health. In fact, IBD is associated with an imbalance of the composition of the intestinal microbiota, process known as dysbiosis, which may promote inflammation. It has been shown that dysbiosis causes a modification of intercellular tight junctions [87], leading to an effective penetration of antigens responsible of the activation of Gut-Associated Lymphoid Tissue (GALT) with the consequent tissue damage [88]. However, at present, it has not been fully clarified if the altered gut microbiota is a cause of disease or a consequence of the inflammatory state of the intestinal environment. In fact, the composition of the intestinal microbiota in patients with IBD and in healthy subjects has been assessed using molecular methods such as 16S rRNA sequence analysis and polymerase chain reaction (PCR). In IBD patients the bacteria concentration was greater than $10^9$/ml in 90–95% of IBD patients, whereas this was true in 35% of healthy controls [89]. However, although there is an increase of bacterial concentration in IBD, the microbial diversity has been reported to be diminished [90], particularly in those patients with active disease. It is interesting that commensals belonging to the *Clostridiales* order, such as *Faecalibacterium* and *Roseburia*, were significantly reduced in patients with ileal CD [91, 92]. These genera are potent producers of short-chain fatty acids, like butyrate, and have shown to exert protective effects in mouse colitis models [93]. In addition, different beneficial effects on immune function have been reported for these bacteria. In this sense, Clostridial groups promote the accumulation of regulatory T cell (Treg) cells in the mouse colon [94]. Furthermore, *Bacteroides fragilis*, which is able to produce polysaccharide A, suppresses IL-17 production and promotes the activity of IL-10-producing $CD4^+$ T cells in mice [95]. Moreover, *Faecalibacterium prausnitzii* is considered a sentinel of 'normal' microbiota, and it stimulates IL-10 production in

peripheral blood mononuclear cells [96], thus promoting in all these cases an intestinal anti-inflammatory status. In fact, it has been described the existence of lower levels of *F. prausnitzii* in CD patients than in healthy controls. Furthermore, in patients who had undergone ileal resection, the depletion of this bacterium was predictive of postoperative disease recurrence [97]. Finally, other studies have revealed a lower concentration of Firmicutes and increased Gammaproteobacteria and Enterobacteriaceae in UC and CD patients [98, 99].

**Altered immune response:** Recent progress with genome-wide association studies has identified many IBD susceptibility genes [100, 101].

In individuals with genetic risk, abnormal interactions between the host immune system and gut microbiota leads to dysregulation of cellular responses such as autophagy and ER stress, and induces an abnormal host immune response in the gut that may result in intestinal inflammation. Under physiological conditions, *lamina propria* hosts a large number of different immune cells, including macrophages, DCs, mast cells, neutrophils, eosinophils, natural killer (NK), NKT cells, T and B cells. All of these cells coexist in a perfect equilibrium that confers tolerance and protection at the same time (Figure 2A). Under inflammatory conditions, there is an infiltration of a large number of activated immune cells into the intestinal mucosa (Figure 2B), in response to the increased expression of inflammatory mediators, chemokine receptors (such as CCR5, CCR6, CCR9) and integrins (like integrin α4β7). Furthermore, fibroblasts and endothelial cell express high levels of chemokines in the mucosa, facilitating intermolecular interactions of leukocytes and their migration from blood to the mucosa, thus promoting local inflammatory response [4-7]. The adaptive immune response can clearly contribute to the pathogenesis of IBD by increasing the release of pro-inflammatory cytokines, which are important to drive the polarization of the T-helper (Th) response, but also to reduce the effectiveness of regulatory T-cells. Several studies have demonstrated that $CD4^+$ T cells isolated from IBD patients produce different type of cytokines: in CD inflamed tissue, these cells produce large amounts of Th1/Th17- associated proinflammatory cytokines (Interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-17A among others); while in

UC patients there is a predominance of Th2-related cytokines (IL-4 and IL-13) and Th17-associated proinflammatory cytokines (IL-17A) [5, 7] (Figure 2B).

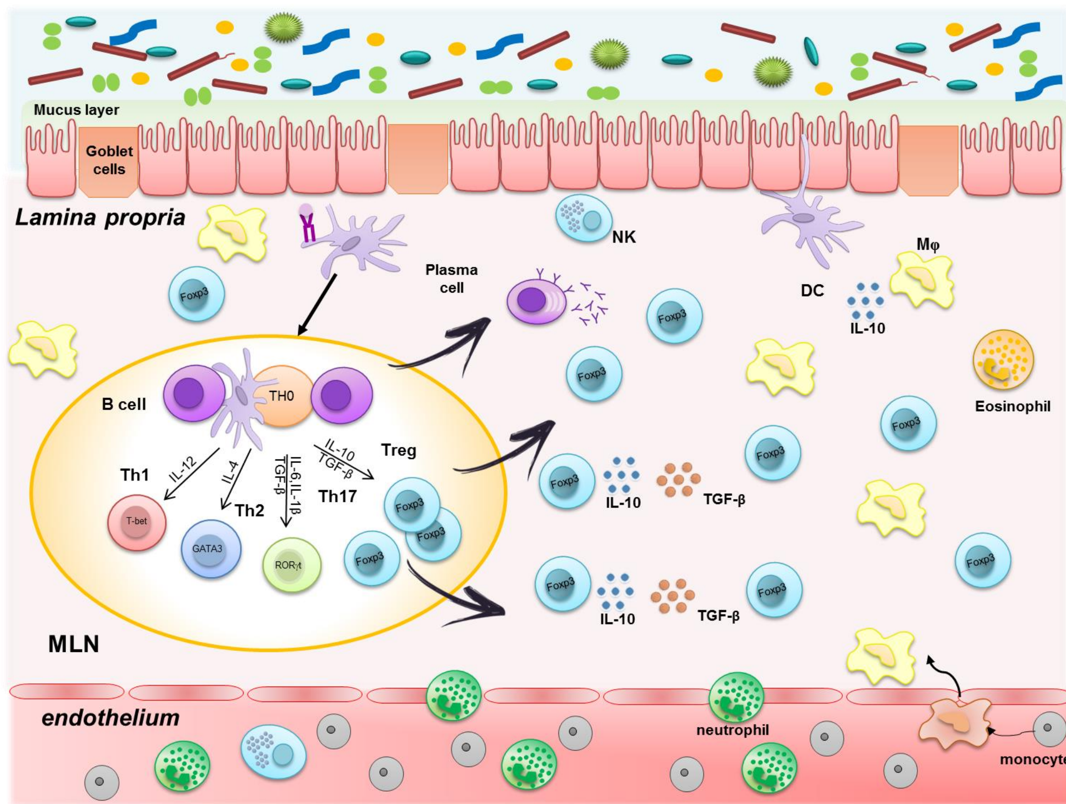


**Figure 2.** Physiopathology of IBD. (A) The intestine is the largest mucosal surface exposed to the external environment. It constitutes an interface between the host and the luminal contents, which include nutrients and the highest count of resident microbes. Thus, the intestinal immune system meets more antigens than any other part of the body and it must discriminate between invasive organisms and harmless antigens, such as food, proteins and commensal bacteria, to prevent infections or preserve the homeostasis. This intestinal homeostasis depends on the dynamic interaction between the microbiota, the intestinal epithelial cells and the resident immune cells, which coordinate a response that keeps the balance between immunity and tolerance.

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## 2. The mucosal immune system

Given the large number of bacteria in the intestinal lumen, the gastrointestinal mucosal immune system must display a delicate homeostatic balance between maintaining tolerance toward commensal microbiota and being able to develop an aggressive immune response against invading pathogens. The mucosal immune system involves three lines of defense: the mucus layer, epithelium, and *lamina propria.*

**Mucus:** A layer of mucus, which constitutes the first line of intestinal defence, protects the surface of the intestine. Mucus is generated by goblet cells in the epithelium, and external and inner parts can be differentiated.

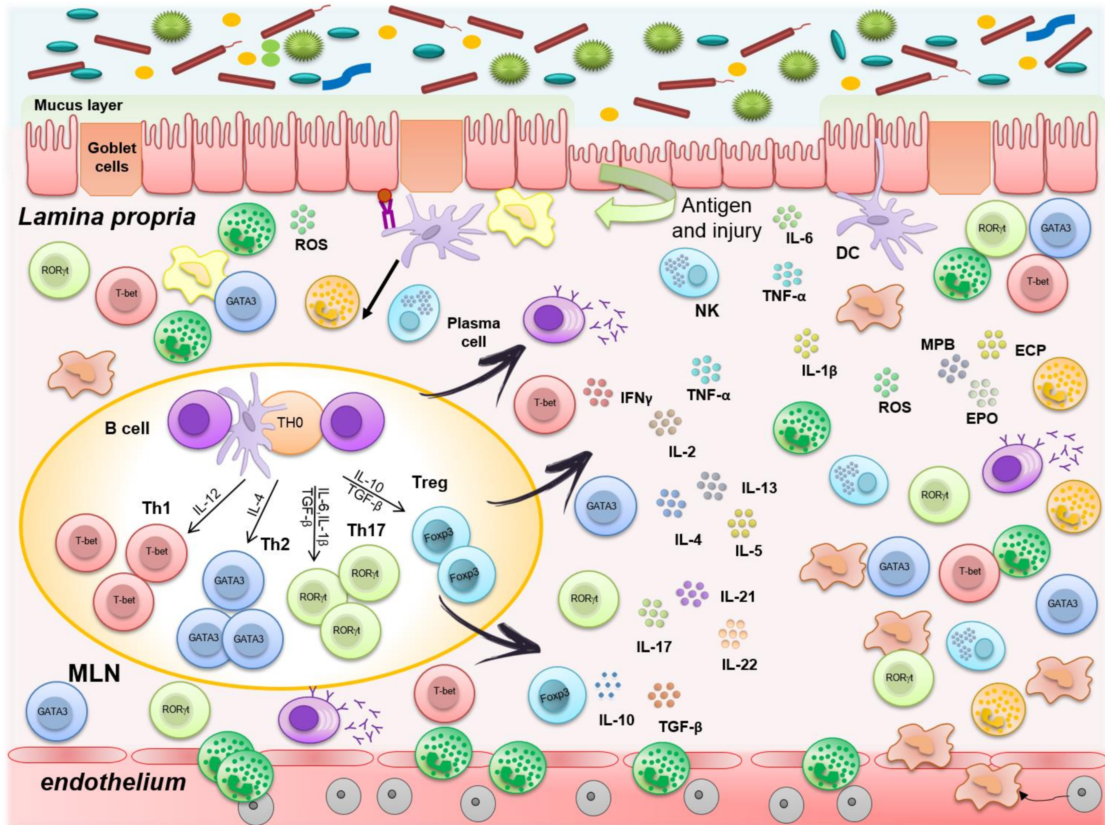


**Figure 2.** Physiopathology of IBD. (B) A breakdown of this balance triggers the chronic inflammatory process that characterizes IBD. There are often several pre-existing conditions that lead to the disease: first of all, a genetic susceptibility of the intestinal immune system to distinguish an environmental antigen presented within the gastrointestinal tract; secondly, the contact with the antigen; and finally, usually due to an alteration of the permeability, the antigen is presented to the gastrointestinal mucosal immune system through its paracellular passage, which triggers the inflammatory cascade. During early inflammation, luminal antigens activate the different innate immune cells located in the intestine, including natural killer cells, mast cells, neutrophils, macrophages and dendritic cells, and maintained inflammatory reaction promotes the activation of the adaptive immune response. Abnormally activated effector CD4$^{+}$ T helper (Th) cells synthesize and release different inflammatory mediators that generate an amplified inflammation that originates chronic tissue injury and epithelial damage.

The inner layer has higher thickness (approximately 100 µm), is adhered to the epithelium, and a lower number of bacteria is present in comparison with the external layer of mucus. Both layers contain antimicrobial substances, and are mainly constituted

by mucins, which play a key role in intestinal homeostasis. Mucin (MUC)-2 gene is the principal secretory of mucin in the intestine and its mutation is associated with IBD, which is supported by studies performed in Muc-2 deficient mice that spontaneously develop colitis. CD patients show decreased MUC1 and MUC4 levels in the ileum, while MUC2, MUC5AC, UC5B, MUC6, and MUC7 are undetectable in the lesions of these patients [102]. UC patients also show decreased MUC2 expression [103-105]. It has been proposed that the decreased levels of mucins cause an impaired mucus layer that leads a direct contact between bacteria and epithelial cells [106].

**Epithelium:** The epithelium is the main component of the epithelial barrier and consists of a monolayer constituted by specialized intestinal epithelial cells (IECs): absorptive enterocytes, goblet cells (mucus producers), enteroendocrine cells (hormone producers) and Paneth cells (growth factor and antimicrobials producers) [107]. All are tied together through the tight junction (TJ) network that separates the body from intestinal lumen components. It is a real physical barrier that regulates the permeability, the production of mucus to form the layer covering the luminal surface of the epithelium, and the recognition of pathogens and production of antimicrobial peptides (AMPs) to ensure effective immunity [108]. Alterations in structure of TJ proteins are associated with the development of IBD [109-111]. Furthermore, it has been shown that TNF-α and IFN-γ, two proinflammatory cytokines whose production is increased in inflammatory conditions, increase TJ permeability and induce apoptosis of IECs [112-114]. This leads to the loss of the epithelial barrier function and induces epithelial damage and ulcers that are present in mucosal inflammation. Moreover, intestinal epithelial cells are also involved in the innate immune response through the expression of membrane TLRs and NOD, which are the most important among intestinal pathogen recognition receptors and essential to maintain homeostasis [115]. These patterns recognize highly conserved "pathogens-associated molecular patterns" (PAMPs), like peptidoglycan and lipopolysaccharide (LPS) among others, which are located on bacteria surface. This binding leads to the activation of immune response through the activation of NF-κB [115, 116].

***Lamina propria***: *Lamina propria* can be considered as the third line of intestinal defence, which contains cells of the innate and the adaptive immune system. The cells from the innate immune system are characterized by their immediate, but non-specific, response against infections; they also determine the beginning of the adaptive response. These cells include macrophages, monocytes, neutrophils, DCs, NK cells and granulocytes. All these cells produce different pro-inflammatory mediators and antimicrobial substances that modulate the phagocytosis of microorganisms and the infected cells. When innate immunity is no longer able to counteract the pathogen aggression, the adaptive immune response is triggered. The adaptive immune response is constituted principally by T and B lymphocytes that generate the effector response mediated by cytokines and antibodies. This response, unlike the innate one, is highly specific and confers lasting immunity, known as memory. Disruption of the innate and acquired gut immune systems may cause the development of chronic intestinal inflammation.

## 2.1. Innate immunity

### Intestinal macrophages

Intestinal macrophages represent the most abundant mononuclear phagocyte population in the steady-state *lamina propria* of the intestine, being, in fact, the largest reservoir of body macrophages [117]. Although macrophages are typically classified in two major subsets depending on their surface markers, M1 and M2 [118], resident macrophages do not fit in this classification because they display hallmarks of both: they constitutively express high levels of MHCII and produce TNF-α [119, 120], features typically of M1 [121], but they also express CD206, CD163, and produce IL-10, which are associated with M2 subtype [122]. Intestinal resident macrophages contribute to immune tolerance via selective inertia, but also protect the organism through the development of the immune response and inflammation in other circumstances. Although resident macrophages have tollerogenic properties, and express low levels of co-

stimulatory molecules, like CD80, CD40 and CD86 [123-125], they are highly active phagocytic cells; thus, they express CD36, receptor responsible of phagocytic uptake of apoptotic cells that has also been reported to display strong bactericidal activity [124-126]. Similarly to DCs, resident macrophages are hyporesponsive to the stimulation of TLR ligands in steady-state [127, 128]. Moreover, resident macrophages display other fundamental functions that are essential for intestinal homeostasis: they act as a non-inflammatory scavengers, contribute to maintain the immune tolerance via production of IL-10 constantly (Figure 3) and also promote the renewal of epithelial cells secreting prostaglandin E2 (PGE2), among other functions [127-130]. All these features give a unique profile of these cells: they ingest and kill microbes without initiating over inflammation, and this acting as a barrier versus the commensal bacteria that have crossed the epithelium layer. Resident macrophages are also involved in sampling the gut luminal content extending their protrusions through the epithelium into the lumen. DCs cells are less frequent at sending the protrusion to capture antigens, but receive them from macrophages via Connexin 43 (Cx43). In fact, DCs of mice lacking Cx43 in CD11c$^{+}$ cells are unable to receive antigens from macrophages [131]. The crosstalk between resident macrophages and DCs seems to be crucial for maintaining intestinal homeostasis.  In fact, in the healthy gut, resident CX3C chemokine receptor 1 (CX3CR1)$^{+}$ macrophages lack expression of the chemokine receptor CCR7 that is required for migration to the mesenteric lymph nodes (MLNs) [132, 133]; however, it has been shown that only CD103$^{+}$ DCs can migrate to MLNs and induce Treg differentiation and imprint on them the expression of the gut homing receptors CCR9 and $\alpha 4\beta 7$ [134-137].

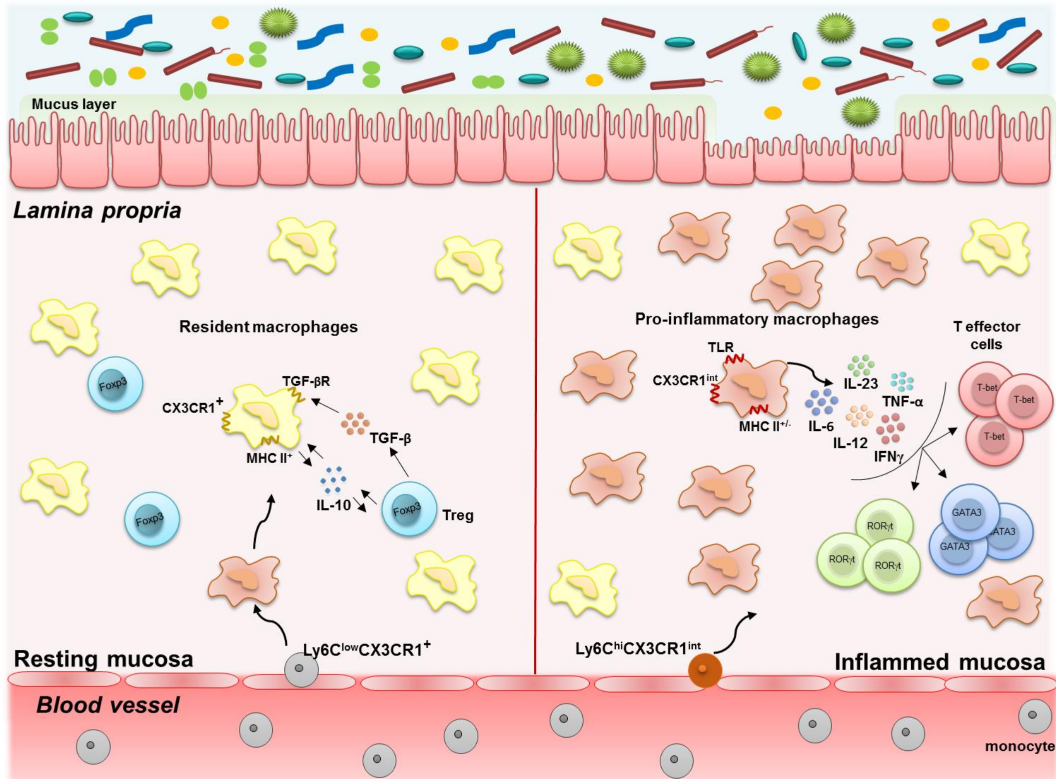


**Figure 3.** Origin and diversity of intestinal macrophages in healthy and inflamed intestine. In resting mucosa, the majority of intestinal macrophages express high levels of CX3CR1, the receptor for the transmembrane chemokine fractalkine (CX3CL1) expressed by intestinal epithelial cells. They produce IL-10 constitutively and are unresponsiveness to TLR or other stimuli. These cells do not produce pro-inflammatory and do not activate the response of T effector cells. IL-10-produced by resident macrophages is needed to maintain the survival of local FoxP3$^{+}$ Treg. Moreover, Treg produce TGF-β which, together with IL-10 and other local factors, maintain resident macrophages in their state of partial inertia. The resident macrophages may be replenished directly from the bloodstream either by resident CX3CR1$^{+}$ Ly6C$^{low}$ monocytes or by inflammatory CX3CR1$^{low}$ Ly6C$^{hi}$ monocytes. After breakdown of the epithelial barrier, or pathogenic invasion, there is an infiltration of large numbers of inflammatory macrophages in the mucosa, which express lower levels of CX3CR1. These CX3CR1$^{int}$ macrophages are derived from recently divided inflammatory CX3CR1$^{low}$ Ly6C$^{hi}$ monocytes in the bloodstream. The CX3CR1$^{int}$ macrophages produce large amounts of pro-inflammatory mediators that drive local inflammation and promote the function of effector T cells.

Of note, resident macrophages seem to maintain functional Treg cells that return from the lymph nodes to the tissue producing IL-10. Recent studies have highlighted the importance of IL-10 to maintain local Treg differentiation in the gut to avoid the activation of inflammatory reactions against food proteins and commensal bacteria [127, 138, 139].

In steady-state, Ly6C$^{hi}$ monocytes constantly enter the normal colonic mucosa and mature into CD64$^{+}$ F4/80$^{hi}$ CX3CR1$^{hi}$ MHCII$^{+}$ cells [119, 140]. These cells are characterized by their anti-inflammatory properties. The development of mature macrophages involves a local differentiation process, in which a series of intermediaries lose Ly6C and upregulate F4/80, CX3CR1, CD163, and CD206, as well as acquiring CD11c and MHCII expression. The maturation process seems to take 5 days and the differentiating monocytes adopt the phenotypic signature of mature colonic macrophages. Among CX3CR1$^{+}$ macrophages, is possible to distinguish two population based on the level of expression of the receptor CX3CR1: CX3CR1$^{hi}$ represent the mucosal resident macrophages with their typical features, as commented before; CX3CR1$^{int}$ represent a small population partially differentiated from Ly6C$^{+}$CCR2$^{+}$ monocytes into CX3CR1$^{hi}$ macrophages. CX3CR1$^{int}$ cells are TLR-responsive with pro-inflammatory characteristic [119]. During intestinal inflammation there are an interruption of the mechanisms that promote the development of anti-inflammatory macrophages, leading to the accumulation of TLR-responsive CX3CR1$^{int}$ macrophages that produce inflammatory mediators instead of the replacement of resident macrophages in resting intestine (figure 3). This is supported by an accumulation of monocytes and cells of first stages of differentiation during the colitis induced by dextran sulphate sodium (DSS) in mice [119].

A similar situation can be seen in T cell dependent colitis in mice [140] and in the inflamed mucosa of Crohn's patients. In the latter, there is an accumulation of CD14$^{hi}$ CD11c$^{hi}$ cells that seems to be the equivalent of Ly6C$^{hi}$ monocyte in mice [141]. In mice, the inflammatory cells of intermediated stages of differentiation up-regulate TNF-α, IL-1 and IL-6 production, as well as the hyper-responsiveness to TLR stimulation. The upregulation of TLRs expression of monocytes and CX3CR1$^{int}$ is associated strongly to microbiota, leads to inflammation in response to luminal materials and plays a key role in IBD.

### Intestinal dendritic cells

DCs, like intestinal resident macrophages, play a key role in maintaining the gut homeostasis. DCs stimulate the primary T-cell response and determine the type of response: against invading pathogens, it will be immunogenic, while against commensal bacteria antigen, it will be tolerogenic [142, 143]. The main function of DCs is to transport the antigen from *lamina propria* into MLNs and Peyer's patches (PP), and then generate the antigen-specific response [144, 145]. DCs have been reported to be located inside the intestinal epithelium, a strategic position from where the cells could potentially survey the luminal microenvironment [132, 146, 147]. This location could explain why these DCs express CD103, an integrin which functions as a receptor for epithelial E-cadherin, and is upregulated upon DC contact with epithelial cells [148-150].

As commented before, it is difficult to distinguish DCs from macrophages in the gut; murine intestinal DC populations are defined by expression of the integrins CD11c (αX) and CD103 (αEβ7) and lack of CX3CR1 [133, 151]. Moreover, unlike intestinal macrophages, DCs are $CD64^-$ [119]. DCs can be divided into $CD11b^+$ and $CD11b^-$ subsets; both populations express CCR7, fundamental marker for their migration to MLNs and to imprint gut-homing marker of Treg [132, 133, 152]. Indeed, DCs upregulate this chemokine receptor before migrating to MLNs, as confirmed in CCR7-deficient DCs that fail to migrate [153]. The $CD11b^-$ subset can drive the polarization of $CD4^+$ T cell into IFN-γ-producing Th1 cells [123, 124]. The other subset, the $CD11b^+$ one, stimulates Th17 and Th1 differentiation. However, both populations can generate Treg response; normal number of Treg cells has been found in mice lacking $CD103^+$ $CD11b^-$ [154] or $CD103^+$ $CD11b^+$ [125, 155] DCs. Moreover, $CD103^+CD11b^-$ cells populate the isolated lymphoid follicles [132], while $CD103^+CD11b^+$ cells occupy the *lamina propria*. The latter migrate to MLN, where they can induce the polarization of Treg via production of retinoic acid (RA, vitamin A derivative), transforming growth factor-β, (TGF-β) [135, 136], and the activity of indoleamine 2,3-dioxygenase (IDO), which converts tryptophan into immunosuppressive metabolites (kynurenines), which inhibit T effector development and promote regulatory T-cell generation [156].

The function of DCs is not cell-autonomous, since depend on the local microenvironment and epithelial cells. Epithelial cells, among other functions, are involved in the tolerance induction. They secrete and release different mediators like TGF-β, IL-10, RA, thymic stromal lymphopoietin (TSLP) and PGE-2. All these mediators, except TSLP, are induced by the presence of microbiota; TSLP is produced constitutively, and through it, the differentiation of tolerogenic phenotype of DCs is induced (also in human system) [149, 157]. NF-κB pathway seems to play an important role in this process; in mice in which IKK-β is deleted, thus leading at an impaired NF-κB signalling, there is a reduced expression of TSLP by epithelial cells and an up-regulation of IL12-p40 by DCs [158]. Thus, intestinal epithelial cells are very important for induction of tolerogenic DCs. In vitro studies have reported that co-culture of bone marrow-derived or splenic DCs with epithelial cell lines induces expression of retinaldehyde dehydrogenase (RALDH), a key enzyme that generates RA; moreover, the co-culture determines the upregulation of CD103, and the ability to generate gut-homing Treg cells, through the secretion of RA and TGF-β [148, 149]. Mice fed with a vitamin-A deficient diet present a reduction of $CD103^{+}CD11b^{+}$ cells [159]. By contrast, PGE2 inhibits the expression of RALDH in DCs [160]. During intestinal inflammation there is an increased production of different pro-inflammatory cytokines, which induce the expression of PGE2 that inhibits RALDH, thus reducing the capacity of $CD103^{+}$ DCs to polarize Treg. In fact, it has been recently described that the responsible of polarization of Th17 cells is only a subpopulation $CD103^{+}$ $CD11b^{+}$ lacking the enzyme involved in RA production. Moreover, these cells express also acyloxyacyl hydrolase (AOAH), an enzyme that inactivates LPS [161].

Thus, DCs are tolerogenic at steady-state, but they have the ability to change their features to potent T cell activators under inflammatory conditions. In experimental colitis there is an accumulation of DCs in MLNs with a pro-inflammatory phenotype. They show lover levels of RALDH, and they promote a Th1 imprinting response instead of a Treg one [162].

### Granulocytes: eosinophils and neutrophils

Recent studies have reported the existence of mucosal eosinophilia in the colonic segments from IBD patients. Similarly, it has been described an increased secretion of eosinophilic products in the colonic tissue of IBD patients, like eosinophilic cationic protein (ECP), major basic protein (MPB) and eosinophil peroxidase (EPO) among others [163]. In addition, it has been described that patients with active IBD show high levels of serum ECP, produced by peripheral eosinophils, when compared to non-active IBD patients [164]. Furthermore, in perfusates from UC patients, there is an increase of PGE2 in rectal and sigmoid colon, being this increase associated with elevations in ECP, myeloperoxidase (MPO) and TNF-α levels [165]. The role of eosinophils in intestinal epithelial integrity seems to be crucial. In fact, in vitro studies have demonstrated that the co-culture of eosinophils and epithelial cells induces a reduction in trans-epithelial resistance, thus indicating that eosinophil-derived products, like MPB, may promote the impairment of the epithelial barrier function [166]. Supporting this, it has been described that MBP-KO mice exposed to oxazolone colitis were relatively protected from colitis compared with wild-type mice [166]. Moreover, eosinophilia has been also observed in the DSS experimental model of mouse colitis, being associated with a release of EPO in the lumen.

On the other hand, polymorphonuclear neutrophils (PMN) play an important role in host innate immune responses and in the pathophysiology of inflammatory disorders, including IBD. In fact, several studies have evidenced the accumulation of a large number of PMN in the intestinal mucosa, which can be used as a marker of the active phase of IBD [167, 168]. Furthermore, in the inflamed mucosa, PMN migration is associated with transient decreases in transepithelial resistance [169, 170], thus affecting epithelial barrier integrity [171]. One of the main features of PMN infiltration, which clearly contributes to the disruption of the epithelial barrier function, is the release of large amount of cytotoxic mediators, such as reactive oxygen and nitrogen species into the intestinal mucosa. PMN activation results in the activation of the cell membrane localized nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which transfers

electrons from NADPH to molecular oxygen, thus generating superoxide radicals ($O_2^-$). $O_2^-$ is rapidly converted to hydrogen peroxide ($H_2O_2$), either by spontaneous dismutation or through catalysis by the enzyme superoxide dismutase (SOD). In addition, in the presence of $Fe^{2+}$, superoxide radicals can be converted, through a non-enzymatic secondary process, into highly reactive hydroxyl radicals (·OH), which are known to depolymerize gastrointestinal mucin, induce lipid peroxidation, oxidize proteins and carbohydrates, and promote deoxyribonucleic acid (DNA) degradation [172]. Moreover, superoxide radicals can act with nitric oxide (NO) and generate highly reactive peroxynitrite ($ONOO^-$). In addition, MPO, a granular enzyme also produced by PMN, can catalyse, in presence of $Cl^-$, the conversion of superoxide radicals into hypochlorous acid, an oxidizing agent that is 100-1000 times more toxic than $O_2^-$ or $H_2O_2$ [173]. It is well known the involvement of reactive oxygen species (ROS) in the pathogenesis of IBD, and their crucial role is further supported by the protective properties of free radical scavangers and other antioxidant in chemically induced colitis [174-176]. Furthermore, either transgenic over-expression of Zinc superoxide dismutase (ZnSOD) or ex vivo extracellular SOD gene transfer resulted in attenuation of DSS-induced colitis in mice [177, 178].

PMN have been also reported to synthesize and secrete a wide variety of pro-inflammatory cytokines, chemokines, growth factors and other inflammatory mediators. One of the most abundant is IL-8 [179], although this chemokine is also produced by macrophages and epithelial cells, PMN represent the main IL-8 expressing cell population at the base of ulcers, in inflammatory exudates on the mucosal surfaces, in crypt abscesses and at the borders of fistula tracts [180]. Also, PMN secrete macrophage inflammatory protein (MIP)-1α and MIP-β, important mediators involved in the recruitment of monocytes, macrophages, eosinophils and lymphocytes. Finally, PMN are also able to produce TNF-α, IL-1α and IL-1β at sites of inflammation, which can actively contribute to the inflamed response of the intestine in IBD patients.

**Natural Killer cells**

NK cells are innate lymphocytes that can regulate immune responses through direct killing of target cells or indirectly by secretion of a variety of cytokines, most notably IFN-γ [181]. The relevance of NK cells to IBD pathogenesis has not been clearly defined. It has been reported that NK depletion in DSS mice colitis promotes a more severe inflammation with high mortality, most probably because the depletion of these cells increases the neutrophil infiltration [182]. In addition, NK cells can inhibit the proliferation and activation of $CD4^+$ T cells [183]. One the most important mediators produced by NK cells is IL-22; this is a particular cytokine since the expression of IL-22 receptor is primarily restricted to epithelial cells and fibroblasts, being these cells the targets for the role of IL-22 in maintaining homeostasis of tissue barriers in the intestine, skin, and lung. Also, IL-22 signalling is important for promoting wound healing and epithelial regeneration [184, 185]. All these data suggest a key role of NK cells in the preservation of epithelial barrier homeostasis [186, 187].

## 2.2. Adaptive immunity

### 2.2.1. T cells

T cells are key players of adaptive immune response that cooperate with other cells and molecules from innate immune system to generate an effective response in order to eliminate the invading pathogens. Upon contact with antigen-presenting cells (APCs), naive $CD4^+$ cells have the potential to differentiate into different Th subtypes: Th1, Th2, induced regulatory T cells (iTreg) or Th17 cells, being this process controlled by the effector cytokines produced by APCs. Each Th subtype exert specific functions: Th1 cells are essential to eliminate intracellular pathogens while Th2 cells mediate allergic reaction and confer protection against parasites, and Th17 cells contribute to remove extracellular bacteria and fungi [188, 189]. A dysregulation of the immune response leads to an abnormal activation of T cells, thus releasing cytokines and chemokines that promote the

beginning of deleterious effects from innate and adaptive components of the immune system, and generating a vicious circle that facilitate the inflammatory process.

### Th1 and Th2 cells

Th1 cells are characterized by the ability to secrete IFN-γ, a potent activator of intracellular killing macrophages, being their main role to protect the host against intracellular pathogens, i.e. intracellular bacteria and viruses that have developed strategies to survive or replicate within macrophages. The Th1 development from naive $CD4^+$ cells implies the sequential actions of STAT1, induced by IFN-γ, and STAT4, induced by IL-12, to promote the expression of the transcription factor T-box (T-bet), expressed in T cells. However, Th2 cells express GATA binding protein 3 (GATA- 3), and are able to secrete IL-4, IL-5 and IL-13, and collaborate to the host defence against helminths. A mutual regulation of Th1 and Th2 polarization is known to be achieved by the production of their main corresponding cytokines, IFN-γ and IL-4, which enforce their own expression while inhibiting alternative commitment, as well as by the Th1- and Th2-specific transcription factors; for instance, T-bet activation can suppress GATA-3 expression [190]. Nevertheless, in addition to their protective functions against invading pathogens, each subtype of these Th cells also participates in the development of different immune-related human conditions: Th1 cells have been reported to play a pathogenic role in organ-specific autoimmune diseases and chronic inflammatory disorders, such as CD, sarcoidosis and atherosclerosis, whereas Th2 cells are clearly involved in allergic disorders [191]. With regard to CD patients, Th1 response is triggered by an increase of mucosal levels of IL-18 and IL-12 [192-194]. Moreover, it has been observed that mucosal T cells derived from CD patients produce higher amounts of IFN-γ and IL-2 than UC patients [195], whereas UC patients produce more IL-5 than CD patients [196]. The hypothesis of a Th1/Th2 cytokine balance has been used to explain the pathogenesis of chronic inflammatory disorders such as IBD. However, the Th1/Th2 paradigm should be reconsidered; it has been shown that UC and CD biopsies cultured ex vivo release high

and comparable amounts of IFN-γ [197]. Moreover, IL-13 has been proposed to act as an anti-inflammatory cytokine, and its levels are reduced in both diseases [198-200].

### Th17 cells

Similarly to Th1 and Th2 cells, Th17 cells have also been reported to display a pathogenic role in IBD [201]. Langrish *et al.* (2005). [202] was the first one who described "Th17 cells" as a new subtype of effector Th cells. Th17 cells expansion was IL-23-dependent, but not driven by IL-12, and they were found to be highly pathogenic and essential for the establishment of organ-specific inflammation. They were further characterized by the expression of the transcription factor retinoic acid orphan receptor (ROR)γt, but not T-bet or GATA-3 [203], and by selectively producing high levels of IL-17A and IL-17F, being crucial for host defence against extracellular pathogens. Monocytes and circulating conventional DCs (cDCs) activated by LPS and peptidoglycan, which produce large amounts of IL-1β and IL-6, but little IL-12, are known to be the most efficient APCs for Th17 differentiation [204]. These cytokines released from APCs transmit their signals by binding to the JAK–STAT pathway [205]. The transcription factor STAT3 plays an important role in Th17 differentiation when activated by both IL-6 and IL-23, and in the expression of other transcription factors for Th17 differentiation and development as well, i.e. RORγt, IRF4 and Batf. For instance, in the absence of STAT3, an impaired RORγt expression may occur, which might lead to increased expression of other transcription factors such as T-bet and Forkhead box P3 (Foxp3) [206]. IL-6 has been found to be essential cytokine for Th17 differentiation of naïve T cells [207], whereas the pro-inflammatory cytokines IL-1β and TNF-α help to increase the efficiency of this process [208]. IL-17 is necessary for IL-17-mediated effector function and the survival of Th17 cells, but not for their differentiation [209]. TGF-β has a role in Th17 differentiation that is dose-dependent: low doses of TGF-β facilitate Th17 differentiation while inhibitory effects are observed at high doses [210, 211] or even indirectly, mainly derived from a strong suppression of Th1 cells proliferation [212]. Th17 produce different cytokines and chemokines, including IL-17A

and IL-17F, IL-21, IL-22, IL-26, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as the chemokine IL-8 and CCL20 [213]. IL-17A and IL-17F, the defining cytokines of this T cell subtype [214, 215], share similar biological activities, by targeting both immune and non-immune cells. Moreover IL-17A induces IL-8 production from epithelial cells, endothelial cells, fibroblasts and macrophages, thus facilitating the recruitment of granulocytes. IL-21 potently induces Th17 differentiation from $CD4^+$ T cells in an autocrine way, amplifying Th17 responses and therefore inducing its own expression in an autocrine loop [216]. Both IL-22, essential for the maintenance of mucosal barrier function [217], and IL-26, produced in the altered innate immune response that occurs during intestinal inflammation [218], can also be produced by other $CD4^+$ T cells subsets besides Th17, Th22 and Th1, respectively, and they are both involved in the development of intestinal inflammation. Finally, the production of either TNF-α or GM-CSF by Th17 cells contributes to the activation, survival and recruitment of neutrophils in different inflammatory conditions [219]. All these findings suggest a functional plasticity of Th17 cells, which, under certain conditions, can shift to the production of Th1, Th2 or iTreg cytokines.

Th17 cells have a dynamic phenotype. It has been shown that in CD patients some Th17 cells can produce IL-17 and IFN-γ, acting like Th1 [220]. More recently, a fraction of Th17 cells that also produce IL-10 has been described [221]. In vitro studies supported this finding as the incubation of Th17 cells with IL-12 induced the production of IFN-γ in addition to IL-17 or instead of IL-17A. This shift of Th17 cells toward the Th1 phenotype has been demonstrated as well in murine experimental models [222, 223]. Thus, the stimulation of human Th17 clones in the presence of IL-12 down-regulates RORγt and up-regulates T-bet, enabling the production of IFN-γ in addition to IL-17A [220]. Furthermore, IL-12 stimulation can also lead to IL-17 repression, therefore indicating that IL-17-secreting T cells can be induced to differentiate into fully polarized Th1 cells [220].

Th17 cells reside mainly at barrier surfaces, particularly in the mucosa of the gut, where they can play their function to protect the host from microorganisms that invade

through the epithelium [207]. As commented above, TGF-β up-regulates RORγt expression [224], the master transcription factor of Th17 differentiation, thus being essential for the development of Th17 cells [209, 225, 226]. Interestingly, TGF-β is also known to induce in naïve T cells the key regulatory transcription factor for the development of Tregs, Foxp3 [227, 228]. However, Bettelli *et al.* (2006) found that the addition of IL-6 and TGF-β to naïve T cells inhibits the generation of Tregs while it induces the expression of IL-23R and RORγt [207, 229], thereby promoting a Th17 phenotype. In addition, it has been demonstrated that TGF-β-induced Foxp3 expression represses IL-23R and RORγt expression, suggesting that the Treg phenotype regulates Th17 cell differentiation [230]. Further support for the reciprocal development of Th17 and Treg cells was derived from studies of retinoic acid function, which induces Foxp3 and inhibits RORγt in Th17-inducing conditions, then promoting the development of Tregs [231, 232].

Finally, the intestinal microbiota plays a major role in inducing the differentiation of Th17 cells in the intestine [211, 212, 233]. Specific bacterial products such as flagellins can be recognized by TLR in APCs and drive Th17 differentiation through IL-23 secretion from DCs. Moreover, bacteria-derived products, like ATP, can also activate the induction of Th17 cells. This process is of great relevance, since commensal gut microbiota, through the expansion of Th17 cells, can regulate the susceptibility to different diseases, including intestinal inflammation.

**Treg cells**

Treg cells are a subset of CD4+ that play a key role in maintaining peripheral tolerance by suppressing abnormal immune response against commensal microbiota or food antigens. Their homeostatic function is to apply a strong negative regulation on other immune cells, like T effectors. Their suppression is mediated by molecules, like cytotoxic T-lymphocyte antigen, or by production of cytokines, such as IL-10 and TGF-β [234]. Treg cells produce and respond to TGF-β, indicating the importance of this anti-

inflammatory cytokine in the maintenance of immune homeostasis [235]. TGF-β signalling is impaired in the inflamed mucosa of human IBD; during inflammation there is an up-regulation of its inhibitory molecule Smad7 [236, 237]. It has been shown that IBD patients showed T effector cells that are unresponsive to Treg cell activation; this situation was reversed when an anti-Smad7 antisense oligonucleotide was used [235]. Treg cells can generate anti-inflammatory effect also by other mechanisms; both mouse and human Treg cells express surface bound enzymes, such as CD39 and CD73, which neutralize the pro-inflammatory effects of extracellular nucleotides such as ATP [238]. It is possible to identify two different populations of Treg: natural regulatory T cell (nTreg) and iTreg. nTreg are known to be generated in the thymus through the IL-2 signalling and phenotypically they are $CD4^{+}CD25^{+}$; the development of iTreg occurs in the peripheral organ/tissue from cells $CD4^{+}CD25^{-}$ in the presence of TGF-β and RA, and normally they acquire CD25 in the sites of inflammation [239, 240]. However, CD25 was not found the unique marker for Treg, because activated T cells also express this marker. Foxp3 is a specific transcription factor expressed by $CD4^{+}CD25^{+}$ Treg thus being the principal marker of these cells. Foxp3 is a regulatory gene for development and function of Treg [241, 242]. Recently, one other distinct subset of Treg, termed type 1 regulatory T cells, has been discovered to be involved in immune regulation in the gut. These cells do not express Foxp3 and display their suppressive function by producing IL-10 and TGF-β [243]. Generally, TGF-β seems essential for the activation of the suppressive capacity of Treg [244]. In the gut there are high levels of TGF-β and this cytokine facilitates the Treg differentiation; in fact, the induction of Foxp3 seems to depend on TGF-β signalling both *in vivo* and *in vitro* [245, 246]. Both nTreg and iTreg secrete the anti-inflammatory cytokine IL-10. Treg are the main population of $CD4^{+}$ lymphocytes that produce IL-10 in the colonic *lamina propria* [247]. Recent studies have highlighted the importance of the anti-inflammatory properties of IL-10 in the human intestine: it is able to inhibit the pro-inflammatory cytokine production, such as TNF-α, to supress indirectly the proliferative responses of antigen specific $CD4^{+}$ T effector cells, and to maintain the persistence of Fopx3 expression in Tregs [248]. However, alterations in frequency and function of Treg have been reported in the pathogenesis of IBD [249-251].

Also intestinal microbiota contributes to the development of Treg, and this is supported by different studies performed in germ-free mice in which there was a reduction in the Treg accumulation in the colon [94]. Multiple mechanisms have been found to be involved in microbiota regulation of Treg differentiation and function. In mice, a deficiency in TLR-9 results in increased frequency of $Foxp3^+$ Treg [252] and a deficiency in TLR-2 results in a reduction of $Foxp3^+$ Tregs [253]. Butyrate, a colon microbial fermentation product, can induce the Treg differentiation in the *lamina propria* and ameliorating Tcell-dependent experimental colitis [254]. Also macrophages and DCs contribute to the Treg development [135, 136]. As commented before, an antagonistic relationship between Treg and Th17 exists [209]. In IBD, the reduced frequency of Treg is associated with increased levels of Th17 [255]. Moreover, cytokines like IL-6, IL-21, IL-23 and IL-27 could inhibit Treg differentiation, but promote the induction of Th1/Th17 cells [256, 257].

### 2.2.2. B cells

B cells are lymphocytes able to produce antibodies. Although their main function is the antibody production, B cells can also act as antigen presenting cells and, moreover, they are able to produce cytokines. Together with other APCs, such as macrophages and DCs, B cells are involved in maintaining mucosal immune homeostasis [258]. They are specialized in immunoglobulin (Ig)A production [259] and the majority of intestinal plasma cells secrete IgA [260]. In the gut lumen, B cells interact with food antigens, commensal bacteria and self-antigens [260], thus operating like a protective barrier for the epithelium against possible pathogens [261]. IgA secretion is controlled by several compounds that are involved also in maintaining intestinal tolerance, including IL-10, TGF-β and RA [259]. Similarly to T cells, B cells have been divided into two subpopulations, a regulatory subset and a pathogenic one. Regulatory B cells, Breg, produce IL-10 that contributes to their suppressive function, and maintain the homeostasis that regulates the balance of the different subsets of T cells (Th1, Th17 and Treg), thus inhibiting the inflammation. Also, they are able to convert effector T cell in Tr1 cells, a

specific subpopulation of Treg cells [262, 263]. All these properties attributed to Breg cells can justify the reported ability of these cells to suppress experimental colitis [262, 263]. The beneficial effects of these cells are supported by the fact that in CD patients there is a reduction of Breg cell population [264]. Recently, it has been reported that IL-33 is an important cytokine for induction of Breg cells; in fact, in IL-10 KO mice the mucosal immune response is attenuated when IL-33-derived B cells are transferred [265]. Other evidences have shown that Breg cells can reduce intestinal inflammation via Treg since these cells can interact directly with T cells and polarize the type of response, although they are not able to induce primary response [266]. Also DCs can activate directly B cells [267, 268] by presenting them processed antigens [269, 270] and thus influencing their differentiation and survival [271]. The survival is promoted by BAFF and APRIL, molecules released by DCs, which activate the IgA production [272, 273]. Finally, macrophages also release BAFF, which can induce B cell differentiation [274].

## 3. Treatment of IBD

The main objectives of pharmacologic therapy in IBD are to promote remission of acute phase of the disease and reduce the incidence of recurrence. This is achieved by inhibiting the exaggerated immune response that occurs in both CD and UC or acting on the largest possible number of pro-inflammatory mediators involved in the pathogenesis of these diseases, or on those who have a key role. Nowadays, the treatments available are anti-inflammatory drugs, immunosuppressive, or biological therapies, which we will discuss below.

### 3.1. Anti-inflammatory drugs

#### Aminosalicylates

Aminosalicylates are drugs characterized by the presence of molecule 5-aminosalicylic acid (5-ASA) or mesalazine in their structure. One of the first drugs that started to be used

is sulfasalazine, which through an azo bond binds one molecule of 5-ASA to another sulfapyridine. The latter acts as carrier molecule to ensure the availability of 5-ASA in the colon [8]. Later on, new ways of transporting the 5-ASA have been developed to solve the problems of sulfapyridine toxicity or to allow its action on different parts of the intestine [8]. Currently, it is available olsalazine, a dimer composed of two molecules of 5-ASA linked by azo bond that requires bacterial reduction to release the active ingredient. Pharmaceutical forms of controlled release also exist, in which 5-ASA is coated with an acrylic resin and dissolves only certain pH values, or sustained release forms, in which microgranules of 5-ASA are formed with a semipermeable membrane of ethylcellulose, which release the compound continuously. Moreover, it is possible to use intrarectal enemas, suppositories, foams or gels to administer 5-ASA topically.

The aminosalicylates are indicated in the outbreak of inflammation of slight intensity (both in CD and in UC) [275, 276] and in the prevention of the reactivation of the UC [277]. However, the chronic use of the therapy with aminosalicylates in the maintenance of the CD recess has not good results, thus it is difficult to support this therapy for long-term. The mechanism of action of the mesalazine is not completely clear. In vitro studies have shown inhibition of the inflammation interfering in the cascade of the arachidonic acid, reducing the liberation of leukotrienes (LT), as the $LTB_4$, PGE2 [278], and ROS [279]. Furthermore, mesalazine induces changes in the production of Igs, a reduced production of pro-inflammatory cytokines as TNF-$\alpha$, IL-1-$\beta$ and IL-2, and a partial inhibition of the expression of platelet activating factor (PAF), leading to a decrease of leukocyte infiltration [280], thus preserving the enterocyte functionality. Aminosalicylates seem to interfere with NF-κB pathway [281] and induce apoptosis in T lymphocytes [282].

**Corticosteroids**

Corticosteroids, such as prednisone, prednisolone or methilprednisolone, are the treatment of choice of moderate and severe outbreaks of the disease and in cases of lack

of response to the salicylates [9]. The main limitation of these drugs is the high risk of adverse reactions of systemic type when are used for long term. For these reasons, corticosteroids for oral and rectal administration have been developed, effective in IBD and with fewer systemic side effects. One of these is budesonide, indicated in ileal or ileocecal CD, of slight or slight - moderate intensity [283], and in active UC [284]. Nevertheless, it is necessary to note that in 20-30 % of the patients have no response, or when the glucocorticoids (corticosteroid) are suspended, they present a reactivation of the disease [285]. Corticosteroids have a broad spectrum of action on the immune system including inhibition of transcription of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-12, IL-8; inhibition of recruitment and proliferation of lymphocytes, monocytes and macrophages, decreased neutrophil migration into inflammatory sites, together with the suppression of arachidonic acid metabolism, stimulation of lymphocyte apoptosis of intestinal *lamina propria* [286]. All these properties make the corticoids the therapy of choice in the treatment of the IBD.

**Immunosuppressive drugs**

Among immunosuppressive drugs, include azathioprine, 6-mercaptopurine, methotrexate, and cyclosporine A (CsA).

Azathioprine, when administered orally, is metabolized into the active metabolite 6-mercaptopurine; thus, it inhibits the cellular synthesis of purine, nucleotide interconversions, the synthesis of RNA and DNA and chromosomal replication [287]. This set of processes inhibits proliferation of activated lymphocytes in IBD and induce apoptosis in these cells [288]. Both azathioprine and 6-mercaptopurine are effective in the induction and maintenance of remission in UC and CD [10]. One of the major side effects of these drugs that could prevent its use in some cases, is the pancreatitis, although it is reversible after discontinuation of treatment [289].

Methotrexate is used through intramuscular administration, in case of intolerance or lack of response to azathioprine or 6-mercaptopurine and is effective both in active

CD, as in the maintenance of remission [11]. In UC, further studies are needed to prove its beneficial effects. The mechanism of action of methotrexate is not fully understood, but it seems to inhibit the synthesis of proi-nflammatory cytokines and promotes lymphocyte apoptosis [283, 290].

CsA has been used mainly in the cortico-refractory outbreaks of CU [291] and in CD [292]. This drug causes a reduction in the activation and proliferation of lymphocytes by blocking the synthesis of IL-2 via calcineurin pathway [293]. The molecule forms a complex with cyclophilin, and then it bonds to calcineurin preventing activation of the transcription of nuclear factor of activated T cells (NFAT). Therefore, NFAT is unable to enter the nucleus and bind DNA blocking the transcription of cytokines such as IL-2 and IFN-γ [293], thus altering the function of T and B cells. Also, it exerts additional effects in neutrophils and mast cells, reducing the infiltration and vascular permeability [294]. CsA also induces apoptosis in T cells by reducing the number of Bcl-2 positive cells [295]; CsA it can be used alone or in combination with other agents, such as rapamycin [296]. The CsA treatment presents side effects in the kidney and neurological toxicity, together with opportunistic infections that may occur [297].

**Biological therapies**

Biological therapies include different strategies, like blocking the action of pro-inflammatory cytokines or interfering in the recruitment of inflammatory cells by antibodies against adhesion molecules.

TNF-α has an important role in the development of IBD, it is a key target in the treatment of these chronic conditions. Infliximab is a monoclonal anti-TNF-α chimeric (mouse-man), and CDP571 and adalimumab are monoclonal antibodies with more humanized proportion. Besides blocking TNF-α, these three drugs are able to induce apoptosis in monocytes and lymphocytes [298]. They are useful compounds for induction of remission in CD and active UC [12, 299, 300]. Infliximab is effective also in maintaining remission in short term [301]. However, the main problem of infliximab is

the development of anti-chimeric antibodies [302]. It would be more appropriate to reserve infliximab as a treatment for patients who cannot tolerate or have not responded to other conventional medical treatments [302].

Moreover, it has been developed antibodies against other pro-inflammatory cytokines, such as MRA, a humanized monoclonal antibody against the soluble IL-6 receptor (IL-6R). It has been reported high concentrations of IL-6R in patients with CD [303], and treatment with MRA has been tested with good results in CD [304]. Furthermore, both fontolizumab, a humanized monoclonal antibody against IFN-γ [305], and J695 (ABT-874) a human monoclonal antibody to IL-12 [306] have shown to exert beneficial effects in the CD. Moreover, anti-CD4 therapy, using monoclonal antibodies as cM -T412, BF-5 and MAX.16H5, acts on $CD4^+$ T cells and induce remission in patients with both CD and UC [307]. In order to inhibit T cell proliferation, it has been developed antibodies to IL-2 receptor, basiliximab or daclizumab, performing well in the UC [308, 309]. Finally, natalizumab is a humanized mouse monoclonal antibody against α4-integrin that inhibits transendothelial migration. It results effective in remission both in CD patients [310] and UC [311].

## 4. Complementary and/or alternative therapy

There are different drugs currently used in IBD, including aminosalicylates, corticosteroids, immunosuppressants or anti-TNF-α agents. Although all of them have shown efficacy in these intestinal conditions, the frequency and severity of adverse effects, inconvenient dosing regimen and partially prohibitive price limit their long term use [13]. For this reason, the development of new therapies that combine efficacy, convenient dosing and lower side effects is an important goal in human IBD therapy. Closely related with all the above, it is interesting to note that there are more and more IBD patients looking for alternative options in their treatment; in fact, a recent study has reported that up to half of these patients have tried or currently use complementary therapy [312]. This can be explained by the absence of complete response to conventional

therapy, the more favorable safety profile of many complementary treatments, and a sense of greater control over their disease [313]. Among the many forms of complementary and/or alternative therapy herbal preparations or their derived compounds display an outstanding position [314]. The rationale for their use is mainly related with safety, since they have been consumed from ancient times, as well as with the presence of different active components that can act simultaneously on different targets in the inflammatory response, including against oxidative stress. Among these active components, polyphenols have been extensively characterized because of their wide spectrum of bioactivity, particularly their potent antioxidant activity. In this context, the evaluation of plant extracts can be considered as an important approach for the development of future treatments in IBD.

### 4.1. Intestinal anti-inflammatory effects of botanical drugs: preclinical and clinical studies

The growing interest about the potential role that medicinal plant extracts may play in intestinal inflammatory conditions has promoted the development of different clinical studies, thus trying to evaluate their potential efficacy and safety. It is important to note that nowadays most botanical drugs go through a similar rigorous testing as pharmaceutical medicines, in an attempt to avoid inconsistent conclusions. Unfortunately, different factors associated with the design, execution and interpretation of these clinical trials make still difficult to easily get clear conclusions with the different strategies to be evaluated against these pathologies [315]. Among these factors, it can be highlighted the clinical heterogeneity of both intestinal conditions, UC and particularly CD, as well as the selection of appropriate therapeutic end points to evaluate the efficacy. In spite of all these concerns, there are positive examples of successful human-controlled trials within the literature of botanical drugs. Although the preclinical studies reporting the beneficial effects of plant extracts on experimental models of colitis are numerous, only a few plant extracts have been used in different clinical assays, which will be described in Table 1.

The main botanical drugs include were *Aloe vera*, *Andrographis paniculata*, *Artemisia absinthium*, *Boswellia serrata*, *Cannabis sativa* and *Curcuma longa*.

***Aloe vera***

*Aloe vera* (Xanthorrhoeaceae) is a tropical plant used in traditional medicine all over the world, mainly for its gel, which is consist on the leaf pulp mucilaginous aqueous extract. The *Aloe vera* juice has been reported to exert anti-inflammatory activity, therefore it has been empirically used for the treatment of UC patients [316] (Table 1), being considered as the single most widely used herbal therapy [317]. These beneficial effects have been related to the immunomodulatory properties ascribed to this gel. This was confirmed when the gel was tested in the DSS model of experimental colitis in rats, since it produced an amelioration of the colonic tissue injury induced by DSS, being this associated with a downregulation of the inflammatory mediators, including cytokines, and an attenuation of the immune cell recruitment [318]. Among the different components of the gel, which comprise acetylated mannans, polymannans, anthraquinone C-glycosides, anthrones, anthraquinones (emodin), and lectins, with reputed biological activities, in the same study it was proposed that the chromone aloesin seemed to play an important role in controlling the intestinal inflammatory process [318].

It was reported that aloesin, mainly, but also aloin and aloe-emodin (Figure 4) were able to reduce MPO activity, an enzyme involved in neutrophil activity, an effect that can account in inhibiting the progression of IBD. Moreover, aloesin is a strong inhibitor of $LTB_4$ that can activate and recruite the inflammatory cells in the tissue injured [319]. Also, these compounds significantly decreased the expression of pro-inflammatory cytokines, like TNF-α and IL-1β, in the colonic segment in a dose-dependent manner, being aloesin again the most effective. However, the mechanism through which it exerts this capacity remains unidentified. It is known that this chromone derivative blocks the activation of the NF-κB pathway and thus inhibiting the expression of related pro-inflammatory genes, including TNF-α [320]. Based on the effect observed in retina gaglion cells [321], the anti-inflammatory effect of Aloe-emodin and aloin metabolite, in

DSS rat colitis, maybe is due to mitogen-activated protein kinases (MAPK) pathway phosphorilation inhibition.

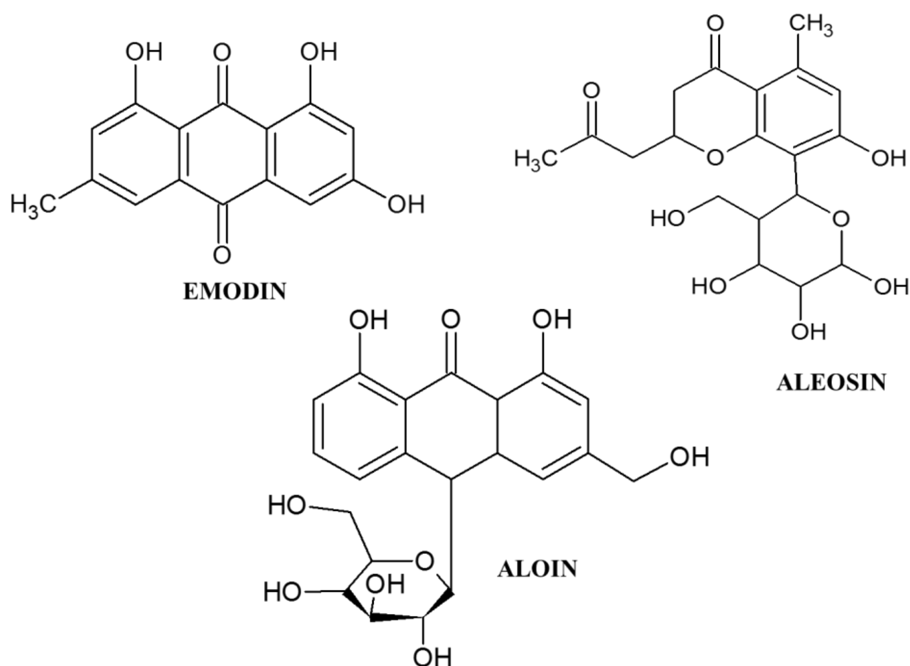


**Figure 4.** Chemical structures of *Aloe vera* compound.

### *Andrographis paniculata*

*Andrographis paniculata* (Acanthaceae) can be mainly found in India and Sri Lanka, as well as in South and South-Eastern Asia, where its extracts are used as anti-inflammatory remedies [322]. HMPL-004 is a proprietary extract from this plant that has been evaluated for its intestinal antiinflammatory effects in human trials [323, 324] (Table 1). The phytochemical analyses of the extracts from *Andrographis paniculata* reveal that the main known components are diterpene lactones, principally andrographolide (Figure 5) and its derivatives, which have been reported to exert antiinflammatory properties through inhibition of the transcription factor NF-κB [325]. Particularly, andrographolide reacts with reduced cysteine 62 of p50 subunit forming a covalent adduct, blocking the

bond of NF-κB oligonucleotide to nuclear proteins. NF-κB activation promoted the increased expression and synthesis of different pro-inflammatory mediators involved in the inflammatory response associated to IBD, including chemokines, cytokines, adhesion molecules… in the different cell types that participate in the altered immune response in these intestinal conditions [326]. For instance, when stimulated-endothelial cells were treated with andrographolide, the reduction of adhesion molecule E-selectin was observed preventing the E-selectin-mediated leukocyte migration [325]. Andrographolide is also able to suppress inducible nitric oxide synthase (iNOS) as observed in RAW 264.7 cells and, as a consequence, there is a reduction of NO production. This inhibition of NO is due to the blockage of the synthesis and also to the reduction of the stability via a post-transcriptional mechanism [327]. Andrographolide prevents the ROS production by neutrophils through the modulation of a protein kinase C (PKC)-pathway. This confers to andrographolide the capacity to downregulate leukocyte integrin Mac-1 ($\alpha_M\beta_2$ CD11bCD18), that it has been reported to be up-regulated by ROS. This reduction leads to reducing neutrophil infiltration and transmigration [328]. Besides, andrographolide exerts immunomodulatory properties, most likely affecting the innate immune cells, including macrophages and dendritic cells, but also T cells, by downregulating the production of pro-inflammatory cytokines [329-331]. It has been observed that andrographolide reduced significantly, in a dose-dependend manner, the IFN-γ production in concanavaline A-stimulated murine T cell in vitro, whereas its effects on IL-2 inhibition were partial. Moreover, it can reduce the ERK1/2 phosphorilation that is associated with a reduction of IFN-γ production [329].

In another study, it has been showed the ability of andrographolide to interfiere with the DCs maturation and with their capacity to present antigen to T cells. When the DCs were treated with the compound and then were mixed with lymphocites for allogenic stimulation, IL-2 release and proliferation were reduced [330]. The extract of *A. paniculada* also contains andrograpanin (Figure 5), a hydrolysate of neoandrographolide (another bicyclic diterpenoid lacton), which also showed anti-inflammatory activity. It was able to reduce the messenger (mRNA) expression of several genes, including TNF-α, IL-6, IL-12p35 and IL-12p40, in a dose-dependent manner. In particular, the reduction

of IL-12p35 and IL-12p40 proteins were lower than their mRNA levels, suggesting that andrograpanin applies the major changes at a post-transcriptional level for these two genes [331].

All these studies could justify the inhibitory effects that the extracts of *Andrographis paniculata* may exert on multiple immune cells (DC, macrophages, T cells) that are implicated in disease development and progression in UC and CD. Supporting this, Michelsen *et al.* (2013). reported the intestinal antiinflammatory effects of the extract HMPL-004 in a T cell driven experimental model of chronic colitis, by inhibiting the proliferation and/or differentiation of naïve T cells, as well as the Th1/Th17 responses that are activated in intestinal inflammation, being these effects associated with reduced expression of the different pro-inflammatory cytokines, including TNF-α, IL-1β, IFN-γ, and IL-22 [332].

### *Artemisia absinthium*

*Artemisia absinthium* (Compositae), commonly known as wormwood, is widely distributed all over the world and it is described in different pharmacopoeias, being leaves and stems used for medicinal purposes.

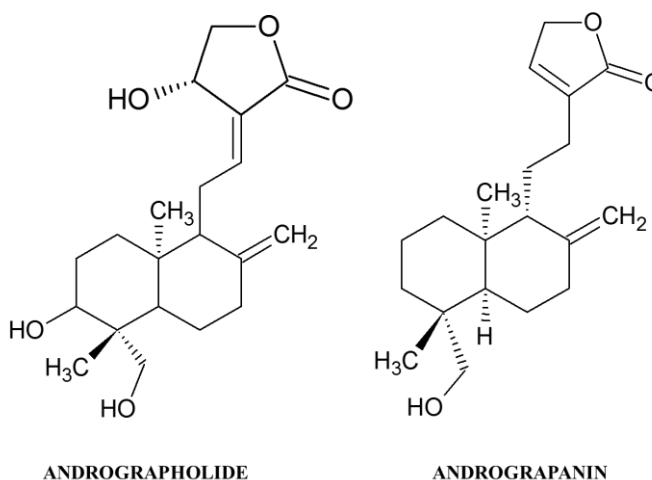


**Figure 5.** Chemical structures of *Andrographis paniculada* compounds.

This botanical drug is usually standardised based on its content in the dimeric guaianolides absinthins [333] being considered as a high-quality wormwood when it contains at least 0.2 % of absinthin. Two different clinical trials have reported the beneficial effect of this botanical drug in CD [334, 335] (Table 1). TNF-α is considered to play a key role in the pathogenesis of CD, which supports the high efficacy obtained with the biologicals acting as TNF-α inhibitors, like infliximab and adalimumab, for severe cases of CD [336]. The results obtained in these clinical trials showed that wormwood administration promoted the clinical improvement of the symptoms in all the patients, whereas no amelioration in the disease was observed in the placebo group. The beneficial effect induced by wormwood was associated with a significant decrease in TNF-α serum levels in comparison with those obtained in the placebo group. The suppression of TNF-α, as well as of others pro-inflammatory cytokines like IL-1β or IL-6, by wormwood extracts has been reported in vitro as well [337]. Among the components of *Artemisia absinthium*, it has been isolated the flavonoid 5, 6, 3',5'-tetramethoxy 7,4'-hydroxyflavone (p7F) (Figure 6) that has been shown to exert anti-inflammatory effects. In fact, p7F was able to inhibit the expression of cyclooxygenase-2 (COX-2) and iNOS in LPS-stimulated RAW 264.7 cells. As a consequence of this inhibition, it has been observed a reduced production of $PGE_2$ and NO in the same cells.

Moreover, p7F decreased the activation of NF-κB, induced by LPS, probably through its anti-oxidant properties [337]. p7F also suppressed the serum levels of TNF-α and inhibited NF-κB activation in vivo [337].

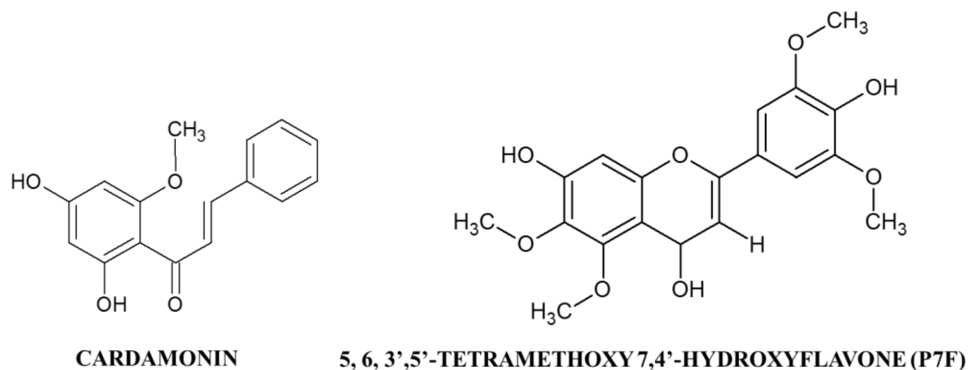


**Figure 6.** Chemical structures of *Artemisia absinthium* compounds.

Anoher compound also isolated from this plant, 20,40-dihydroxy-60-methoxychalcone, known as cardamonin (Figure 6), has shown to dose-dependtly inhibit NO relase and iNOS expression in LPS-stimulated RAW 264.7, as well as NF-κB activation [338].

All these results would support the use of *Artemisia absinthium*, at least as complementary therapy, in IBD.

***Boswellia serrata***

The oleo-gum resin from *Boswellia serrata* (Burseraceae), or Indian frankincense, is a traditional Ayurvedic remedy used to treat inflammatory diseases, including UC. As claimed by a survey performed in Germany, approximately 36% of IBD patients have been administered with *Boswellia serrata* extracts to treat their intestinal condition, reporting positive therapeutic effects [339], and they have been assayed in different clinical trials [340-342] (Table 1).

Among the different chemical compounds of this resin, triterpens are the most abundant (30-60 %, depending on its origin), being boswellic acids the major constituents (Figure 7), which are thought to largely contribute to the pharmacological activities such anti-inflammatory and antiarthritic effects ascribed to this crude drug [343]. In vivo experiments performed in the acetic acid model of rat colitis have proposed that the antioxidant properties of the extracts from *Boswellia serrata* may also account for their intestinal anti-inflammatory effect [344, 345]. It has been shown that *Boswellia serrata* extract reduced the lipid peroxidation, while increased the levels of SOD, thus ameliorating the oxidative stress associated to intestinal inflammation. Additional mechanisms can also account in its beneficial effects. Thus, in vitro experiments have shown that these compounds decreased the leukotriene formation by blocking the 5-lipoxygenase pathway which can account in the beneficial effect showed by this botanical drug since leukotriens have been clearly involved in the pathogenesis of IBD [346].

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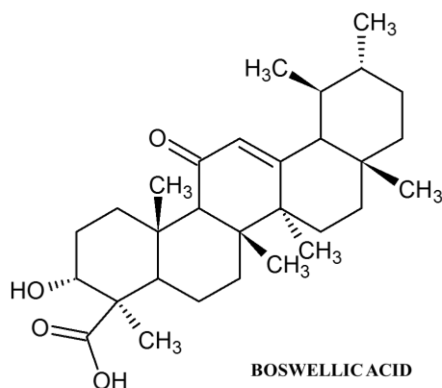


**Figure 7.** Chemical structure of Boswellic acid.

Similarly, the anti-inflammatory effects of boswellic acids seem to involve the inhibition of different cellular pathways including those related to the transcription factor NF-κB activation, which has been described to induce the expression and/or to activate the function of many pro-inflammatory cytokines, like TNF-α, IL-1β, IL-6, that are crucial for the development and the maintenance of intestinal inflammation [347, 348]. Surprisingly, contradictory results have been reported when the effectiveness of boswellia extracts were assayed in DSS- or trinitrobenzene sulfonic acid (TNBS)-induced experimental models, since no efficacy was demonstrated in ameliorating colitis in these models [349]. Furthermore, in the same study, it was demonstrated the ability of different boswellic acids to enhance the basal and the IL-1β -stimulated NF-κB activity in intestinal epithelial cells, as well as the potential hepatotoxic effect of boswellia, claiming for an special attention with the use of this botanical drug in IBD and other inflammatory disorders [349]. On the contrary, a semi-synthetic form of acetyl-11-keto-β-boswellic acid lessened the disease activity index in DSS colitis, and it managed to diminish the recruitment of leucocytes and platelets, maybe due to its ability to prevent P-selectin up-regulation, thus protecting colonic mucosa from DSS insult. The beneficial effects showed by this derivative were similar to those obtained with the standard corticosteroid dexamethasone [350].

### *Cannabis sativa*

*Cannabis sativa* (Cannabaceae) has been long employed for the treatment of different diseases, especially for chronic pain and different neurological conditions [351, 352]. Moreover, this botanical drug has treated different gastrointestinal conditions including anorexia, emesis, abdominal pain, diarrhea, and diabetic gastroparesis [353]. It has been reported to contain over 60 different cannabinoid compounds, which are responsible for the biological activities reported for *Cannabis sativa* [354]. In addition, experimental evidence suggests that the endogenous cannabinoid system is involved in most of the major immune events, including those located in the gastrointestinal tract [355, 356]. For this reason, it was proposed that the activation of this system by cannabinoids might have a therapeutic role in human IBD [357]. However, and although its use is common in IBD patients, there are few controlled studies that evaluate the exact role of cannabis in IBD [358-360] (Table 1).

The mechanisms involved in the intestinal anti-inflammatory effects of cannabis can be related to the capacity of cannabinoids to down-regulate the production and release of different pro-inflammatory mediators including TNF-α, IL-1β and nitric oxide, thus restoring the altered immune response that occurs in IBD [361]. Most probably, these effects would be related to cannabinoid receptors type 1 (CB1) activation that mediates essential protective signals and counteracts pro-inflammatory pathways, since it has been reported that the severity of two different experimental models of colitis, induced by the intrarectal infusion of 2,4-dinitrobenzene sulfonic acid (DNBS) or by oral administration of DSS, are higher in CB1-deficient mice ($CB1^{-/-}$) than in wild-type [362]. Lack of CB1 receptors rendered mice more sensitive to inflammatory insults, indicating a protective role of the CB1 receptors during inflammation induction. Consistently, the administration of a specific CB1 antagonist to these wild-type mice before colitis induction resulted in a similar degree of intestinal damage to that seen in $CB1^{-/-}$ mice; whereas the administration of a cannabinoid receptor agonist protected from DNBS-induced colitis in mice [362]. Supporting these observations, both Δ-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Figure 8), two of the main components of cannabis, were able to exert beneficial

effects in the TNBS model of acute colitis in rats, with a similar efficacy to that shown by sulphasalazine, which was used as a positive control [363]. Particularly, treatment with THC and combined treatment with CBD was able to reduce macroscopic damage score. Both alone and in combination, THC and CBD reduced the MPO activity similarly to treatment with sulphasalazine [363].

Unfortunately, the main concern with the use of cannabis in IBD can be derived from the indiscriminate binding of the cannabinoids to the receptors in the central nervous system, which may result in serious side effects including dizziness, dry mouth, nausea, fatigue, somnolence, euphoria, vomiting, disorientation, drowsiness, confusion, loss of balance, and even hallucination [364].

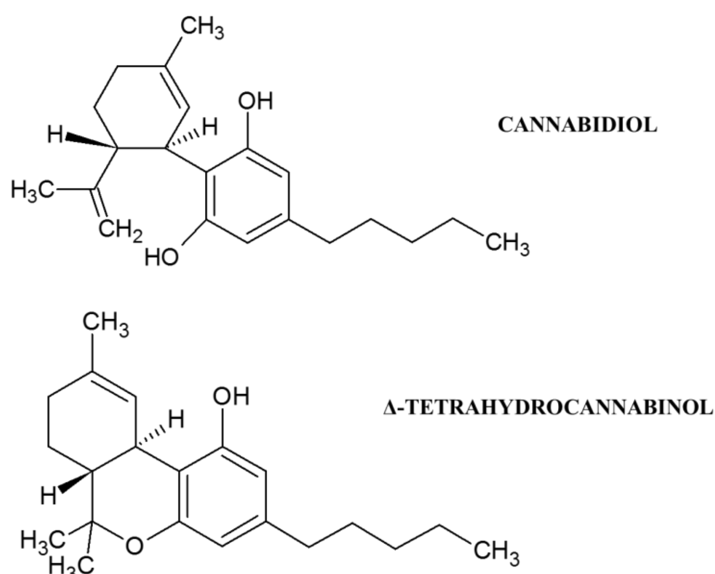


**Figure 8.** Chemical structures of *Cannabis sativa* compounds.

It is evident that the manipulation of the endocannabinoid system can be helpful for the management of human IBD; however, additional research needs to be carried out to consider cannabis (and cannabinoids) as a suitable medicine in these gastrointestinal conditions. Maybe, the use of specific cannabinoids can constitute an attractive alternative. This may be the case of cannabidiol, which possesses anti-inflammatory and

immunomodulatory properties, as demonstrated in experimental models of rodent colitis, but it lacks of any cognitive and psychoactive actions [365].

### *Curcuma longa*

*Curcuma longa* (Zingiberaceae) is commonly known as Turmeric. It is an Indian spice obtained from the rhizomes of the plant, which has been long used in Ayurvedic medicine for the handling of different inflammatory diseases [366]. Curcumin (Figure 9) is the main active component of turmeric that also provides its vibrant yellow colour [367]. Numerous pharmacological activities have been reported for curcumin, including antioxidant, antimicrobial, anticancer and anti-inflammatory properties. Regarding the latter, curcumin has been suggested to exert positive effects in IBD, although just a few clinical studies have shown the power of curcumin to prevent and/or ameliorate this condition.

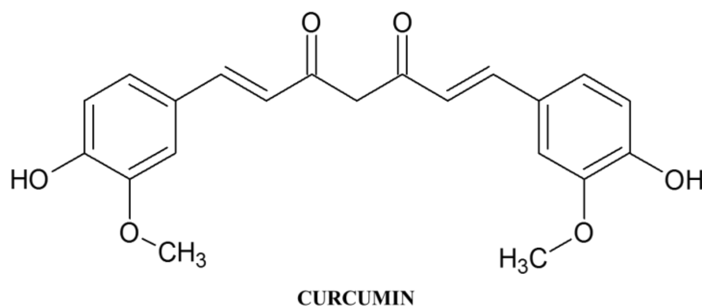


**Figure 9.** Chemical structure of curcumin.

Clinical studies showed that the concurrent administration of curcumin and standard drugs improved their efficacy [368, 369] (Table 1) and seemed to be well-tolerated after a prolonged administration, which can be considered as a secure medication for maintaining remission and preventing relapse [370].

The preclinical studies performed with curcumin have shown its efficacy in different experimental models of colitis, either chemically-induced or in knock out mice, and following different protocols of administration, that is, preventative or curative [371-376]. Besides, all these studies have rendered important information about the possible mechanisms implicated in these beneficial effects. Among these, the antioxidant properties of curcumin, derived from its ability to scavenge free radicals, may play a prominent role, given the oxidative stress that characterizes these intestinal conditions [377]. In addition, curcumin can also modify multiple signaling pathways, especially the kinases MAPK and ERK, thus affecting the expression of different proteins implicated in the intestinal inflammatory cascade, like MPO, COX-2, iNOS or lipoxygenase (LOX) [378]. Additionally, a modulation of the altered immune response has been proposed to occur after curcumin administration in experimental colitis, by attenuating the production of pro-inflammatory cytokines, like TNF-α, IL-1β, IL-12 or IFN-γ, but increasing of the expression of anti-inflammatory cytokines [372, 375]. Other mechanism involved in the beneficial effects of curcumin can be related to an inhibitory effect of NF-κB activity [379]. In fact, it has been reported that elevated levels of NF-κB in IBD proportionately amplified the production of inflammatory cytokines and resulted in mucosal damage, which in turn can up-regulate the production of this transcription factor, promoting a recurring feedback loop of inflammation [380]. In consequence, the ability of curcumin to modulate NF-κB activation may prevent the inflammatory response, thus preventing colonic mucosa damage.

In conclusion, the results reviewed show that botanical drugs might prompt clinical remission and a clinical response in IBD patients. In particular, botanical drugs significantly induce clinical remission in CD patients and clinical response in UC patients; however, there was not a significantly induction of clinical remission in UC patients and an obvious clinical response in CD patients. The results of sub-analyses taking into account the plant type demonstrate that only *Artemisia absinthium* and *Boswellia serrata* were able to induce clinical remission, while *Aloe vera* induced a clinical response. However, none of the plants confirmed endoscopic or histological efficacy. On the other hand, none of the plants produced any adverse effects in

comparison with placebo. So, although some of the botanical drugs may have clinical efficacy in patients with IBD, there is too limited evidence to make any strong conclusions. However, botanical drugs could still be safer than synthetic drugs, despite the fact that they are not completely devoid of risk.

### 4.2. Perspectives

The potential additional benefits of botanical drugs could be that patients accept them, besides their efficacy, acceptable safety, and comparatively low cost. Patients all over the world seem to be prone to use botanical drugs, and their efficacy has been now evaluated in multitude of clinical trials in the management of UC. However, the evidences are still partial, intricate, and puzzling, and unquestionably related to both benefits and side effects.

First of all, there should be a deeper knowledge of their composition, the active compounds that are responsible of their properties, and their side effects or toxicity. It is also important to control the harvest of the right plants, their quality, and the later processing to ensure the stability of the active components. Therefore, there is a need for a regulation that establishes the quality standards of the botanical drugs that are sold.

Secondly, further controlled clinical trials, with larger number of patients, are required to evaluate the potential efficacy and toxicity of botanical drugs in the treatment of UC. In this regard, it is also important to let the doctors know any evidence so they can prescribe these botanical drugs with the maximal guarantee.

**Table 1.** Clinical trials of botanical drugs in patients with inflammatory bowel disease

| Herbal preparation | Study design | Nr. Patients | IBD type | Dose | Comparator | Frequency | Endpoint | Ref. |
|---|---|---|---|---|---|---|---|---|
| ***Aloe vera*** | Randomized, Double-blind controlled study | 44 | UC | 100 ml twice/day | Placebo | 4 weeks | *Aloe vera* produced a significantly better clinical response than in those receiving placebo. The Simple Clinical Colitis Activity Index and histological scores decreased significantly during treatment with *aloe vera* but not with placebo. | [316] |
| ***Andrographis paniculada*** (HMPL-004) | Randomized Double-blind multicentre | 120 | UC | 1.2 g/day | Mesalazine (4.5 mg/day) | 8 weeks | There were no significant difference between the two treated groups when considering the clinical efficacy rates or the safety profile. | [323] |
| | Randomized Double-blind placebo-controlled | 224 | UC | 1.2 g/day and 1.8 g/day | Placebo | 8 weeks | Patients treated with the extract, mainly at the highest doses, were more likely to achieve clinical response than those receiving placebo, whereas the incidence of adverse events was similar among groups, although the occurrence of rash was higher in the HMPL-004 extract groups. | [324] |
| ***Artemisia absinthium*** | Randomized Double-blind multicentre | 40 | CD | 3 x 500 mg/day | Placebo | 10 weeks | After 8 weeks of treatment with wormwood, there was almost complete remission of symptoms in 65% of the patients, whereas no beneficial effect was observed in those receiving the placebo. | [334] |
| | Randomized Double-blind multicentre | 20 | CD | 3 x 750 mg/day (in addition to standard therapy) | Stardard therapy + Placebo | 6 weeks | Wormwood administration promoted the clinical improvement of the symptoms in all the patients. The beneficial effect was associated with a significant decrease in TNFα serum levels in comparison with those obtained in the placebo group, where no amelioration in the disease was observed. | [335] |

| Herbal preparation | Study design | Nr. Patients | IBD type | Dose | Comparator | Frequency | Endpoint | Ref. |
|---|---|---|---|---|---|---|---|---|
| ***Broswellia serrata*** (Gum resin) | - | - | UC | 750 mg (3 x 250 mg) | Sulfasalazine 3g (3 x 1g) | 6 weeks | All parameters tested improved after treatment with *Boswellia serrata* gum resin, the results being similar compared to controls: 82% out of treated patients went into remission; in case of sulfasalazine remission rate was 75%. | [340] |
| (Gum resin) | - | 30 | UC | 900 mg (3 x 300 mg) | Sulfasalazine 3g (3 x 1g) | 6 weeks | Patients showed an improvement in several parameters: stool properties, histopathology as well as scanning electron microscopy, besides haemoglobin, serum iron, calcium, phosphorus, proteins, total leukocytes and eosinophils. The remission was higher in patients treated with *Boswellia serrata* | [341] |
| (Boswelan) | Randomized Double-blind multicentre placebo-controlled | 82 | CD | 2.4 g/day | Placebo | 12 months (52 weeks) | Boswelan showed a safety profile during the long-term therapy but the results obtained did not show a higher efficacy when compared with placebo. | [342] |
| ***Cannabis sativa*** | Retrospective Observation study | 30 | CD | - | - | - | Cannabis administration was associated with an improvement in disease activity and a reduction in the need of other medications, as well as a reduced risk of surgery. | [359] |
| | Prospective Placebo-controled | 21 | CD | 2 cigarettes containing 115mg of THC /day | Placebo | 8 weeks | It has been reported a significant amelioration of the CD activity index in the majority of the subjects after cannabis treatment in comparison with placebo administration; in fact, complete remission was achieved in half of the subjects in the cannabis group, whereas it only occurred in 10% of the placebo group patients | [360] |

| Herbal preparation | Study design | Nr. Patients | IBD type | Dose | Comparator | Frequency | Endpoint | Ref. |
|---|---|---|---|---|---|---|---|---|
| ***Curcuma longa*** | Open-label pilot study | 5 | UC | 1.100g/day (550mg x2) for 1 month, then 1.650 g/day (550 mg x3) for 1 month | - | 2 months | The results from this study revealed that the treatment of these patients with curcumin for two months resulted in an overall improvement in all the patients, as evidenced by amelioration of the serological parameters evaluated (erythrocyte sedimentation rate and C-reactive protein) as well as the disease activity index followed, together with a reduction in the dose of medication, or even suppression. In the CD group, all patients also referred fewer bowel movements, less diarrhoea, as well as less abdominal pain and cramping | [368] |
| | Open-label pilot study | 5 | CD | 1.080 g/day (360mg x3) for 1 month, then 1.440 g/day for two months | - | 3 months | | |
| | Randomized Double-blind multicentre placebo-controlled | 89 | UC | 2 g/day plus sulfasalazine or mesalazine | Placebo plus sulfasalazine or mesalazine | 6 months | The relapse rate was significantly higher in the placebo group, receiving only the aminosalicylate (20.5%), than in the curcumin-treated cohorts (4.7%). During the period of the study it was reported a marked reduction of the disease-associated clinical activity index and the endoscopic index scores | [369] |

# AIMS

## AIMS

The term inflammatory bowel disease (IBD) mainly includes ulcerative colitis and Crohn's disease, and refers to a chronic disease of the digestive tract, typically characterized by chronic and spontaneously relapsing inflammation. At present, the etiology of IBD has not been fully elucidated; however, it most probably appears to be related to a combination of genetic and environmental factors in which an abnormal exacerbated immune response of the intestine to otherwise innocuous stimuli takes place [381]. As a result, there is an up-regulation of the synthesis and release of different proinflammatory mediators, including reactive oxygen and nitrogen metabolites, eicosanoids, chemokines and cytokines, which actively contribute to the pathogenic cascade that initiates and perpetuates the inflammatory response in the gut [382]. Since a specific causal treatment for human IBD is not available yet, IBD treatment pursues two clear goals: firstly, to promote the remission of symptoms during the acute flare [383]; and secondly, to maintain the remission and control the chronic inflammation to prevent or delay the reactivation of the intestinal inflammatory process. It is evident that down-regulation of the exacerbated immune response is essential for the treatment of IBD patients. Actually, this is the main aim of the pharmacological therapy, which includes aminosalicylates, immunosuppressants (glucocorticoids, azathioprine, metothrexate and cyclosporine A), and biologicals (infliximab or adalimumab) [384]. However, and although these drugs have shown efficacy, the rate of adverse effects is high, which may even limit their required long-term use [13]. As a consequence, there is an increasing interest for the development of new therapies that combine efficacy and safety in human IBD therapy.

In this context, the use of alternative therapies has emerged as a common approach in gastrointestinal diseases [385]; in fact, a study reported that almost half of IBD patients have tried or currently use complementary therapy [312]. Different factors can contribute to this situation, including the lack of a complete response to standard therapy, the general feeling about a more favourable safety profile of complementary treatments, in combination with the appreciation of a better control over their disease [313, 317, 383].

Among the different forms of complementary and/or alternative therapy, the botanical drugs are especially relevant for the treatment of these intestinal conditions [386]. This can be mainly related to their safety, since they have been consumed from ancient times, as well as with their reputed efficacy, most probably due to the presence of different active components that can act simultaneously on different targets in the inflammatory response. However, most of these uses have an empirical basis, and in consequence, it is necessary to proper evaluate these botanical drugs to consider them as an adequate strategy to the treat IBD.

The Mediterranean region in general, and Andalusia in particular, has been long considered as an important source of medicinal plants used in different inflammatory complaints, including those affecting the gastrointestinal tract, from ancient times. Among these, one of the most interesting families that provides a greater number of species to the catalogue of popular herbal medicine is Lamiaceae. In this context, based on ethnopharmacological studies previously reported by the research group from the department of Botany at the University of Granada, we have selected four plants for the preclinical evaluation of the intestinal anti-inflammatory activity. These plants were: *Phlomis lychnitis, Phlomis purpurea, Lavandula dentata* and *Lavandula stoechas.*

The general aim of this Thesis was to evaluate the efficacy of the corresponding extracts in experimental models of rodent colitis, and compared it with that obtained with a standardized extract form *Thymus serpyllum* (*Serpylli herba* extract, from PhytoLab GmbH & Co KG (Vestenbergsgreuth, Germany).

With this aim, the particular objectives proposed were:

1. To evaluate the intestinal anti-inflammatory effect of different doses of the extracts of *Serpylli herba, Phlomis lychnitis, Phlomis purpurea, Lavandula dentata* and *Lavandula stoechas*. For this purpose, orally administered extracts were tested as curative treatment in the trinitrobencenesulphonic acid (TNBS) acute colitis model in rats, in the dextrane sodium sulfate (DSS) model in mice.

These experimental models are used in the preclinical assays for the study of new therapeutic strategies potentially applicable to treatment of human IBD.

2. To establish the mechanisms involved in the beneficial effects through the in vitro studies performed in different cells actively involved in the intestinal immune response. It was evaluated the anti-oxidant capacity of the different extracts and their impact on immune response and the intestinal epithelial integrity.

The results of this Thesis would contribute to an understanding of the potential use of medicinal plants as intestinal anti-inflammatory agents in this pathology.

# MATERIALS & METHODS

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## 1. Reagents

All chemicals were purchased from Sigma Chemical (Madrid, Spain), unless otherwise stated.

## 2. Plant material and extract preparation

The preparation of the aqueous extract of *Serpylli herba* was performed by Finzelberg GmbH & Co KG (Andernach, Germany) and provided by PhytoLab GmbH & Co KG (Vestenbergsgreuth, Germany). Briefly, 10 kg of dried *S. herba* was extracted twice by percolation using 12 l and 10 l of water at 60°C. The extract was filtered through a 250 µm sieve and condensed to a dry matter content of about 50%. Nearly 1.3 kg of dry extract equivalent was then re-diluted to 10% of dry matter content and extracted three-times with 4 l of n-heptane for de-fatting (~ 1% oily substances). The water phase (roughly 1 kg dry extract equivalent) was condensed and Arabic gum and maltodextrin (15% each) were added as drying excipients, homogenized and the mixture dried in a vacuum drying chamber at 50°C. Finally the extract preparation was milled to a homogeneous powder. The final extract is characterized as follows: 70% native extract (DERnative 5:1), 15% Arabic gum and 15% maltodextrin, content of essential oil <0.1%, of this <0.01% thymol/carvacrol, and rosmarinic acid of 1.8%, being the presence of the latter confirmed by HPLC analysis.

The aerial parts of *Phlomis purpurea* and *Phlomis lychnitis* were collected in May 2011, the first one in the area of Velez de Benaudalla (Granada, Spain), and the second one in the area of Sierra del Jaral (Granada, Spain). The aerial parts of *Lavandula dentata* and *Lavandula stoechas* were collected, in the area of Calahonda (Granada, Spain) and in the area of Lújar (Granada, Spain) respectively, in June 2012. All plants were identified and authenticated by Department of Botany of University of Granada. Voucher specimens

corresponding to *Phlomis lychnitis L.* (GDA 59100), *Phlomis purpurea L.* (GDA 59099), *Lavandula dentata L.* (GDA 60249) and *Lavandula stoechas L.* (GDA 60250) were deposited in the herbarium of the University of Granada (Spain). The plant extracts were prepared as follows: 5 g of ground plant material was mixed with washed sea sand (Panreac Quimica S.A.U., Spain) and extracted with 30 ml of methanol 50% (v/v) at 1,500 pound per square inch (PSI) and 80ºC for 10 min in a accelerated solvent extraction (ASE) 200 extraction system (Dionex Corporation, USA). After two cycles of extraction, liquid extracts were pooled and the solvent evaporated under vacuum at 60ºC. Extraction efficiency (expressed as percentage w/w) was 21% for *Phlomis lychnitis*, 18% for *Phlomis purpurea*, 20% for *Lavandula dentata* and 14% for *Lavandula stoechas*. The polyphenolic content of the extracts was determined by the Folin–Ciocalteu method, as previously described [387] using gallic acid as standard, and the results were expressed as equivalent to grams of gallic acid per 100 g of extract (%); these values were 12.8% for *P. lychnitis*, 13.8% for *P. purpurea*, 15.6% for *L. dentata* and 18.8% for *L. stoechas*.

## 3. Analysis by reversed-phase (RP) high-performance liquid chromatography (HPLC) coupled to diode array detector (DAD) and high-resolution mass spectrometry (MS).

Methanol, ultrapure water, acetonitrile, and glacial acetic acid were purchased from Fisher Chemicals (ThermoFisher, Waltham, MA, USA). Solvents used for extraction and analysis were of analytical and HPLC-MS grades, respectively.

ASE plant extracts were dissolved at 5 mg/ml using an aqueous solution of methanol at 75% (v/v), sonicated and filtered (0.2 μm pore size, 13 mm, polytetrafluoroethylene). Analyses were made with an Agilent 1200 series rapid resolution (Palo Alto, CA, USA) equipped with a vacuum degasser, a binary pump, an autosampler, a column heater, and a DAD. The analytical method was based on method described by Lopez-Cobo *et al.* (2010). [388], with some modifications. For that, the separation was carried out with an Agilent Poroshell 120 EC-C18 column (4.6 × 100 mm,

2.7 μm) and at 25ºC. A gradient elution was programmed using acidified water (1% acetic acid) as mobile phase A, and acetonitrile as mobile phase B. The program was as follows: 10–15% B in 4 min, 15–16% B in 1 min, 16–18% B in 3 min, 18–20% B in 4 min, 20–22% B in 1 min, 22–25% B in 1 min, 25–28% B in 2 min, 28–30% in 1 min, 30–31% in 1 min, 31–32% B in 1 min, 32–50% B in 5 min, 50–75% B in 3 min, 75–100% B in 5 min, 2 min at 100% B, 100–10% B in 2 min and, finally 10% B was maintained during 3 min (initial conditions). The flow rate was set at 0.800 ml/min throughout the gradient. The UV spectra were recorded from 190 to 600 nm, and the chromatograms were registered at 240, 280, 330, 360 and 520 nm.

The system was coupled to a 6540 Agilent Ultra-High-Definition (UHD) Accurate-Mass quadrupole-time-of-flight (QTOF), which was equipped with an Agilent Dual Jet Stream electrospray ionization (AJS ESI) interface. The MS operating conditions were as follows: gas temperature, 360°C; drying gas, nitrogen at 12 l/min; nebulizer pressure, 50 psig; sheath gas temperature, 370°C; sheath gas flow, nitrogen at 12 l/min. The voltages were optimized and set with the appropriate polarity for working in the negative ionization mode. MS spectra were acquired over a mass range from *m/z* 70 to 1700. For further MS/MS experiments, auto-MS [389] mode was also employed, using nitrogen as collision gas and a collision energy ramp from 25-35 eV. Reference mass correction of each sample was performed with a continuous infusion of Agilent TOF mixture containing two mass references. The detection window was set to 100 ppm. Data acquisition (2.5 Hz) in the centroid mode was governed *via* the Agilent MassHunter Workstation.

Data analysis was performed on a Mass Hunter Qualitative Analysis B.06.00 (Agilent technologies, Palo Alto, CA, USA). For characterization, the isotope model selected was common organic molecules with a peak spacing tolerance of *m/z* 0.0025 and 7 ppm. Then, the compound characterization was done by taking into account the generation of molecular formula candidate with a mass error limit of 5 ppm. It was also performed considering the retention time (RT), experimental and theoretical masses, isotopic pattern and MS/MS spectra. The MS score related to the mass error, isotope

abundance and isotope spacing for the generated molecular formula, was set at ≥80. Studies on Lamiaceae family, as well as several chemical structure databases were consulted such as Reaxys and KNApSAcK Core System

## 4. Evaluation of the antiradical activity of the extracts by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The antiradical activity of the extracts was determined spectrophotometrically in a MRXetc (DYNEX Technologies GmbH, Denkendorf, Germany), by monitoring the disappearance of DPPH at 515 nm, according to a previously described procedure [14]. The antioxidant activity was expressed as IC50 values (in mg/ml), which were calculated by a non-linear regression with a one phase exponential association equation using GraphPad Prism version 5.0 (La Jolla, CA, USA).

## 5. Trinitrobenzene sulphonic acid (TNBS) model of rat colitis

This study was carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health.

Female Wistar rats (180–200 g) obtained from Janvier (St Berthevin Cedex, France) were housed in makrolon cages and maintained in an air-conditioned atmosphere with a 12-h light-dark cycle, and they were provided with free access to tap water and food. The rats were randomly assigned to different experimental groups (n= 10). An untreated TNBS control group and a non-colitic group were included for reference, which received the vehicle used, Carboxymethyl cellulose (CMC) or water), to administer the test compounds. A positive group was added, constituted by colitic rats treated with sulfasalazine (SAZ) (30mg/kg) or dexamethasone (DEX) (1.2mg/kg). Different experimental designs are shown in figure 10. Colonic inflammation was induced in the control and the treated groups. The rats were fasted overnight, anesthetized with halothane and given 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm through the anus. During and after TNBS

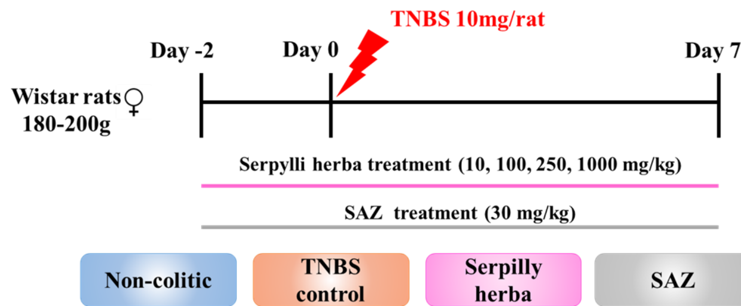
administration, the rats were kept in a head-down position until they recovered from anesthesia, and then they were returned to their cages. Rats from the non-colitic group were administered intracolonically 0.25 ml of phosphate buffered saline instead of TNBS [15]. Either the extracts or sulphasalazine or dexamethasone were administered from the day of the colitis induction, or two days before in case of *Serpylli herba*, until the day before of the sacrifice of the rats by oral gavage, which took place seven days after the induction of the colonic damage. All the rats were sacrificed with an overdose of halothane. Animal body weights, occurrence of diarrhea, and water and food intake were recorded daily throughout all the experiments.

Once the animals were sacrificed, the colon was removed aseptically and placed on an icecold plate, longitudinally opened and cleaned from their luminal contents with cold saline. Afterwards, the colonic segment was weighed and its length measured. Each colon was scored for macroscopically visible damage on a 0–10 scale by two observers unaware of the treatment, according to the criteria described by Bell *et al.* (1995) [16].

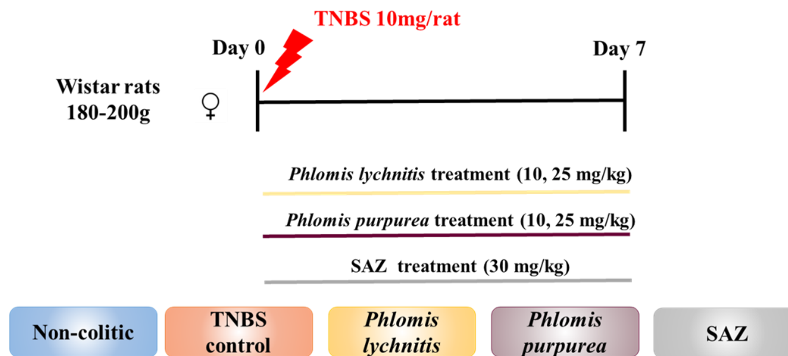
**Table 2**. Criteria for assessment of macroscopic colonic damage in rat TNBS induced colitis described by Bell *et al.* (1995) [16].

| Score | Criteria |
|---|---|
| **0** | No damage |
| **1** | Hyperaemia, no ulcers |
| **2** | Linear ulcer with no significant inflammation |
| **3** | Linear ulcer with inflammation at one site |
| **4** | Two or more sites of ulceration/inflammation |
| **5** | Two or more major sites of ulceration and inflammation or one site of ulceration/inflammation extending along the length of the colon |
| **6-10** | If damage covers along the length of the colon, the score is increased by 1 for each additional centimetre of involvement |

A)




B)




C)

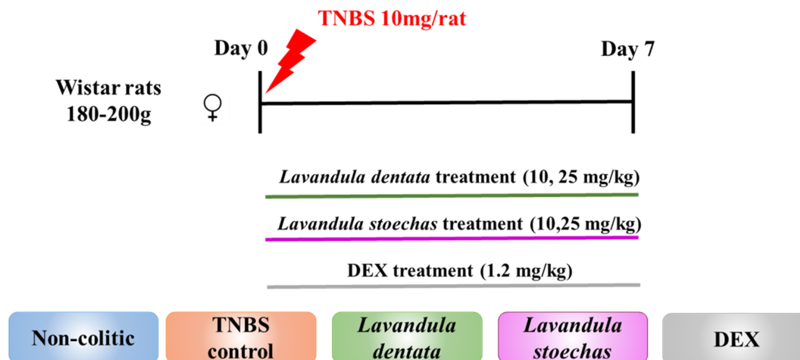



**Figure 10.** Experimental designs in TNBS model of rat colitis. A) Experimental design of *Serpylli herba* treatment. The treatment started three days before the induction of colitis through the administration of TNBS; four experimental groups received *Serpylli herba* at 10, 100, 250 and 100 mg/kg, and the last group received sulphasalazine (SAZ) at 30 mg/kg, which was used as the positive control. B) Experimental design of *Phlomis lychnitis* and *Phlomis purpurea* treatments. The treatment started the same day of induction of colitis and both extracts were administered at two doses: 10 and 25 mg/kg. Another group received sulphasalazine (SAZ) at 200 mg/kg, which was used as the positive control. C) Experimental design of *Lavandula dentata* and *Lavandula stoechas*. The treatment starts the same day of induction of colitis and both extracts were administered at two doses: 10 and 25 mg/kg. Another group received dexamethasone (DEX) at 1.2 mg/kg, which was used as the positive control.

Representative whole gut specimens were taken from a region of the inflamed colon corresponding to the adjacent segment to the gross macroscopic damage and were fixed in 4% buffered formaldehyde for the histological studies. Equivalent colonic segments were also obtained from the non-colitic group. The colon was subsequently minced, aliquoted and kept frozen at -80ºC until biochemical determinations and RNA extraction were performed.

## 6. Dextran sodium sulfate (DSS) model of mouse colitis

Female C57BL/6J mice (7–9 weeks old; approximately 20 g) obtained from Harlan (Barcelona, Spain) were housed in makrolon cages and maintained in an air-conditioned atmosphere with a 12-h light-dark cycle, and they were provided with free access to tap water and food. The mice were randomly assigned to two different groups: non-colitic (n = 10) and DSS colitic groups (n = 40). The colitis was induced by adding DSS (36–50 kDa, MP Biomedicals, Ontario, USA) in the drinking water at the concentration of 3% for a period of 5 days, after which DSS was removed. Colitic mice were divided in four groups of 10 animals each: two of them received the extract of *S. herba* for oral favage, at doses of 100 or 250 mg/kg, one group was treated with sulphasalazine at the dose of 50 mg/kg, and the remaining group (colitic control mice) was given the vehicle (200 µl of CMC). (Figue 11).

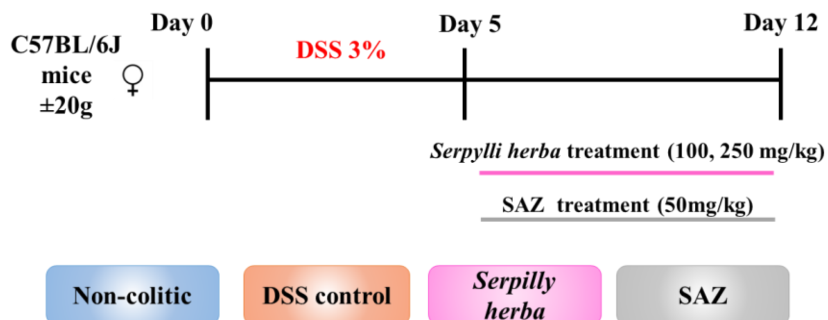



**Figure 11.** Experimental design of *Serpylli herba* in experimental model of colitis induced by DSS in mice.

Female CX3CR1$^{GFP}$ mice (8-10 weeks old; approximately 21 g) [390] were assigned to two different groups: non-colitic (n=8) and DSS colitic-group (n=24). The colitis was induced by adding DSS (36–50 kDa, MP Biomedicals, Ontario, USA) in the drinking water at a concentration of 3% for a period of 5 days after which DSS was removed. Colitic mice were divided in three groups of 8 animals each: two of them received the extract of *L. dentata* and *L. stoechas* respectively, both at doses of 10mg/kg, and the remaining group (colitic control mice) was given the vehicle (100 µl of $H_2O$) (Figure 12).

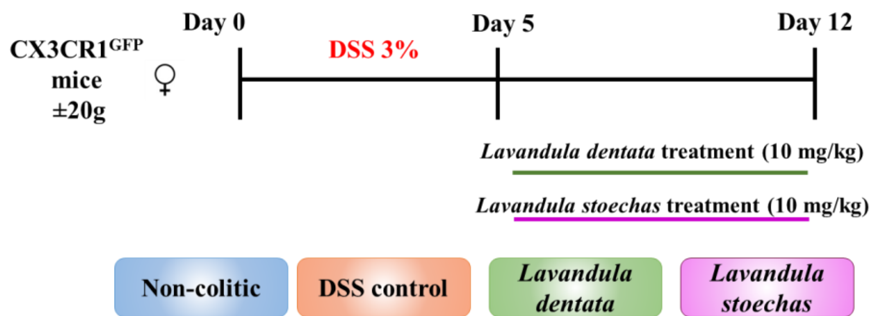



**Figure 12.** Experimental design of *Lavandula dentata* and *Lavandula stoechas* in experimental model of colitis induced by DSS in mice.

All mice were sacrificed 12 days after the beginning of the experiment. Animal body weight, the presence of gross blood in the feces and stool consistency were evaluated daily for each mouse by an observer unaware of the treatment. These parameters were assigned a score according to the criteria proposed previously by Cooper *et al.* (1993) [20] (Table 3) and used to calculate an average daily disease activity index (DAI).

Once the animals were sacrificed, the colon was removed aseptically, and representative whole gut specimens (0.5 cm length) were taken from the distal inflamed region and were fixed in 4% buffered formaldehyde for the histological studies; equivalent colonic segments were also obtained from the non-colitic group. The remaining colonic tissue was subsequently sectioned in different longitudinal fragments

to be used for biochemical determinations, RNA isolation or isolation of *lamina propria* cells.

**Table 3.** Scoring of disease activity index (DAI) described by Cooper *et al.* (1993) [20] .

| Score | Weight loss | Stool consistency | Rectal bleeding |
|---|---|---|---|
| **0** | None | Normal | Normal |
| **1** | 1 - 5 % | | |
| **2** | 5- 10 % | Loose stools | |
| **3** | 10 – 20 % | | |
| **4** | > 20 % | Diarrhoea | Gross bleeding |

## 7. Effects of the extracts on carrageenan-induced paw edema in mice

Swiss albino mice (35-40g) were obtained from Janvier (St BerthevinCedex, France). Animals were housed in makrolon cages and maintained in an air-conditioned atmosphere with a 12-h light-dark cycle with standard diet and water *ad libitum*. The mice were randomly assigned to nine groups (n = 10). An untreated carrageenan control group and a healthy group were included for reference, which received orally the vehicle used (water) to administer the test compounds. Six of them were orally treated with the extracts (dissolved in 1 ml of water): *Lavandula dentata* (10, 25 and 100 mg/kg) or *Lavandula stoechas* (10, 25 and 100 mg/kg), and the remaining received treatment with diclofenac (10 mg/kg). 60 min after the administration of the test substances, the paw edema was induced in all experimental groups, except in the healthy group, by a subplantar injection of 40 µl of 1% Type I carrageenan solution into the right hind paw [391], whereas 40µL of saline solution was injected into the left hind paw of each mouse. The thicknesses of injected paws were measured by using a digital caliper every 60 min for 6h. The percentage of inhibitory values of edema in carrageenan-induced hind paw edema test were calculated. Animals were killed by cervical dislocation after the final assessment

and the paw removed by cutting at the tibio-tarsal level, which were homogenized and kept frozen at -80ºC until biochemical determinations were performed.

## 8. Histological studies

Cross-sections were selected and embedded in paraffin. Full-thickness sections of 5 µm were obtained at different levels and stained with hematoxylin and eosin. The histological damage was evaluated by a pathologist observer, who was blinded to the experimental groups, according to the criteria previously described by Camuesco *et al.* (2004) [17] (Table 3).

## 9. Biochemical determinations in colonic tissue.

### Myeloperoxidase activity

MPO activity was measured according to the technique described by Krawisz *et al.* (1984) [18]. Colonic specimens where homogenized in 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0) and MPO activity in supernatant was measured and calculated from the absorbance (at 460 nm) changes that resulted from decomposition of $H_2O_2$ in the presence of O-dianisidine; the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 µmol hydrogen peroxide/min at 25ºC.

### Glutathione content

Total glutathione (GSH) content was quantified with the recycling assay described by Anderson *et al.* (1985) [19] in which it is sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) and reduced by NADPH in the presence of glutathione reductase (Boehringer Mannheim, Barcelona, Spain). The rate of 2-nitro-5- thiobenzoic acid formation was monitored at 412 nm and the glutathione present was evaluated by

comparison of that result with a standard curve; the results were expressed as nmol/g wet tissue.

**Table 4.** Scoring criteria of full-thickness distal colon sections adapted from Camuesco *et al.*, (2004) [17]
.

| **Mucosal epithelium and *lamina propria*** |
| --- |
| Ulceration: none (0); mild surface (0-25%) (1); moderate (25-50%) (2); severe (50-75%) (3); extensive-full thickness (more 75%) (4). |
| Polymorphonuclear cell infiltrate |
| Mononuclear cell infiltrate and fibrosis |
| Edema and dilation of lacteals |
| **Crypts** |
| Mitotic Activity: lower third (0); mild mid third (1); moderate mid third (2); upper third (3) |
| Dilations |
| Goblet cell depletion |
| **Submucosa** |
| Polymorphonuclear cell infiltrate |
| Mononuclear cell infiltrate |
| Edema |
| Vascularity |
| **Muscular layer** |
| Polymorphonuclear cell infiltrate |
| Mononuclear cell infiltrate |
| Edema |
| Infiltration in the serosa |

Scoring scale: 0 none; 1 slight; 2 mild; 3 moderate; 4 severe. Maximum score: 59.

### Cytokines production

Colonic samples for the determination of colonic cytokine production were immediately weighed, minced on an ice-cold plate and suspended (1:5 w/v) in a lysis buffer containing 20 mM HEPES (pH 7.5), 10 mM ethylene glycol- bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid, 40 mM β-glycerophosphate, 2.5 mM magnesium chloride, 1% Igepal®, 1 mM dithiothreitol, 500 μM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/m iodoacetamide and 2 mM sodium orthovanadate. The tubes were placed in an orbital rotor (4 °C) for 20 min and centrifuged at 9000g for 10 min at 4 °C; the supernatants were frozen at − 80 °C until assay. The cytokines were quantified by enzyme-linked immunoabsorbent assay (ELISA) (R&D Systems Europe, Abingdon, UK) and the results were expressed as pg/g wet tissue.

### iNOS and COX-2 expressions

In order to evaluate tissue iNOS and COX-2 expression, the samples were homogenized (1/5w/v) in phosphate buffered saline supplemented with 0.1% sodium dodecylsulfate (SDS), 0.1% sodium deoxycholate, 1% TritonX-100 and protease and phosphatase inhibitor (aprotinin, leupeptin and PMSF). For the Western blot, 100 mg of proteins were boiled at 95ºC in Laemmli SDS loading buffer and separated on 7.5% SDS-PAGE. The proteins were then electrotransferred to nitrocellulose membranes (PROTAN &, Schleicher & Schuell, Germany). The membranes were blocked for at least 1 h at room temperature in Tris buffered saline-0.1% Tween-20 (TBS-T) 5% non-fat dry milk and then incubated with TBS-T containing 5% BSA and the dilution (1:2000) of iNOS or COX-2 antibody (Transduction Laboratories, Becton Dickinson Biosciences, Madrid, Spain). After three washes of 5 min each with TBS-T, the membranes were incubated with 5% non-fat dry milk and peroxidase-conjugated anti-rabbit IgG antibody for 1 h (1:5000). After three washes of 5 min with TBS-T, ECL detection was performed (NEN Life Science Products, Zaventem, Belgium). Control of protein loading and transfer was conducted by detection of the β-actin levels. The cytokine IL-1β was quantified by ELISA

(R&D Systems Europe, Abingdom, UK) and the results were expressed as pg/g wet tissue.

### Analysis of gene expression in samples by RT-PCR and real time PCR

Total RNA (from colon, hind paw or cells) was extracted using TRIzol® Reagent (Invitrogen LifeTechnologies, Paisley, U.K.), following the manufacturer's instructions, and was reverse transcribed using oligo (dT) primers (Promega, Southampton, UK). Semi-quantitative Reverse Transcription-Polymerase chain reaction (RT-PCR) was performed using specific primers (Table 4). RT-PCR analysis was performed at cycles below 35 using primers for the housekeeping gene β-actin for comparative reference. RT-PCR reaction was performed using Go Taq® DNA Polymerase (Promega, Southampton, UK) in accordance with the manufacturer's recommendations. RT-PCR mixtures were denatured at 95 °C for 3 min, followed by 22 to 35 cycles of denaturation at 94 °C for 1 min, annealing at 55–60 °C for 45 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. RT-PCR products were analyzed by electrophoresis in a 1% agarose gel containing ethidium bromide.

Real time quantitative PCR (qPCR) amplification and detection was performed on optical-grade 48well plates in a EcoTM Real-Time PCR System (Illumina, CA, USA) with 20 ng of cDNA, the KAPA SYBRs FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA) and specific primers at their annealing temperature (Ta) (Table 4). To normalize mRNA expression, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. The mRNA relative quantitation was calculated using the ΔΔCt method.

### Isolation of *lamina propria* cells

The large intestines of mice were removed and washed in Phosphate Buffered Saline (PBS) (Life Technologies/Invitrogen, Paisley, U.K.), and the fat was removed. The intestines were opened longitudinally, washed in 2% fetal bovine serum (FBS) (Life Technologies/Invitrogen, Paisley, U.K.) in Hank's Balanced Salt Solution (HBSS) (Life Technologies/Invitrogen, Paisley, U.K.), and cut into 0.5 cm sections. Tissue was then placed in 10 ml HBSS/2% FBS and shaken vigorously, and the supernatant was discarded. A total of 10 ml fresh calcium/magnesium-free HBSS (Life Technologies/Invitrogen, Paisley, U.K.) containing 2 mM EDTA was then added, the tube was placed in a shaking water bath for 15 min at 37˚C and shaken vigorously, and the supernatant discarded.

**Table 5.** Primer sequences used in RT-PCR and qPCR assays for in vitro studies and the in vivo models, DSS model of mice colitis, TNBS model of rat colitis and carrageenan-induced paw edema in mice.

| Gene | Organism | Sequence 5'-3' | Annealing T °C |
|---|---|---|---|
| GAPDH | Rat | FW:CCATCACCATCTTCCAGGAG<br>RV:CCTGCTTCACCACCTTCTTG | 60 |
| IL-1β | Rat | FW: GATCTTTGAAGAAGAGCCCG<br>RV: AACTATGTCCCGACCATTGC | 59 |
| Il-17 | Rat | FW:TGGACTCTGAGCCGCAATGAGG<br>RV: GACGCATGGCGGACAATAGAGG | 60 |
| TNF-α | Rat | FW:GTCTTTGAGATCCATGCCATTG<br>RV:AGACCCTCACACTCAGATCA | 57 |
| IL-6 | Rat | FW: CTTCCAGCCAGTTGCCTTCTTG<br>RV: TGGTCTGTTGTGGGTGGTATCC | 60 |
| CINC-1 | Rat | FW: CCGAAGTCATAGCCACACTCAAG<br>RV: TCACCAGACAGACGCCATCG | 60 |
| ICAM-1 | Rat | FW: GTGAACTGCTCTTCCTCTTG<br>RV: AGTGGTCTGCTGTCTTCC | 60 |
| MCP-1 | Rat | FW: TCTTCCTCCACCACTATGC<br>RV: TCTCCAGCCGACTCFATTG | 60 |
| VILLIN | Rat | FW:TGCTACCTGCTGCTCTATACCTAC<br>RV:CTGGCTCGTCGTTGTACTTCTG | 59 |

| | | | |
|---|---|---|---|
| TFF-3 | Rat | FW: ATGGAGACCAGAGCCTTCTG<br>RV: ACAGCCTTGTGCTGACTGTA | 60 |
| MUC-2 | Rat | FW: ACCACCATTACCACCACCTCAG<br>RV: CGATCACCACCATTGCCACTG | 60 |
| MUC-3 | Rat | FW: CACAAAGGCAAGAGTCCAGA<br>RV: ACTGTCCTTGGTGCTGCTGAATG | 60 |
| β-Actin | Mouse | FW: CACAAAGGCAAGAGTCCAGA<br>RV: ACTGTCCTTGGTGCTGCTGAATG | 55 |
| IL-1β | Mouse | FW: TGATGAGAATGACCTCTTCT<br>RV: CTTCTTCAAAGATGAAGGAAA | 60 |
| Il-6 | Mouse | FW: TAGTCCTTCCTACCCCAATTTCC<br>RV: TTGGTCCTTAGCCACTCCTTCC | 60 |
| IL-12 | Mouse | FW: CATCGATGAGCTGATGCAGT<br>RV: CAGATAGCCCATCACCCTGT | 62 |
| IL-17 | Mouse | FW: CCTGGGTGAGCCGACAGAAGC<br>RV: CCACTCCTGGAACCTAAGCAC | 56 |
| TNF-α | Mouse | FW: AACTAGTGGTGCCAGCCGAT<br>RV: CTTCACAGAGCAATGACTCC | 60 |
| IFN-γ | Mouse | FW: GGAGGAACTGGCAAAAGGATGGT<br>RV: TTGGGACAATCTCTTCCCCAC | 55 |
| IL-10 | Mouse | FW: TGCCTGCTCTTACTGACTGG<br>RV: TCATTTCCGATAAGGCTTGG | 63 |
| MIP-2 | Mouse | FW: AGTTAGCCTTGCCTTTGTTCAG<br>RV: CAGTGAGCTGCGCTGTCCAATG | 57 |
| ICAM | Mouse | FW: CAGTCCGCTGTGCTTTGAGA<br>RV: CGGAAACGAATACACGGTGAT | 62 |
| MUC-3 | Mouse | FW: CGTGGTCAACTGCGAGAATGG<br>RV: CGGCTCTATCTCTACGCTCTCC | 56 |
| iNOS | Mouse | FW: GTTGAAGACTGAGACTCTGG<br>RV: ACTAGGCTACTCCGTGGA | 67 |
| COX-2 | Mouse | FW: GGGTTGCTGGGGGAAGAAATG<br>RV: GGTGGCTGTTTTGGTAGGCTG | 60 |
| MMP-9 | Mouse | FW: TGGGGGGCAACTCGGC<br>RV: GGAATGATCTAAGCCCAG | 60 |

A total of 10 ml calcium/magnesium-free HBSS was then added, the tube was shaken vigorously, and the supernatant discarded. This step was repeated, and the remaining tissue was digested with 1.25 mg/ml collagenase D (Roche Diagnostic Systems, Mannheim, Germany), 0.85 mg/ml collagenase V, 1 mg/ml dispase (Life Technologies/Invitrogen, Paisley, U.K.), and 30 U/ml DNase (Roche Diagnostic Systems, Mannheim, Germany) in complete Roswell Park Memorial Institute medium (RPMI) 1640 for 30–40 min in a shaking water bath until complete digestion of the tissue. At the start of and every 5–10 min during the incubation, the tube was shaken vigorously, and the final supernatant was passed through a Nitex mesh (Cadisch & Sons, London, U.K.).

**Flow cytometry**

Aliquots $4x10^4$ cells, of each sample, were blocked with purified anti-mouse CD16/CD32 (eBioscience Inc., San Diego California, USA) and the cells were used for surface staining for 30 min at 4˚C. For intracellular staining, cells were first activated in vitro by TLR ligands in the presence of 10 mg/ml brefeldin A for 4.5 h. The cells were then fixed, permeabilized with the Fix/Perm Fixation/Permeabilization kit (eBioscience Inc., San Diego, CA, USA) and stained for cell-surface and intracellular markers. The antibodies for suface staining were: CD45 (PercP, Clone 30-F11, (Biolegend, San Diego, CA, USA)), CD11c (PE-Cy7, Clone N418 (Biolegend, San Diego, CA, USA)), LyC6 (Alexafluor 700, Clone HK1.4, (Biolegend, San Diego, CA, USA)), F4/80 (V450, Clone BM8 (eBioscience, San Diego, CA, USA)), MHCII (Brilliant Violet 500, Clone M5/114.1.2 (Biolegend, San Diego, CA, USA)), CD11b (Brilliant Violet 605, Clone M1/70 ((Biolegend, San Diego, CA, USA)), Viability dye-(APC-Cy7, Clone N418 (eBioscience Inc., San Diego California, USA)).

All cells were analyzed on LSR II or FACSAria I (BD Biosciences, Oxford, UK) using FlowJo software (Tree Star, Ashland, OR, USA).

## 10. In vitro evaluation of the effects of the extracts on immune cells

### Bone marrow-derived macrophages (BMDM)

BMDM were generated as described previously [392] with some modifications. Bone marrow was isolated from femurs of 6-week-old BALB/c mice (Janvier, St Berthevin Cedex, France). Mice were sacrificed by cervical dislocation, the adherent tissue was removed and both femurs were dissected. The bone ends were cut off, and the marrow tissue was flushed by irrigation with Dulbecco's Modified Eagle Medium (DMEM). The marrow plugs were dispersed by passing them through a 25-gauge needle, and the cells were suspended by vigorous pipetting and washed. Cells were cultured in 150 mm Petri dishes with 40 ml of DMEM containing 20% FBS and 30% Lcell-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF) [393]. Cells were incubated at 37ºC in a humidified 5% $CO_2$ atmosphere. After 6 days of culture, a homogeneous population of adherent macrophages was obtained. Cells were incubated in the presence or the absence of *Serpylli herba* extract at different concentrations (0.1 µg/ml to 40 µg/ml) for 1 hour before stimulation with LPS (Escherichia coli 055:B5) (10 ng/ml). After 24 h, supernatants were collected and centrifuged at 10,000g for 5 min. An aliquot was used to determine nitrite levels by Griess assay, and another was stored at −80 ◦C until IL-6 determination by ELISA (R&D Systems Europe, Abingdom, UK) was performed.

### Epithelial cell lines

The murine epithelial cell line CMT-93 and human epithelial cell line Caco-2 were obtained from the Cell Culture Unit of the University of Granada (Granada, Spain) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum FBS and 2 mM l-glutamine, in a humidified 5% CO2 atmosphere at 37ºC. CMT-93 and Caco-2 cells were seeded onto 24-well plates at a density of $5 \times 10^5$ cells per well and grown until confluence.

CMT-93 and Caco-2 cells were cultured with *L. dentata* and *L. stoechas* extracts at different concentrations (0.1 µg/ml to 100 µg/ml) for 2 h and then stimulated with LPS

(10 μg/ml for CMT-93 for 72h) or IL-1β (1ng/ml for Caco-2 for 24h). Afterwards, supernatants were collected and centrifuged at 10,000g for 5 min.

### Peripheral blood mononuclear cells mixed lymphocyte reaction

Buffy coats were obtained from healthy donors with informed consent for research use. Peripheral blood mononuclear cells (PBMCs) were separated with Ficoll (GE Healthcare, Madrid, Sapin) gradient centrifugation and then resuspended and cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) containing 10% FBS, 1% glutamine, 1% pyruvate, 1% non-essential AA and 1% penicillin-treptomycin. PBMCs were incubated with different concentrations (0.001-10 μg/ml) of *L. dentata* and *L. stoechas* extracts and stimulated with LPS (100 ng/ml) during 24 hours. After that, the levels of TNF-α, IL-12p40 and IL-10 were evaluated in the supernatants by ELISA (R&D systems). PBMCs from two different donors were co-cultured (1:1) in presence of *L. dentata* and *L. stoechas* extracts at different concentrations (0.001-10 μg/ml). After 5 days supernatants were collected and IFN-γ levels were measured by ELISA (R&D Systems, Abingdon, UK).

### Dendritic cells derived from human peripheral blood monocytes

DCs were derived from human peripheral blood monocytes selected with anti-CD14 antibodies coupled to magnetic beads (Miltenyi, Bologna, Italy). $CD14^+$ cells were incubated for 6 days in complete medium containing GM-CSF (5 ng/ml; BD Biosciences, Madrid, Spain) and IL-4 (2.5 ng/ml; BD Biosciences, Madrid, Spain) in order to obtain immature monocytes-derived dendritic cells moDCs.

MoDCs were incubated with *L. dentata* and *L. stoechas* extracts for two hours and then stimulated with LPS at 100 ng/ml for 24 h or *Salmonella* FB62(1h) (1:1 bacteria: DC). After extensive wash, *Salmonella* FB62-stimulated cells were incubated for 24 hours in the presence of gentamicin (100 μg/ml). Supernatants were tested for cytokine

abundance (IL-12p70 and IL-10) and cells were analyzed by flow cytometry for activation and maturation markers: HLA-DR (PerCp, Clone L243 (Biolegend, San Diego, CA, USA)), CD1a (PE-Cy7, Clone HI149 (Biolegend, San Diego, CA, USA)), CD80 (PE, Clone L307.4), CD83 (APC, Clone HB15c (BD Pharmigen$^{TM}$, San Jose, CA, USA)), CD86 (FITC, Clone 2331 (FUN-1) (BD Pharmigen$^{TM}$, San Jose, CA, USA)).

## 11. Statistics

All results are expressed as the mean ± SEM. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. Differences between proportions were analyzed with the chi-squared test. All statistical analyses were carried out with the GraphPad Prism version 5.0 (La Jolla, CA, USA), with statistical significance set at $P < 0.05$.

# RESULTS

# RESULTS

## 1. *Serpylli herba*

### 1.1. Intestine anti-inflammatory effect of *Serpylli herba* extract on TBNS rat colitis

In the course of the experiment, the inflammatory status induced after the administration of TNBS to rats was evidenced by anorexia, since there was a reduced food intake in the TNBS control group when compared to normal rats (data not shown). Correspondingly, colitic animals from control group showed a reduced body weight gain with time, being of 14.1 ± 2.9 % seven days after colitic induction, in contrast with the higher weight gain observed in non colitic rats (7.8 ± 1.4%; $P<0.01$ *vs*. control colitic group) (Figure 13). Once the rats were sacrificed, the colonic tissue from control colitic rats appeared inflamed and necrosed, typically extending 5–6 cm along the large intestine, thus obtaining a mean ± SEM score value of 8.1 ± 0.3 (Table 6). In addition, hyperemia, and focal adhesions to adjacent organs were also observed in most of the rats from this group. Bowel wall thickening was clearly evidenced by an almost three-fold increase in the colonic weight/length ratio in rats from the untreated colitic group when compared to non-colitic rats (Table 6).

The administration of the different doses of *Serpylli herba* extract, as well as of SAZ, for two days before colitis induction did not result in any symptom of diarrhea or affect weight evolution (data not shown). However, whereas the lowest (10 mg/kg) and the highest (1000 mg/kg) doses of the extract were devoid of any significant effect, the treatment of colitic rats with the doses of 100 or 250 mg/kg ameliorated the weight decrease observed in the untreated colitic group, being statistically significant 6 and 7 days after colitis induction (Figure 13); a similar impact on colonic impact was observed after treatment of colitic rats with sulphasalazine (Figure 13).

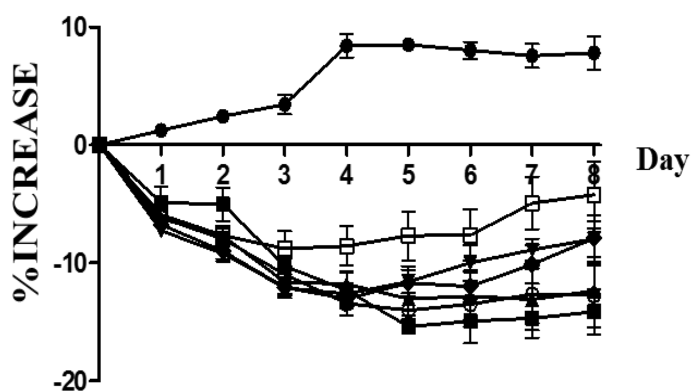



**Figure 13.** Effects of different doses of *Serpylli herba* extract and sulphasalazine (SAZ) on weight evolution in TNBS rat colitis. Data are expressed as means ± SEM. (●) Non colitic group; (■) TNBS control group; (▲) *Serpylli herba* 10 mg/kg; (▼) *Serpylli herba* 100 mg/kg; (♦) *Serpylli herba* 250 mg/kg; (○) *Serpylli herba* 1000 mg/kg; (□) Sulphasalazine 30 mg/kg. All colitic groups significantly differ from the non-colitic group (P<0.05). The treatments show significant differences (P<0.05) in comparison with the control group from the following days: day 4 sulphasalazine 30 mg/kg, day 6 *Serpylli herba* 100 mg/kg, day 7 *Serpylli herba* 250 mg/kg.

The macroscopic analysis of the colonic samples revealed the beneficial effect exerted by the different doses of the extract in this model of experimental colitis. Thus, a significant reduction in the extension of colonic damage was observed in treated colitic rats with the extract, at the doses of 10, 100 and 250 mg/kg, resulting in a lower macroscopic score than in untreated control colitic group (Table 6). When the colonic weight/length ratio was evaluated, as a consistent index of edematous tissue, it was only significantly reduced in the group of colitic rats treated with 100 mg/kg of the extract (Table 6). However, the results obtained in this assay revealed that sulphasalazine (30 mg/kg) did not show significant modifications of these macroscopic parameters in comparison with control colitic group (Table 6).

The histological assessment of colonic samples from the TNBS control group revealed an ulceration and necrosis of epithelial cells higher than 50% of surface in the majority of animals. The colon showed a very severe transmural inflammation constituted by infiltration of granulocytes cells predominately neutrophils in *lamina propria* and a mixture of granulocytes and mononuclear cells (macrophages, lymphocytes and plasmatic cells) in submucosa, muscularis and serosa layers. Edema was also evident in most of the samples. The grade of lesion was severe or very severe, giving a score value of 28.0 ± 0.9 (Figure 14).

**Table 6.** Effects of several doses of *Serpylli herba* extract on colonic macroscopic damage score, weight/length ratio, myeloperoxidase (MPO) activity and glutathione (GSH) content in TNBS experimental rat colitis.

| Group (n=10) | Damage Score (0-10) | Weight/length (mg/cm) | MPO (mU/g tissue) | GSH (nmol/g tissue) |
|---|---|---|---|---|
| Non-colitic | 0 | 71.2 ± 3.1 | 5.3 ± 0.8 | 639 ± 39 |
| TNBS control | 8.1 ± 0.3 | 203.5 ± 10.8 | 362.3 ± 44.5 | 89 ± 15 |
| *Serpylli herba* (10 mg/kg) | 7.2 ± 0.3* | 172.9 ± 14.7 | 240.1 ± 29.0* | 198 ± 30* |
| *Serpylli herba* (100 mg/kg) | 6.9 ±0.3* | 149.7 ± 10.0* | 319.8 ± 48.2 | 329 ±45* |
| *Serpylli herba* (250 mg/kg) | 7.0 ±0.4* | 170.5 ± 18.5 | 307.0 ± 34.6 | 275 ± 61* |
| *Serpylli herba* (1000 mg/kg) | 7.5 ± 0.4 | 204.2 ± 19.4 | 206.0 ± 48.9* | 251 ± 44* |
| SAZ (30 mg/kg) | 7.8 ±0.3 | 198.3 ± 17.3 | 188.0 ± 8.4* | 149 ± 27 |

Data are expressed as mean ± SEM. *$P<0.05$ *vs.* TNBS control group. All colitic groups statistically differ from non-colitic group ($P<0.05$).

The treatment with *Serpylli herba* extract showed a significant recovery of the intestinal histology compared to control group, although this was more evident in animals treated at doses of 100, 250 and 1000 mg/kg. The improvement of the inflammatory process was observed mainly in submucosa and muscularis layers. In the group of animals treated with the dose of 10 mg/kg, ulceration was present in 50% of intestinal epithelium with a severe leukocyte infiltration, primarily composed by neutrophils in *lamina propria*, although the presence of inflammatory cells was lower than in the control group, both in submucosa and muscularis mucosa, where the severity of the lesion was only severe in a few cases. Hyperplasia of mucosal epithelium and depletion of goblet cells was slight or moderate. The lower inflammatory infiltrate in submucosa and muscularis was also observed with 100 and 250 mg/kg doses, being in both groups the lesions quite similar. Neutrophils were the predominant cells in the *lamina propria*, or even the only cells, showing a degree of ulceration lower than those found in control and 10 mg/kg treated groups. Crypt epithelium showed a slight hyperplasia and depletion of goblet cells. On the other hand, a moderate-severe mixture of chronic and acute inflammation was

observed in submucosa and muscularis layers. Finally, the group receiving the dose of 1000 mg/kg presented a slight increase of transmural inflammation with a spreading of infiltrative cells into the serosa; however, the severity of inflammatory lesions was also lower than those from control. The surface of ulceration was between 25-50% of mucosal epithelium with a slight hyperplasia and goblet cell depletion, most frequently at the margin of the ulcers (Figure 14). The sulphasalazine-treated group also presented a significant recovery of the colonic lesions induced by TNBS. Thus, the severity of ulceration and inflammatory infiltrate in *lamina propria* were slight or moderate, and lymphocytes and macrophages were observed in submucosa and in lower number in muscularis. Only few animals also presented a small and focal cell infiltrate in the serosa. The epithelium adjacent to ulcer remained unaffected (Figure 14). The beneficial histological effects of the extract were confirmed biochemically. The colonic damage induced by TNBS was associated with an increased MPO activity in comparison with non-colitic rats, indicating the massive neutrophil infiltration into the inflamed tissue, as revealed by the histological studies. Although all the assayed doses of the extract reduced this enzyme activity when compared with the untreated colitic control group, only the doses of 10 and 1000 mg/kg achieved statistical significance, which suggested a lower leukocyte infiltration in the colonic tissue, thus confirming the observations from the histological studies (Table 6). Similarly, the colonic MPO activity from the group of colitic rats treated with sulphasalazine was also significantly reduced, being similar to that obtained with an extract dose of 100 mg/kg (Table 6).

Correspondingly, the colonic glutathione content, which is depleted in colitic rats as a consequence of the colonic oxidative stress that occurs in the inflammatory process, was significantly increased at all doses of the extract, but not significantly increased with sulphasalazine. These data reveal an improvement in the altered oxidative status in colitic rats treated with the extract of *Serpylli herba* (Table 6).

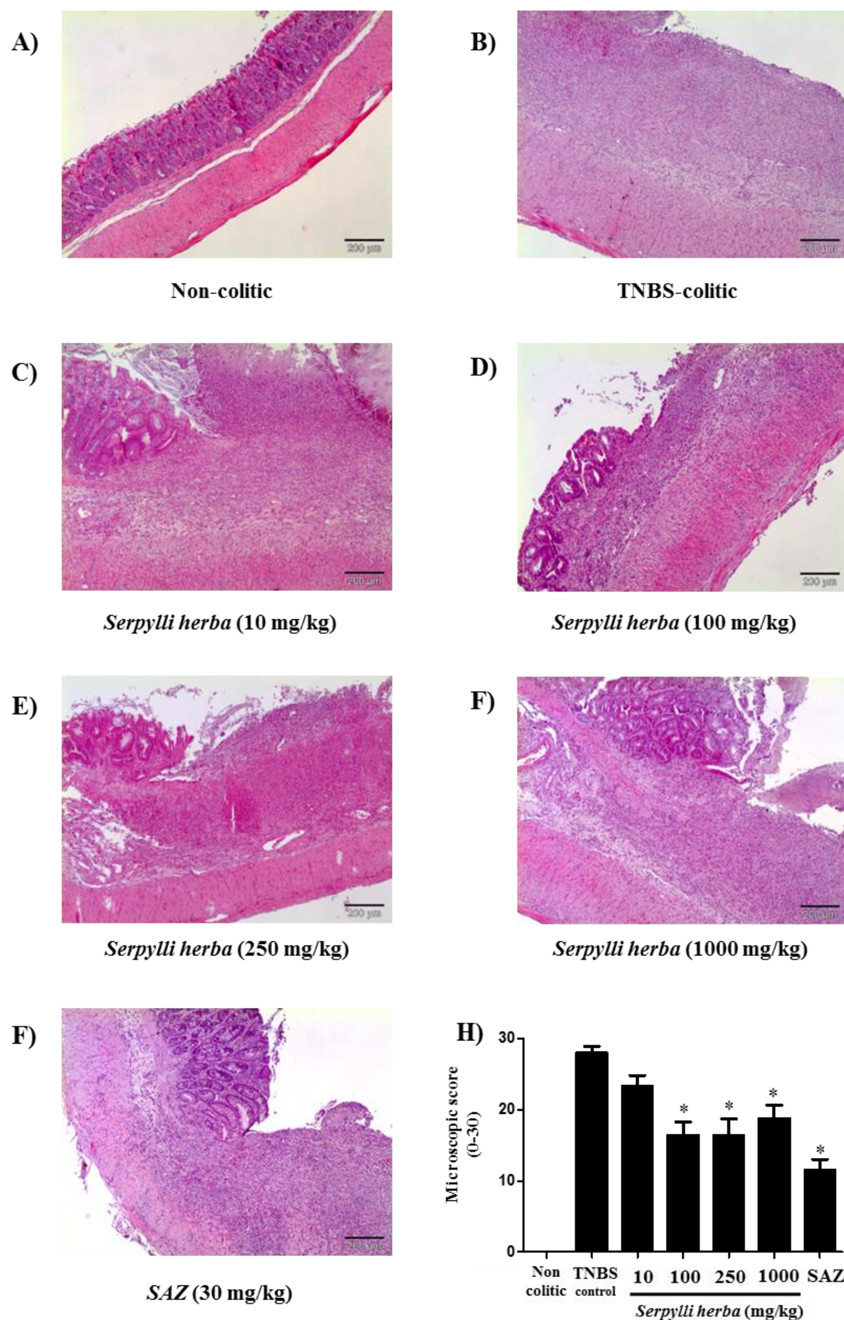



**Figure 14.** Histological sections of colonic tissue stained with hematoxylin and eosin showing the effects of different doses of *Serpylli herba* extract and sulphasalazine (SAZ) (30 mg/kg) in TNBS rat colitis: A) Non-colitic; B) TNBS-control; C) *Serpylli herba* (10 mg/kg); D) *Serpylli herba* (100 mg/kg); E) *Serpylli herba* (250 mg/kg); F) *Serpylli herba* (1000 mg/kg); G) sulphasalazine (SAZ) (30 mg/kg) and H) microscopic score assigned according the criteria previously described [17]. Data are expressed as means ± SEM. *P<0.05 *vs*. TNBS control group.

The colonic inflammatory status in TNBS-induced colitic rats was also characterized by altered expression of different colonic markers. Both colonic iNOS and COX-2 protein expressions were increased in colitic control rats when compared with non-colitic animals (Figure 15). Whereas all the assayed doses of the extract *of Serpylli herba* significantly reduced the expression of COX-2 in colitic rats, only the two highest doses (250 and 1000 mg/kg) did ameliorate the increased expression of iNOS. Sulphasalazine (30 mg/kg) was able to downregulate the expression of both inducible enzymes (Figure 15).

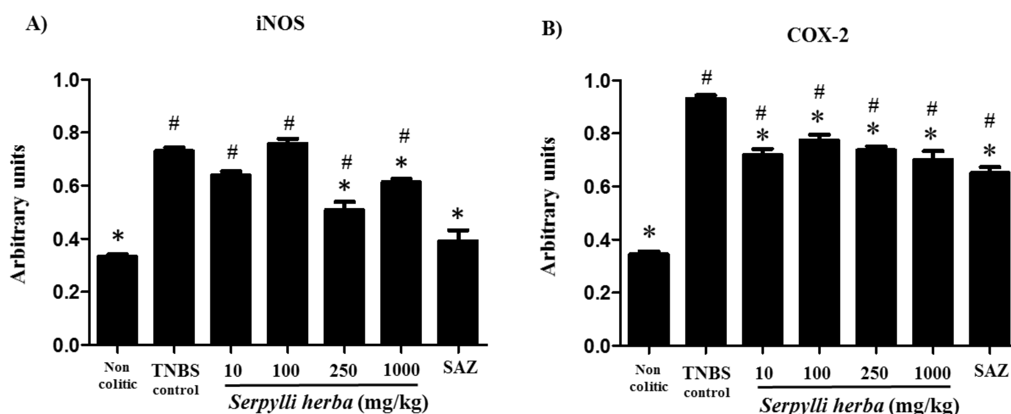



**Figure 15.** Effects of different doses of *Serpylli herba* extract (10, 100, 250 and 1000 mg/kg) and sulphasalazine (SAZ) (30 mg/kg) on colonic iNOS (A) and COX-2 (B) expressions examined by western blot in TNBS rat colitis. Data are expressed as means ± SEM. *$P<0.05$ *vs*. TNBS control group. $^{\#}P<0.05$ *vs*. non-colitic group.

In addition, the production of the chemotactic eicosanoid $LTB_4$, as well as the cytokines TNF-α, IL-1β and IL-10 were quantitated by ELISA in the colonic samples to better characterize the intestinal anti-inflammatory effect exerted by the extract of *Serpylli herba*. The colonic inflammatory status was associated with increased levels of the pro-inflammatory mediators $LTB_4$, TNF-α, and IL-1β, together with a decrease in the production of the anti-inflammatory cytokine IL-10 (Figure 16). Treatment of colitic rats with the extract resulted in a clear beneficial effect on these inflammatory markers; although not all the doses assayed showed the same profile. In comparison with the

untreated colitic group, the administration of the highest doses (250 and 1000 mg/kg) of the extract significantly reduced the levels of $LTB_4$, TNF-α, and IL-1β, without showing any significant effect on IL-10 production, the latter being significantly reduced when doses of 10 and 100 mg/kg were applied (Figure 16). The treatment of colitic rats with sulphasalazine (30 mg/kg) was associated with a significant reduction in the colonic levels of TNF-α (Figure 16).

Finally, the expression of three other pro-inflammatory mediators, i.e. the cytokine IL-17, the intercellular adhesion molecule-1 (ICAM-1) and the monocyte chemotactic protein-1 (MCP-1), were also analyzed in the TNBS-colitic rats by RT-PCR. The results show that colonic inflammation significantly increased the expression of these markers in comparison with non-colitic rats (Figure 16). Treatment with all doses of *Serpylli herba* extract, as well as with 30 mg/kg of sulphasalazine, significantly ameliorated the increased expression of either IL-17 or ICAM-1 when compared with untreated colitic rats; however, the expression of MCP-1 was only reduced in those colitic rats treated with sulphasalazine (Figure 16).

### 1.2. Intestinal anti-inflammatory effect of *Serpylli herba* extract on DSS mouse colitis

To confirm the anti-inflammatory effect of the extract of *Serpylli herba* observed in the TNBS-colitis in rats, we evaluated it in the DSS model of colitis in mice, following a curative treatment protocol, that is, the administration of the extract started once the colitis had been induced and maintained for 7 days after removal of the DSS from the drinking water. Both doses of the extract (100 and 250 mg/kg) did not significantly attenuate the body weight loss that occurred during the time-course of the experiment in comparison with untreated colitic mice (not shown). However, macroscopic evaluation of colonic segments showed that the high dose assayed significantly reduced colonic weight/length ratio (30.2 ± 1.2 mg/mm) in comparison with control mice (34.9 ± 2.0 mg/mm; $p<0.05$), a parameter that has been suggested to be directly correlated with the

severity of colonic damage in the DSS-induced colitis [376].Histological assessment of colonic samples from the DSS control group revealed an ulceration of epithelial cells, which typically comprised between 50% and 75% of the surface of colonic segments. Hyperplasia and dilation of crypts were observed in all samples, and this was associated with goblet cell depletion. A chronic inflammatory cell infiltration into the *lamina propria* was observed and edema was observed between the mucosa and muscularis layers of the intestine. The grade of lesion was moderate, giving a score value of 20.9 ± 1.3 (Figure 17). In contrast, in the groups of colitic mice treated with *Serpylli herba* extract, there was a recovery from intestinal damage as evidenced by the mucosal re-epithelization and the decrease in the inflammatory infiltrate both in *lamina propria* and submucosa. In these groups of mice, only 3-4 mice out of 10 howed mucosal ulceration affecting more than 25% of the surface, and this was evaluated as slight or moderate howed mucosal ulceration affecting more than 25% of the surface, and this was evaluated as slight or moderate.

In the areas of ulceration, the inflammatory infiltrate was lower than that observed in untreated colitic mice. In the mucosa, the process of recovery was evidenced by the hyperplasia in the basal areas of the crypts, although goblet cells still appear depleted from their mucin content. Consequently, the grade of lesion was significantly lower than that obtained in the control group, giving score values of 10.5 ± 1.2 and 8.9 ± 1.1 for 100 and 250 mg/kg, respectively ($p<0.05$ *vs* control group) (Figure 17). When the colonic segments from colitic mice treated with the control drug sulphasalazine (50 mg/kg), a recovery of the intestinal segments was also observed, showing a histological damage score value of 9.3 ± 1.4, significantly lower than the control group ($p<0.05$) (Figure 17).

Furthermore, biochemical analysis of the colonic segments confirmed *Serpylli herba* extract effects on the immune response significantly reduced the colonic production levels of pro-inflammatory cytokines IL-1β and IL-6, which was increased in colitic mice in comparison with the non-colitic group (Figure 18); this effect was similar to that obtained after treatment of colitic mice with sulphasalazine (Figure 18).

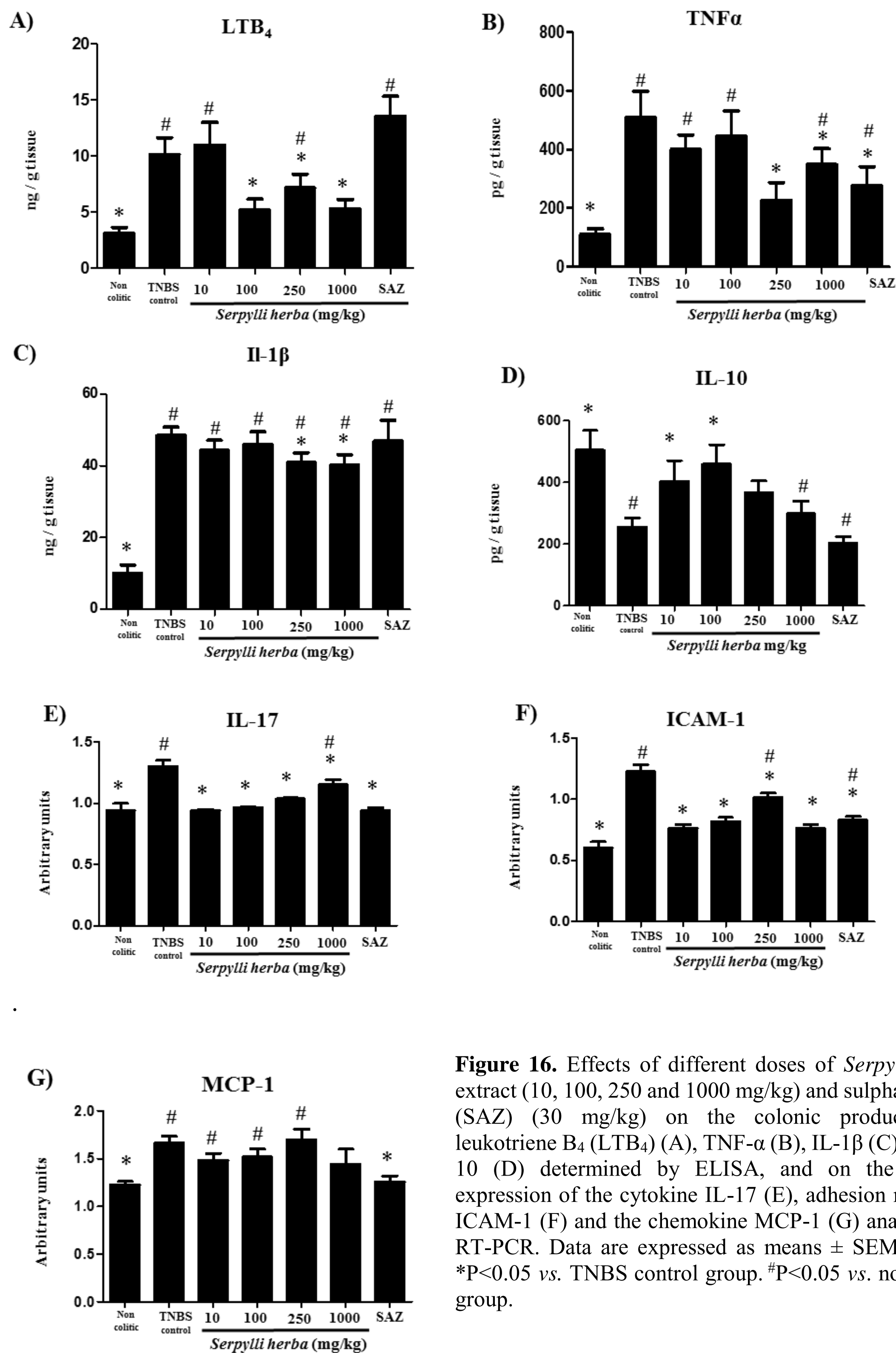



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**Figure 16.** Effects of different doses of *Serpylli herba* extract (10, 100, 250 and 1000 mg/kg) and sulphasalazine (SAZ) (30 mg/kg) on the colonic production of leukotriene $B_4$ ($LTB_4$) (A), TNF-α (B), IL-1β (C), and IL-10 (D) determined by ELISA, and on the colonic expression of the cytokine IL-17 (E), adhesion molecule ICAM-1 (F) and the chemokine MCP-1 (G) analyzed by RT-PCR. Data are expressed as means ± SEM (n=10); *P<0.05 *vs.* TNBS control group. #P<0.05 *vs*. non-colitic group.

Furthermore, evaluation of the expression of different colonic inflammatory markers by PCR showed that the intestinal anti-inflammatory effect of *Serpylli herba* extract was associated with a significant reduction in the colonic expression of the cytokines TNF-α, IFN-γ, IL-17 and of the adhesion molecule ICAM-1, as well as with an increased expression of the anti-inflammatory cytokine IL-10, when compared with the untreated colitic group (Figure 18); however, administration of sulphasalazine to colitic mice resulted in significant modifications only when IL-10 and ICAM-1 were evaluated

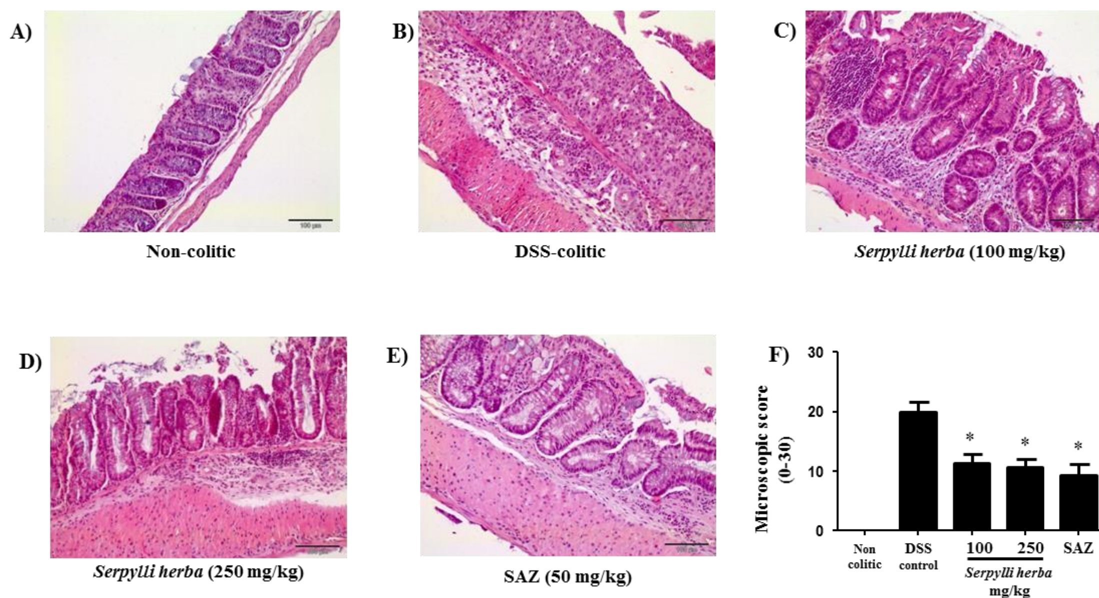


**Figure 17.** Histological sections of colonic tissue stained with hematoxylin and eosin showing the effects of different doses of *Serpylli herba* extract and sulphasalazine (SAZ) (50 mg/kg) in DSS mouse colitis: A) Non-colitic; B) DSS-control; C) *Serpylli herba* (100 mg/kg); D) *Serpylli herba* (250 mg/kg); E) sulphasalazine (SAZ) (50 mg/kg) and F) microscopic score assigned according the criteria previously described [17]. Data are expressed as means ± SEM. *P<0.05 *vs*. DSS control group.

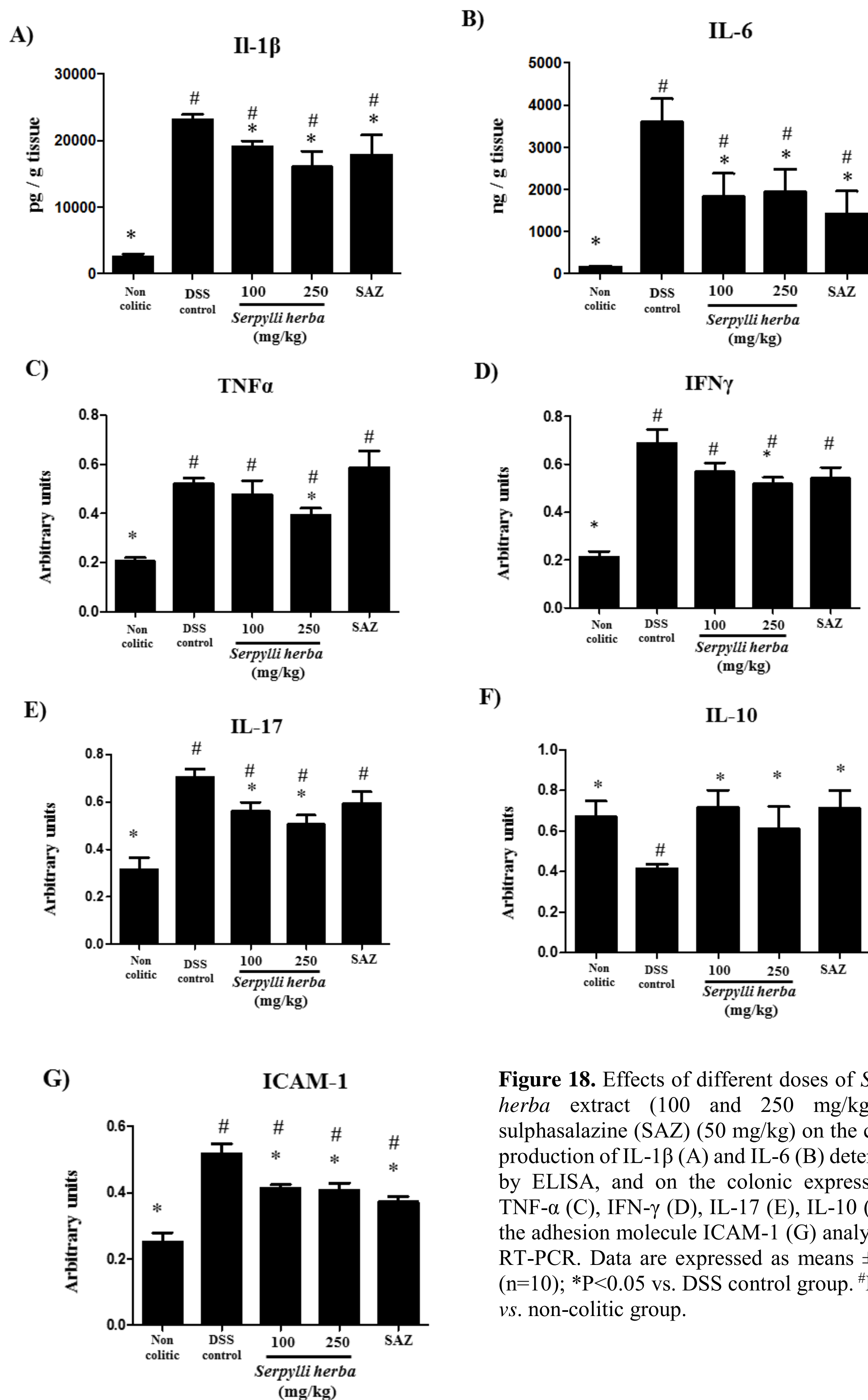


**Figure 18.** Effects of different doses of *Serpylli herba* extract (100 and 250 mg/kg) and sulphasalazine (SAZ) (50 mg/kg) on the colonic production of IL-1β (A) and IL-6 (B) determined by ELISA, and on the colonic expression of TNF-α (C), IFN-γ (D), IL-17 (E), IL-10 (F) and the adhesion molecule ICAM-1 (G) analyzed by RT-PCR. Data are expressed as means ± SEM (n=10); *P<0.05 vs. DSS control group. #P<0.05 *vs*. non-colitic group.

## In vitro effects of *Serpylli herba* extract in BMDM

In order to further characterize the involvement of the immunomodulatory properties of *Serpylli herba* extract in its intestinal anti-inflammatory effect, we tested its effect in LPS-stimulated BMDM. Macrophages are considered the main source of the pro-inflammatory mediators in IBD, thus actively contributing to the pathology of these intestinal conditions [394]. Different concentrations of the extract were assayed and the results revealed that *Serpylli herba* extract pre-treatment inhibited dose-dependently LPS induced nitrite and IL-6 production (Figure 19), without affecting cell viability (data not shown).

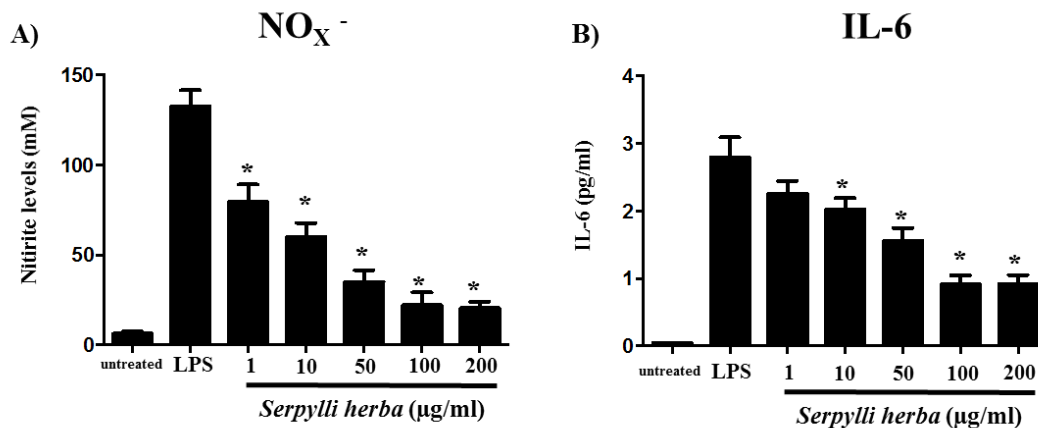


**Figure 19.** Effects of different concentrations of *Serpylli herba* extract on nitrite levels (A) and on IL-6 production (B) in BMDM cells stimulated with LPS (10 ng/ml). Data are expressed as means ± SEM. The experiments were performed three times, with each individual treatment being run in triplicate.

## 2. *Phlomis* sp. plants

### 2.1. Antiradical activity of the extracts

Both extracts showed similar antiradical effect in the DPPH assay, being their IC50 values 0.17± 0.05 for *P. lychnitis* and 0.15 ± 0.02 mg/ml for *P. purpurea*.

### 2.2. Intestinal anti-inflammatory effect of the extracts in the TNBS model of rat colitis

Similarly to that described above, the intracolonic administration of TNBS/ethanol resulted in colonic inflammation, which was seven days later characterized by severe necrosis of the mucosa, typically extending 4 to 6 cm along the colon, bowel wall thickening, hyperemia and focal adhesions to adjacent organs. In the course of the experiment, anorexia was evidenced due to the reduced food intake in the TNBS control group in comparison with non-colitic to normal rats (data not shown). Correspondingly, colitic animals from control group showed a gradual body weight lost with time, being that of 4.52 ± 4% seven days after colitic induction, in contrast with the weight gain observed in healthy rats (13.9 ± 0.5%; $P<0.01$ *vs*. control colitic group).

Although the treatment of colitic rats with the extracts of *P. purpurea* or *P. lychnitis* did not show any effect on food intake or weight evolution (data not shown), the macroscopic analysis of the colonic samples revealed that both of them had an evident anti-inflammatory effect. Thus, a significant reduction in the extension of colonic damage was observed in treated animals, especially in those that received the lowest dose of the extracts (10 mg/kg). This resulted in a lower macroscopic score when compared to the untreated control colitic group (Table 7). Similarly, the colonic weight/length ratio, as a reliable index of edema tissue, was significantly reduced in colitic rats treated with any of these extracts and at both doses assayed, being the greater reduction achieved with the extract of *P. purpurea* (Table 7).

**Table 7.** Effects of extracts of *P. lychnitis* and *P. purpurea* on colonic macroscopic damage score, weight/length ratio, myeloperoxidase (MPO) activity and glutathione (GSH) content in trinitrobenzenesulphonic acid (TNBS) experimental rat colitis..

| Group (n=10) | Damage Score (0-10) | Weight/length (mg/cm) | MPO (mU/g tissue) | GSH (nmol/g tissue) |
|---|---|---|---|---|
| Non-colitic | 0 | 77.2 ± 4.4 | 34.0 ± 7.5 | 1716 ± 54 |
| TNBS control | 6.9 ± 0.4 | 266.2 ± 42.1 | 394.6 ± 57.6 | 959 ± 118 |
| *P. lychnitis* (10 mg/kg) | 5.2 ± 0.4* | 158.2 ± 30.4* | 213.4 ± 49.1* | 1340 ± 83* |
| *P. lychnitis* (25 mg/kg) | 6.1 ±0.4* | 175.1 ± 24.0* | 177.5 ± 40.2* | 1029 ±147[#] |
| *P. purpurea* (10 mg/kg) | 4.5 ±0.6* | 97.1 ± 7.5* | 223.7 ± 58.5* | 1355 ± 115* |
| *P. purpurea* (25 mg/kg) | 5.8 ± 0.5* | 123.5 ± 11.4* | 226.8 ± 55.9* | 1183 ± 125 |
| Sulphasalazine (200 mg/kg) | 6.4 ±0.3 | 182.3 ± 28.9 | 262.0 ± 28.6 | 880 ± 55 |

Data are expressed as mean ± SEM. **P*<0.05 *vs.* TNBS control group. All colitic groups statistically differ from non-colitic group (P<0.05).

On the contrary, sulphasalazine (200 mg/kg) treatment did not significantly modify any of these macroscopic parameters in comparison with control colitic group (Table 7).

The microscopic evaluation of the colonic specimens confirmed the intestinal anti-inflammatory effect of the extracts (Figure 20). In comparison with the normal architecture of the colon (Figure 20A), the colonic samples from the TNBS control group (Figure 20B) showed intense transmural disruption of colonic tissue, in which an extensive ulceration and inflammation involved all the intestinal layers. The epithelial ulceration of the mucosa typically affected over 75% of the surface, and it was associated with diffuse leukocyte infiltration, mainly composed of neutrophils in the mucosa layer and, to a lesser extent, lymphocytes in the submucosa. This inflammatory process was also associated with severe goblet cell depletion. In this group of rats, the grade of lesion was considered as severe or very severe, with a score value (mean ± SEM) of 30.0 ± 4.4 (Figure 20H). On the contrary, the histological assessment of the colonic specimens from

rats treated with either *P. lychnitis* (Figures 20C and 20D) or *P. purpurea* (Figures 20E and 20F) revealed a pronounced recovery of the colonic tissue, with a significantly reduced score in comparison to untreated rats (Figure 20H). The transmural involvement of the lesions was reduced and most of the samples showed a restoration of the epithelial cell layer. Only a maximum of 25% of the epithelium was affected in contrast to the extensive ulceration observed in non-treated animals. In addition, many of the goblet cells appeared replenished with their mucin content. In addition, the improvement in colonic histology was accompanied by a reduction in the inflammatory infiltrate, which was slight to moderate with a patchy distribution, although neutrophils were also the predominant cell type. However, colonic samples from sulphasalazine-treated group showed a lower recovery, with a similar degree of ulceration and goblet cell depletion to that observed in the TNBS control group (Figure 20G).

The beneficial effects showed by *P. purpurea* or *P. lychnitis* were confirmed biochemically. Thus, the colonic damage induced by TNBS was associated with an increased MPO activity in comparison with non-colitic rats, being indicative of the massive neutrophil infiltration that takes place into the inflamed tissue. Both plant extracts significantly reduced this enzyme activity more than a 50% (Table 7), confirming the lower leukocyte infiltration in the colonic tissue observed in the histological studies.

Similarly, the colonic glutathione content, which is depleted in colitic rats as a consequence of the colonic oxidative stress that occurs in the inflammatory process, was significantly increased in those rats treated with the dose of 10 mg/kg of both extracts, thus revealing an improvement in the altered oxidative status (Table 7). Whereas sulphasalazine was able to significantly reduce colonic MPO activity in colitic rats, it was devoid of any significant effect on the glutathione content (Table 7).

The colonic inflammatory status was also characterized by increased colonic iNOS protein expression in comparison with non-colitic animals. The treatment of colitic rats with either *P. lychnitis* or *P. purpurea* extracts resulted in a down-regulation of iNOS expression, showing the latter a greater effect (Figure 21). On the contrary, sulphasalazine did not show any significant effect on this protein expression (Figure 21).

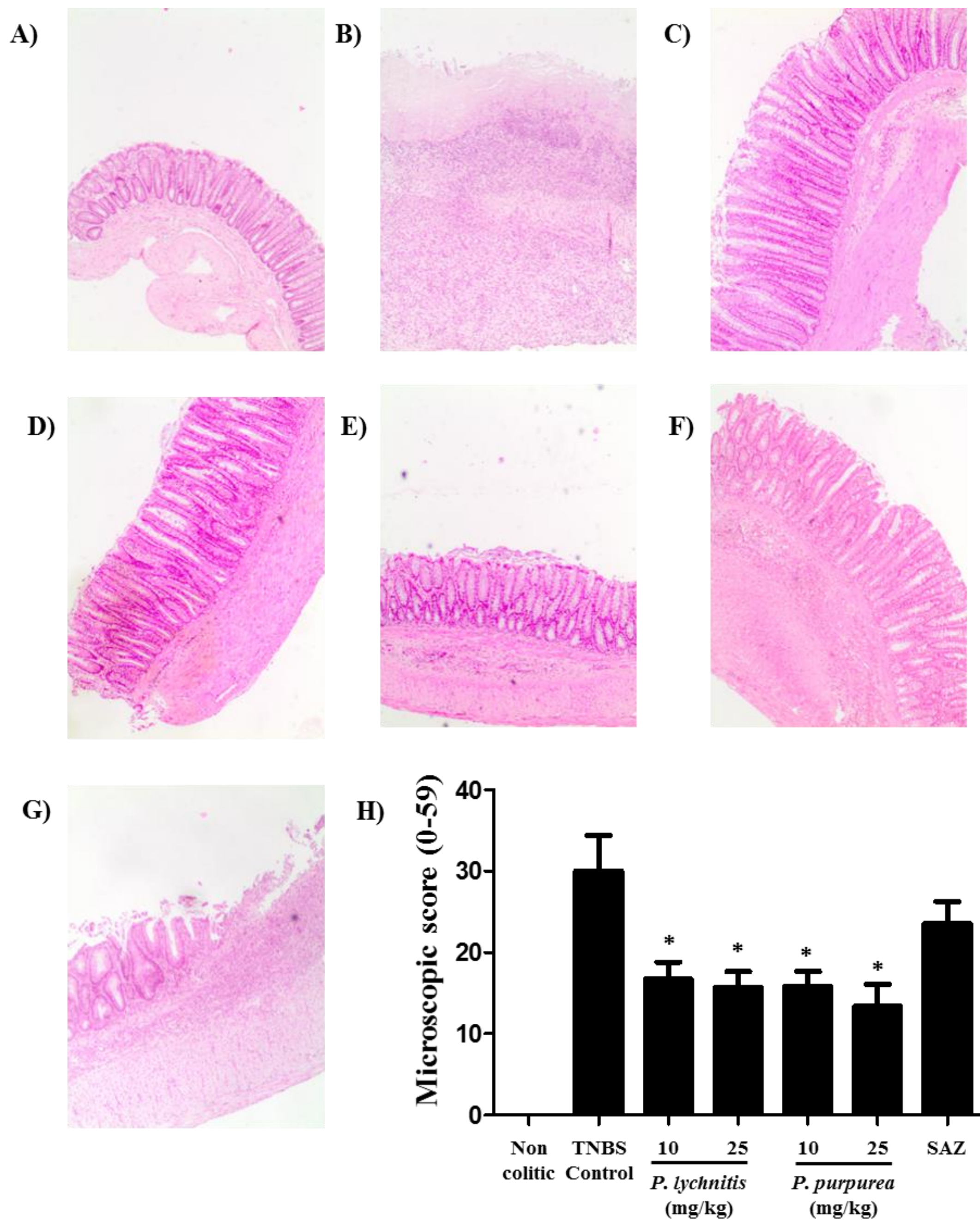


**Figure 20.** Histological sections of colonic tissue stained with hematoxylin and eosin showing the effects of *P. lychnitis* (10 and 25 mg/kg) and *P. purpurea* (10 and 25 mg/kg) extracts, and sulphasalazine (SAZ) (200 mg/kg), in trinitrobenzenesulphonic acid (TNBS) rat colitis: A) Non-colitic; B) TNBS-control; C) *P. lychnitis* (10 mg/kg); D) *P. lychnitis* (25 mg/kg); E) *P. purpurea* (10 mg/kg); F) *P. purpurea* (25 mg/kg); G) sulphasalazine (SAZ) (200 mg/kg) and H) microscopic score assigned according the criteria previously described [17]; Data are expressed as means ± SEM. *P<0.05 *vs.* TNBS control group (n=10).

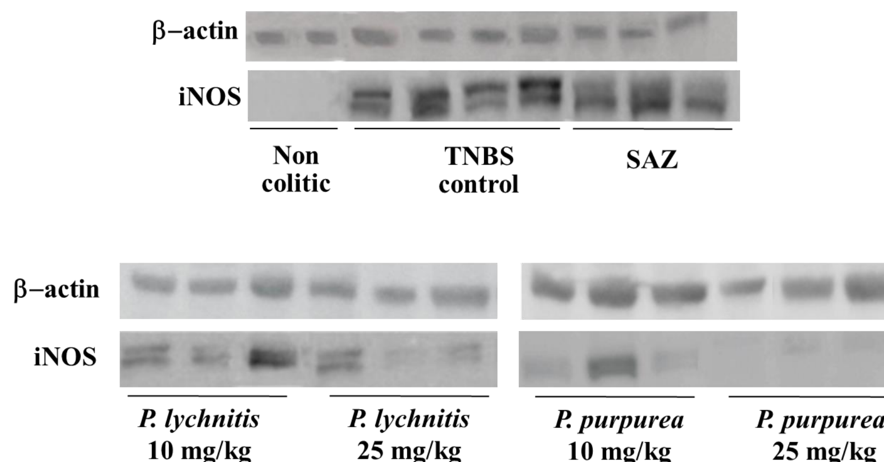


**Figure 21.** Effects of *P. lychnitis* (10 and 25 mg/kg) and *P. purpurea* (10 and 25 mg/kg) extracts, and sulphasalazine (SAZ) (200 mg/kg), on colonic iNOS expression examined by western blot in trinitrobenzenesulphonic acid (TNBS) rat colitis.

Other markers of inflammation, including pro-inflammatory cytokines (IL-1β, TNF-α and IL-17), MCP-1 and Cytokine-induced neutrophil chemoattractant 1 (CINC-1), ICAM-1, as well as proteins involved in epithelial integrity (villin, MUC-2, MUC-3 and trefoil factor (TFF-3)) were analyzed in the colonic samples to better characterize the differences between the intestine anti-inflammatory effect exerted by the two extracts assayed (Figures 22-24). As expected, the expression of the pro-inflammatory cytokines was upregulated in control colitic rats in comparison with the non-colitic group.

Although both extracts exerted an intestine anti-inflammatory effect, only the administration of *P. purpurea* extract was able to reduce the expression of IL-1β (at the dose of 25 mg/kg), both at the mRNA and protein levels, TNF-α (at the dose of 25 mg/kg) and IL-17 (at both doses assayed), indicating an inhibitory effect on the altered immune response that characterizes the colonic inflammatory process (Figure 22).

Similarly, only the treatment of colitic rats with *P. purpurea* extract resulted in a significant reduction of the expression of the chemokines CINC-1 (at the dose of 25 mg/kg) and MCP-1 (at both doses), increased in untreated colitic rats. However, when *P.*

*lychnitis* extract was evaluated only a trend in reducing the expression of MCP-1 was observed, without any statistical difference (Figure 23).

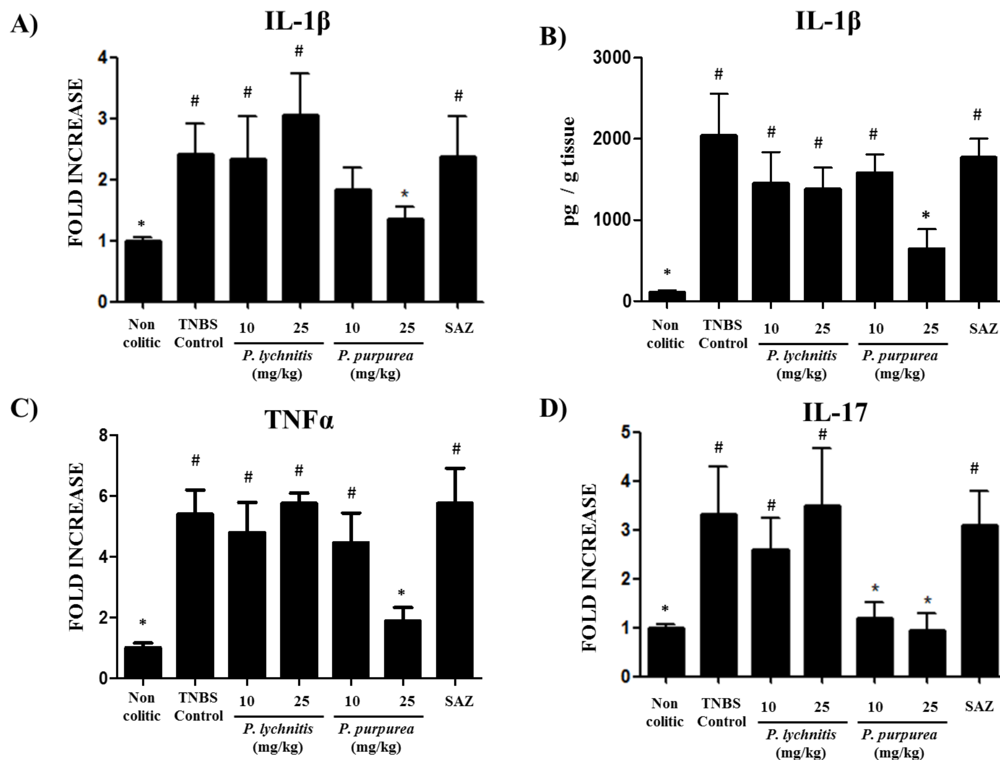


**Figure 22.** Effects of *P. lychnitis* (10 and 25 mg/kg) and *P. purpurea* (10 and 25 mg/kg) extracts, and sulphasalazine (SAZ), on colonic gene expression in trinitrobenzenesulphonic acid (TNBS) rat colitis of the cytokines (A) IL-1β (real-time PCR), (B) IL-1β (ELISA), (C) TNF-α (real-time PCR) and (D) IL-17 (real-time PCR); data are expressed as means ± SEM (n=10); *P<0.05 *vs*. TNBS control group; #P<0.05 *vs*. non-colitic group.

However, when the expression of proteins involved in colonic epithelial integrity was evaluated, both extracts showed a beneficial impact in restoring their significantly reduced expression found in colitic rats compared to the healthy ones. The administration of *P. lychnitis* extract resulted in a significant increased expression of the mucins MUC-2 and MUC-3 in the colonic tissue at both doses assayed, whereas only the highest dose was able to increase villin expression, and TFF-3 expression was not affected. However, when *P. purpurea* extract treatment to colitic rats was considered, all these markers of

epithelial integrity were significantly increased, by both doses when MUC-2 was evaluated, and only by the dose of 25 mg/kg when MUC-3, villin and TFF-3 were analyzed (Figure 24).

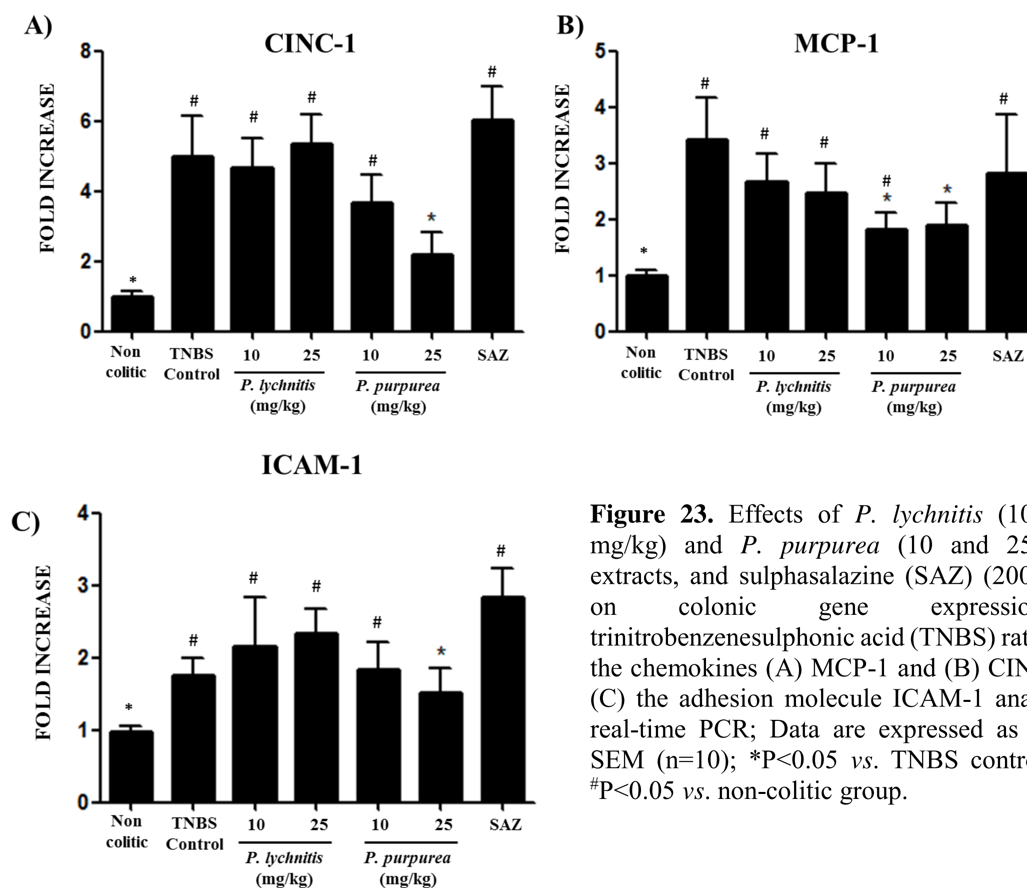


**Figure 23.** Effects of *P. lychnitis* (10 and 25 mg/kg) and *P. purpurea* (10 and 25 mg/kg) extracts, and sulphasalazine (SAZ) (200 mg/kg), on colonic gene expression in trinitrobenzenesulphonic acid (TNBS) rat colitis of the chemokines (A) MCP-1 and (B) CINC-1, and (C) the adhesion molecule ICAM-1 analyzed by real-time PCR; Data are expressed as means ± SEM (n=10); *P<0.05 *vs*. TNBS control group; #P<0.05 *vs*. non-colitic group.

In this study, sulphasalazine was only able to significantly increase the expression of the mucins MUC-2 and MUC-3 in the colonic tissue, in comparison with the corresponding colitic control group, without observing a significant improvement in any of the other markers studied (Figures 22-24)

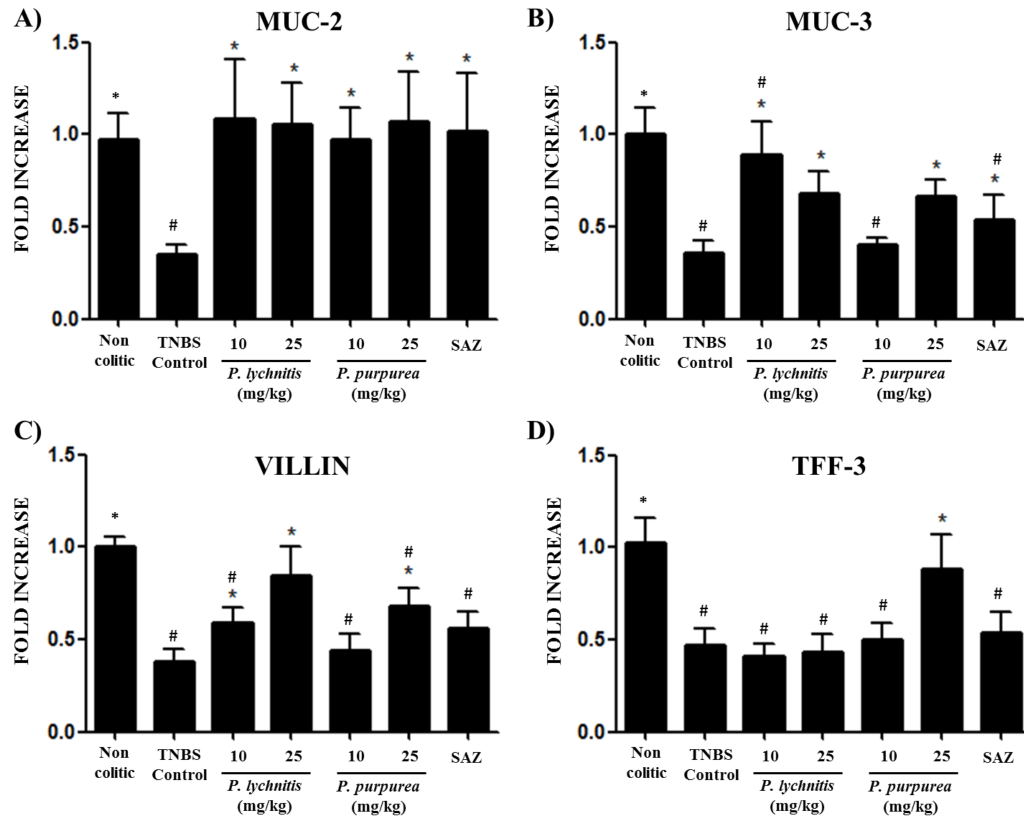


**Figure 24.** Effects of *P. lychnitis* (10 and 25 mg/kg) and *P. purpurea* (10 and 25 mg/kg) extracts, and sulphasalazine (SAZ) (200 mg/kg) on colonic gene expression in trinitrobenzenesulphonic acid (TNBS) rat colitis of the colonic barrier function mediators (A) MUC-2, (B) MUC-3, (C) villin and (D) TFF-3 analyzed by real-time PCR; Data are expressed as means ± SEM (n=10); *P<0.05 *vs.* TNBS control group; #P<0.05 *vs.* non-colitic group.

## 3. *Lavandula* sp. plants

### 3.1. Chemical analysis of *Lavandula dentata* and *Lavandula stoechas* by RP-HPLC-DAD-MS

The qualitative extracts profiles obtained by RP-HPLC-DAD-MS are shown in Figure 25. On the one hand, these figures depict the base peak chromatogram (BPC) in the negative ionization mode obtained by MS, showing regions of a representative chromatogram with compounds with the highest intensity. On the other hand, these figures also show the chromatograms of the extracts at 280 nm, at which most phenolic compounds absorb.

The main phenolic compounds from the ASE extracts of *Lavandula dentata* and *Lavandula stoechas* were characterized on the basis of their global spectrometric data. Confirmation of the phenolic compounds was made through a comparison with standards, whenever these were available in-house. Alternatively, in the absence of standards, a "tentative" characterization procedure was performed. In this sense, the UV-Vis data obtained by the DAD was a valuable tool for classifying phenolic compounds into families and subfamilies, according to the presence of characteristic absorption bands. For example, hydroxycinnamic acid derivatives and flavones have a strong UV band I absorption (from 310-346 nm, approximately) and weaker band II absorption close to 280 nm, whereas hydroxybenzoic acid derivatives have a strong UV band II absorption [389]. In this regard, the wavelengths of maximum absorption for the characterized phenolic compounds are depicted in Table 8. On the other hand, the QTOF mass analyzer delivers accurate mass measurements and isotopic fidelity, which allows the molecular formula of the target compound to be obtained [395]. Therefore, in order to procure confident formula assignments for target molecular ions, the lower mass error value and the higher MS score the better (Table 8). Afterwards, databases as well as literature were consulted for the retrieval of chemical structure information taking the MS and UV-Vis data into account. Finally, using MS/MS spectra, the structure of the parent compound may be tentatively confirmed through studying the fragmentation pattern. In addition, the RT served as criterion of polarity and elution order, which was used for the numbering of the

compounds (Figure 25 and Table 8). As an example, Figure 4 shows the UV, MS spectrum highlighting the deprotonated molecular ion ([M-H]-) and MS/MS spectrum showing the fragment ions for rosmarinic acid (14).

The results showed that Lavandula phenolic compounds eluted between organic acids and triterpenoids (Figure 25). They belonged to three phenolic classes that are widespread in plants: hydroxybenzoic acids (Table 8. Compound n. 2), hydroxycinnamic acids (Table 8. Compounds n. 1, 4, 8, and 13-15) and flavones (Table 8. Compounds n. 3, 5-7, 9-12, and 16), which is a flavonoid subclass. The presence of the caffeic acid dimer rosmarinic acid is characteristic of the family Lamiaceae, and as well flavones [396]. Interestingly, yunnaneic acid F (Table 8. Compound n. 8) and salvianolic acid B (Table 8. Compound n. 15) have only been described in the genus *Salvia* [397]. So, to our knowledge, the occurrence of this type of caffeic acid trimer and tetramer, respectively, is reported here for the first time in the genus *Lavandula.*

In addition, with the exception of salvianolic acid B and isoscutellarein 8-O-glucuronide, the rest of phenolic compounds were present in both extracts, but the chromatographic peaks presented different signal intensities. Preliminarily, that means quantitative differences (Figure 25).

### 3.2. In vitro effects of the extracts on cell activity involved in the immune response

In these assays, different doses of *L. stoechas* and *L. dentata* extracts were evaluated in vitro in different cell types involved in the immune response: epithelial cells (CMT-93 and Caco-2 cells), primary bone marrow-derived macrophages (BMDM), peripheral blood monocyte cells (PBMCs) and monocyte derived dendritic cells (moDCs). Cell viability was not affected by any of the doses assayed for both plant extracts (data not shown).

**Table 8.** Main phenolic compounds characterized in *L. dentata* (LD) and *L. stoechas* (LS) extracts

| Nº | RT (min) | $[M-H]^-$ | Formula | Score | Error (ppm) | UV (nm) | Main fragments *via* MS/MS | Proposed compound | Presence LD | LS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Hydroxybenzoic acids | | | |
| 2 | 4.58 | 325.0936 | C1518O8 | 97.5 | -1.99 | 264, 298, 305 | 163.0396, 119.0502 | Coumaric acid hexoside 1[a] | + | + |
| | | | | | | | Hydroxycinnamic acids and derivatives | | | |
| 1 | 2.38 | 197.046 | C9H10O5 | 98.3 | -2.16 | 280 | 135.0450, 123.0450, 72.9931 | 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid[a] | + | + |
| 4 | 6.83 | 327.1097 | C15H20O8 | 93.7 | -3.34 | 270 | 165.0551, 147.0446, 121.0658, 106.0416 | Hydroxy hydrocinnamic acid glucoside[b] | + | + |
| 8 | 10.21 | 597.1255 | C29H26O14 | 98.6 | -0.56 | 275, 329 | 329.1011, 311.0904, 267.1005, 197.0438, 179.0332, 135.0434 | Yunnaneic acid F[a] | + | + |
| 13 | 15.38 | 193.0512 | C10H10O4 | 98.3 | -2.98 | 327 | 178.0278, 161.0248, 133.0298 | Methyl caffeate[a] | + | + |
| 14 | 15.57 | 359.0783 | C18H16O8 | 96.9 | -2.43 | 288, 329 | 197.0436, 179.0328, 161.0223, 135.0430 | Rosmarinic acid[c] | + | + |
| 15 | 17.40 | 717.1468 | C36H30O16 | 99.0 | -0.79 | 286, 310 | 519.0403, 339.0502, 321.0403, 295.0600, 197.0441, 185.0232 | Salvianolic acid B[a] | - | + |
| | | | | | | | Flavonoids (flavones) | | | |
| 3 | 6.32 | 593.1515 | C27H30O15 | 98.9 | -0.67 | 270, 340 | 503.1182, 473.1080, 383.0760, 353.0654 | Apigenin di-*C*-hexoside[c] | + | + |
| 5 | 9.78 | 637.1056 | C27H26O18 | 98.1 | -1.45 | 268, 341 | 461.0730, 285.0403, 175.0239, 133.0278, 113.0234 | Luteolin 7,4'-di-glucuronide[a] | + | + |
| 6 | 9.97 | 431.0988 | C21H20O10 | 99.2 | -0.91 | 268, 338 | 341.0649, 311.0546, 283.0592, 269.0431, 117.0325 | Apigenin *C*-hexoside isomer 1[c] | + | + |
| 7 | 10.15 | 431.0989 | C21H20O10 | 99.0 | -0.95 | 271, 338 | 341.0668, 311.0561, 283.0610, 269.0459, 117.0344 | Apigenin *C*-hexoside isomer 2[c] | + | + |
| 9 | 10.70 | 461.0724 | C21H18O12 | 98.7 | 0.44 | 334 | 285.0405, 166.9982, 151.0333, 117.0194, 113.0240 | Isoscutellarein 8-*O*-glucuronide[a] | + | - |
| 10 | 10.88 | 461.0728 | C21H18O12 | 99.1 | -0.45 | 269, 346 | 285.0412, 151.0037, 133.0295, 113.0240 | Luteolin 7-*O*-glucuronide[c] | + | + |
| 11 | 11.06 | 447.093 | C21H20O11 | 98.5 | 0.85 | 346 | 285.0383, 256.0349, 151.0011, 133.0269, 107.0113 | Luteolin 7-*O*-glucoside[c] | + | + |
| 12 | 14.34 | 431.0982 | C21H20O10 | 99.3 | 0.38 | 267, 337 | 268.0363, 239.0327, 151.0021, 117.0326, 107.0128 | Apigenin 7-*O*-glucoside[c] | + | + |
| 16 | 26.57 | 283.0626 | C16H12O5 | 93.2 | -4.50 | 267, 334 | 268.0379, 240.0422, 151.0033, 117.0343 | Genkwanin[c] | + | + |

+: detected; -: not detected.

[a]These compounds were reported here in *L. dentata* and *L. stoechas* for the first time, but previously described in other Laminacee species [396-400].
[b]This compound was reported in the family of *Ranuculaceae* [401].
[c]These compounds were reported in *L. dentata* and/or *L. stoechas* before [396, 401].

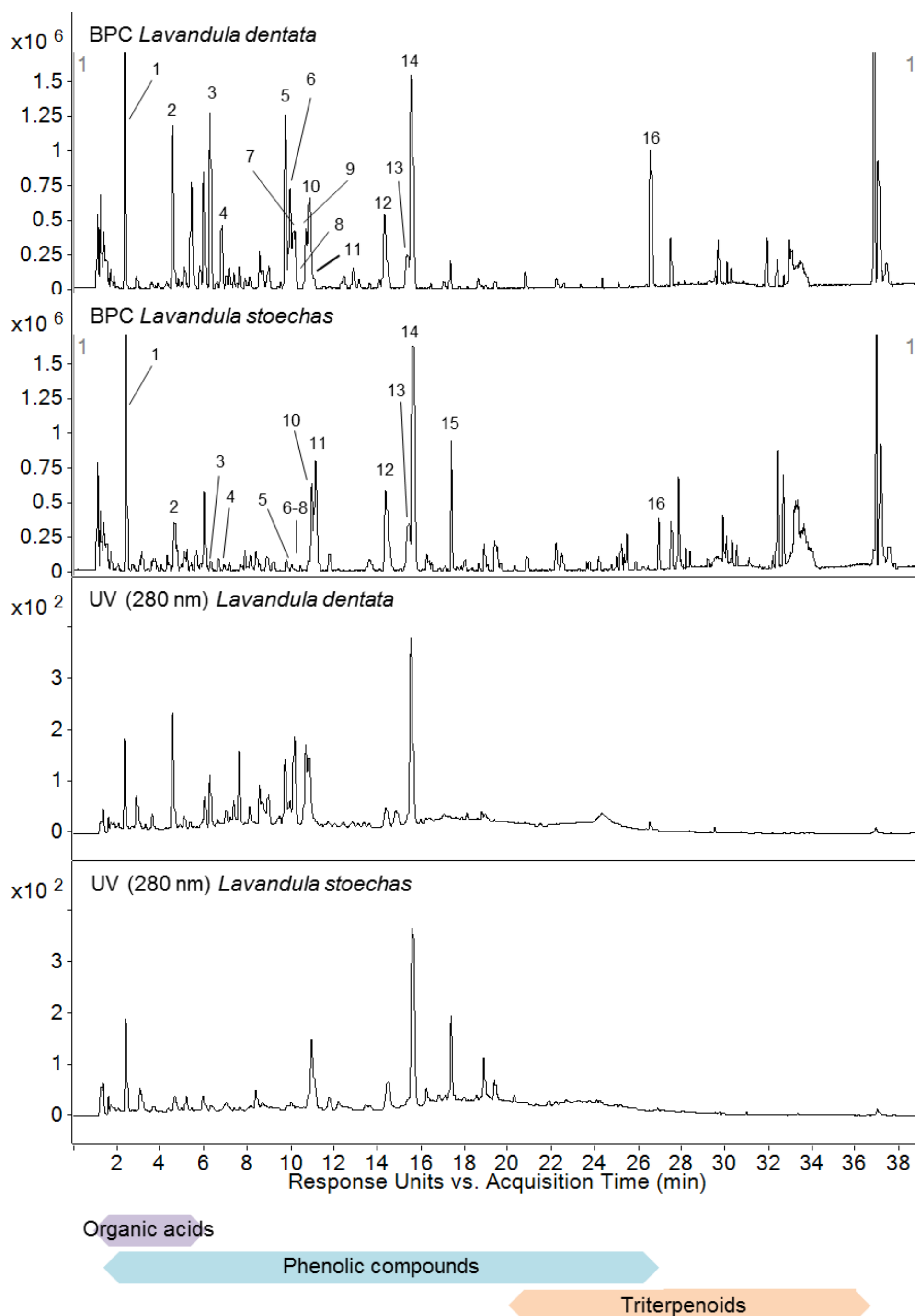


**Figure 25.** A) Base peak chromatogram (BPC) obtained by reversed-phase (RP) high-performance liquid chromatography (HPLC) coupled to diode array detector (DAD) and high-resolution mass spectrometry (MS) of *L. dentata* extract. B) BPC obtained from RP-HPLC-DAD-MS of *L. stoechas* extract. C) The UV spectra of *L. dentata* extract. D) The UV spectra of *L. stoechas* extract.

## BMDM

BMDM represent a homogenous, non transformed population of macrophages. After their incubation with LPS for 24 h, an increased release of the cytokines IL-6 and nitric NO was observed in the culture medium (Figure 26).

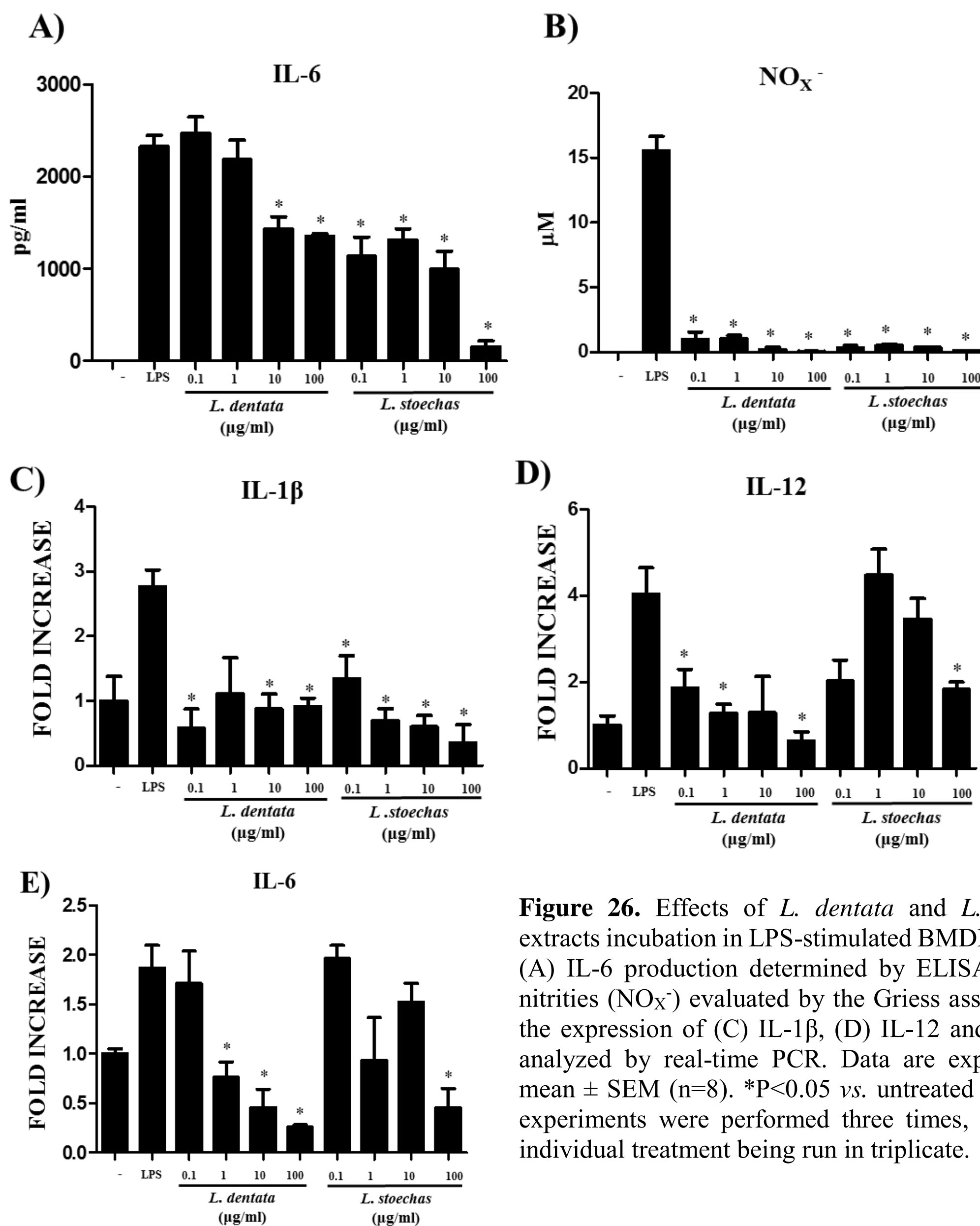


**Figure 26.** Effects of *L. dentata* and *L. stoechas* extracts incubation in LPS-stimulated BMDM cells on (A) IL-6 production determined by ELISA and (B) nitrities ($NO_X^-$) evaluated by the Griess assay and on the expression of (C) IL-1β, (D) IL-12 and (E) IL-6 analyzed by real-time PCR. Data are expressed as mean ± SEM (n=8). *P<0.05 *vs.* untreated cells. The experiments were performed three times, with each individual treatment being run in triplicate.

The incubation of these cells with the extracts of *L. dentata* or *L. stoechas* inhibited the LPS activated production of NO at all concentrations assayed, with a similar efficacy for both plants. When IL-6 production was considered, both extracts reduced the release of the cytokine. However, *L. stoechas* showed a higher efficacy than *L. dentata*, since this inhibitory effect was observed with all the concentrations of *L. stoechas* assayed, whereas only 10 and 100 mg/ml of *L. dentata* showed significant effect (Figure 26). Similarly, the mRNA expressions of IL-1β, IL-6 and IL-12 were enhanced in LPS-stimulated BMDM, which were significantly reduced after incubation with the extracts (Figure 26). All the doses assayed of both *L. stoechas* or *L. dentata* extracts showed a similar inhibitory efficacy when IL-1β expression was evaluated; however, the extract of *L. dentata*, at concentrations of 1, 10 and 100 μg/ml, inhibited the expression of both IL-6 and IL-12 in these stimulated cells, whereas *L. stoechas* showed significant inhibitory effect of the expression of these cytokines only when the highest dose was assayed.

**CMT-93**

Mouse rectum carcinoma cell line CMT-93 were used like a model of intestinal epithelial cells. The incubation of CMT-93 cells with LPS increased the release of the pro-inflammatory cytokines IL-6 and TNF-α (Figure 27).

When these cells were previously incubated with different doses of the extracts from *L. stoechas* or *L. dentata*, the increased production of TNF-α was significantly reduced dose-dependently. Similarly, both extracts were able to inhibit the increased IL-6 production, but although *L. dentata* only showed significant inhibitory effect at the concentration of 100 μg/ml, *L. stoechas* reduced this production at the doses of 10 and 100 μg/ml. The qPCR analysis of the mRNA expression of different inflammatory markers in these cells corroborated these results.

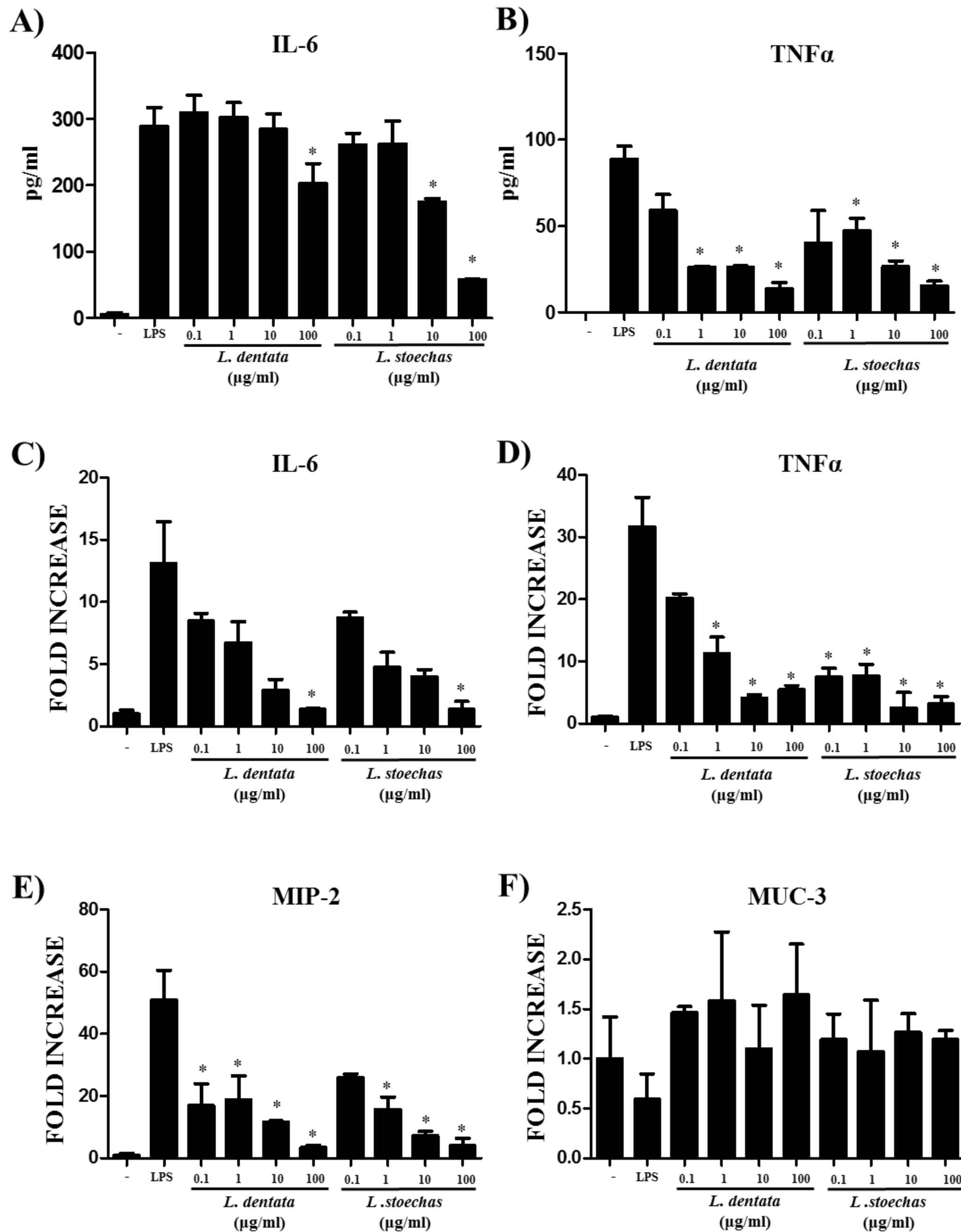


**Figure 27.** Effects of *L. dentata* and *L. stoechas* extracts incubation in LPS-stimulated CMT-93 cells on (A) IL-6 and (B) TNF-α production, determined by ELISA, and on the expression of (C) IL-6, (D) TNF-α, (E) the chemokine MIP-2 and (F) the mucin MUC-3, analyzed by real-time PCR. Data are expressed as mean ± SEM (n=8). *P<0.05 *vs.* untreated cells. The experiments were performed three times, with each individual treatment being run in triplicate.

Thus, the mRNA expressions of IL-6, TNF-α and MIP-2 were increased in LPS-stimulated epithelial cells compared with the non-stimulated cells, whereas MUC-3 expression trended to diminish. Both extracts were able to reduce the IL-6 expression, although only the highest concentration assayed (100 µg/ml) reached statistical significance. Correspondingly, TNF-α expression was also reduced, at all the concentrations of both extracts, except for the lowest of *L. dentata*. When MIP-2 expression was evaluated, the different concentrations tested, excepting the lowest one (0.1 µg/ml), showed an inhibitory effect. Finally, the incorporation of the extracts to the cell cultures resulted in a trend to increase the expression of MUC-3 compared with LPS-treated cells, although no statistical differences were observed.

### Caco-2

The human colon adenocarcinoma cell line Caco-2 was used as another model of intestinal epithelial cells. Incubation of these cells with IL-1β induces the secretion of IL-8, a pro-inflammatory chemokine that is released by intestinal epithelial cells and increases inflammatory cells migration from blood stream into the mucosa and submucosa during chronic IBD, enhancing intestinal tissue destruction [402].

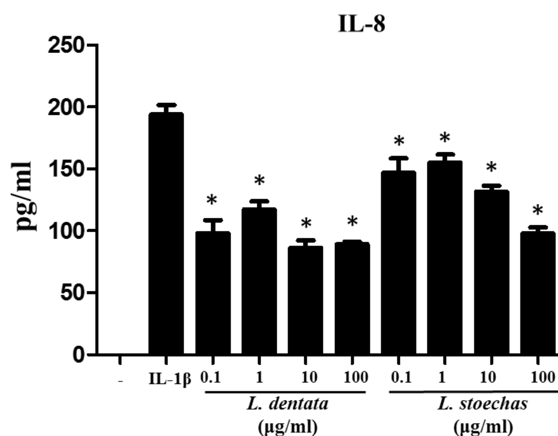


**Figure 28.** Effect of *L. dentata* and *L. stoechas* on IL-8 production in Caco-2 cells stimulated with IL-1β (1ng/ml). Data are expressed as means ± SEM. *P<0.005 *vs.* untreated cells. The experiments were performed three times, with each individual treatment being run in triplicate.

The pre-treatment of these cells with *L. dentata* or *L. stoechas* resulted in a reduction of IL-8 production significantly when compared with the IL-1β-stimulated cells without extract (Figure 28).

**PBMCs**

PBMCs include lymphocytes T, lymphocytes B, NK cells, monocytes, and dendritic cells. The stimulation of human PBMCs, isolated from blood of donors, with LPS during 24 hours resulted in an increased production of pro-inflammatory mediators, such as subunit beta of interleukin 12 (IL-12p40) and TNF-α. When these cells were treated with *L. dentata* or *L. stoechas* extracts, the production of these pro-inflammatory cytokines was reduced dose-dependently. The treatment with *L. dentata* was able to significantly reduce IL-12p40 levels at all concentrations assayed, whereas the significantly reduction of TNF-α production was only obtained at 1 and 10 μg/ml concentrations. Similarly, *L. stoechas* was able to significantly reduce TNF-α levels at all concentrations tested, and IL-12p40 production at 0.1, 1 and 10 μg/ml concentrations (Figure 29).

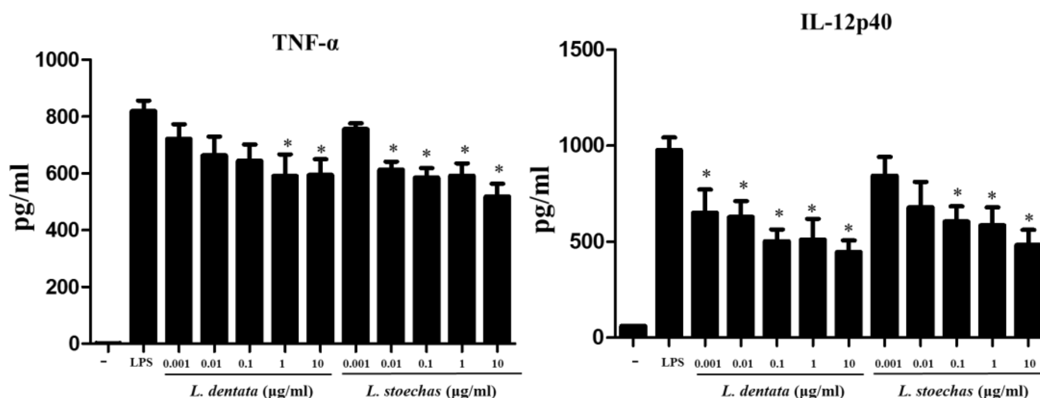


**Figure 29.** Effect of *L. dentata* and *L. stoechas* on pro-inflammatory cytokines, TNF-α and IL-12p40, in PBMCs stimulated with LPS at 100ng/ml. Data are expressed as means ± SEM. *P<0.005 *vs*. untreated cells. The experiments were performed three times, with each individual treatment being run in triplicate.

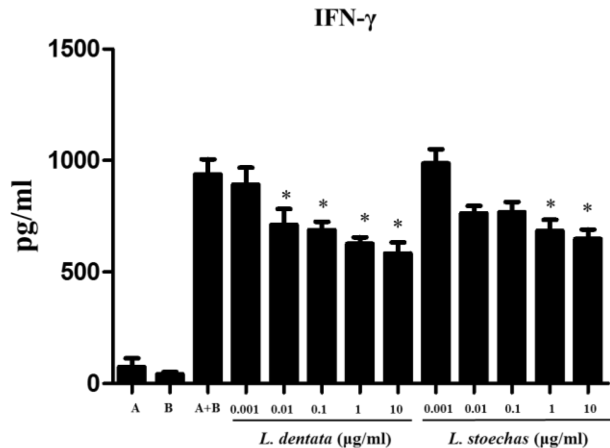


**Figure 30.** IFN-γ production in a bidirectional mixed lymphocyte reaction in the presence of *L. dentata* and *L. stoechas*. Data are expressed as means ± SEM. *P<0.005 *vs.* untreated cells. The experiments were performed three times, with each individual treatment being run in triplicate.

When lymphocytes from genetically different individuals are mixed together in tissue culture blast transformation occurs, a reaction known as the mixed lymphocyte reaction (MLR). Both extracts were tested in a co-colture of PBMCs from different donors. As result of bidirectional mixed lymphocyte reaction, there was an increase in IFN-γ production that was reduced in a dose-dependent manner when the extracts were added to the culture media. *L. dentata* was able to significantly reduce the production of IFN-γ at all concentration assayed, except to the lowest one; whereas *L. stoechas* did it only at the highest concentrations (1 and 10 µg/ml) (Figure 30).

#### MoDCs

MoDCs were derived by monocyte isolated from human blood. MoDCs were incubated with both extracts and then stimulated with live *Salmonella typhimurium* (FB62), which has been reported to induce a strong inflammatory response in DCs [403]. As shown in Figure 31, the incubation of these cells with FB62 drastically increased the production of the pro-inflammatory cytokine IL-12p70, which was significantly reduced when the extracts of *L. dentata* or *L. stoechas* were added to the culture media, being the highest concentrations the most effective for both extracts.

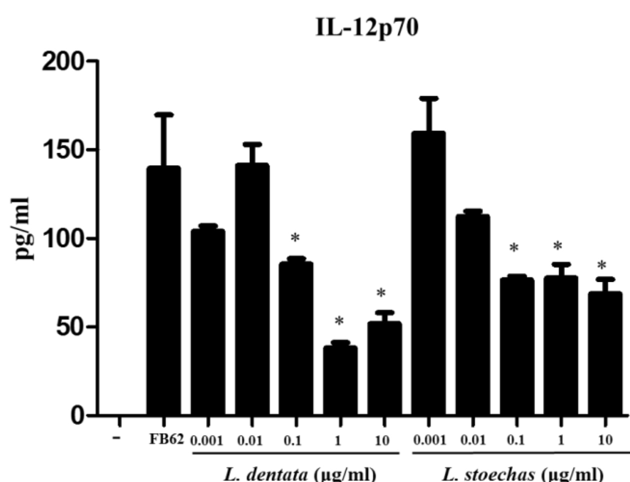


**Figure 31.** IL-12p70 secretion by moDCs conditioned with *L. dentata* and *L. stoechas* and then infected with *Salmonella typhimurium* (FB62). Data are expressed as means ± SEM. *P<0.005 *vs.* untreated cells. The experiments were performed three times, which each individual treatment being run in triplicate.

Then, the co-stimulatory molecules of these cells were analysed in the same experimental conditions. The stimulation with FB62 resulted in an increased presence of the co-stimulatory molecules CD80, CD83 and HLA-DR. When these cells were incubated with both extracts, they were able to reduce the expression of these molecules significantly at the highest concentration used in the previous studies (10 µg/ml). In fact, *L. dentata* seemed to restore these levels to the normal values (Figure 32).

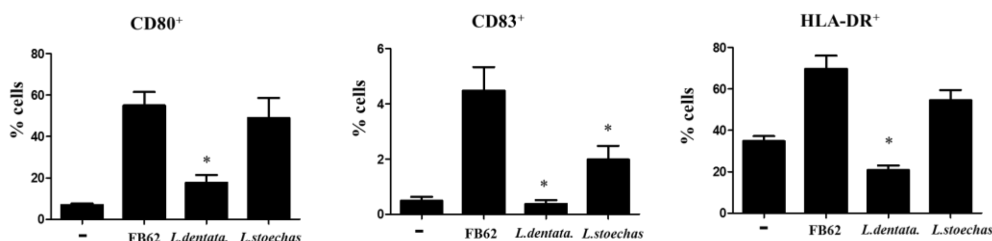


**Figure 32.** The expression of co-stimulatory molecules CD80, CD86 and HLA-DR on moDC surface. The cells were treated with *L. dentata* and *L. stoechas* and then infected with *Salmonella thyphimurium* (FB62). Data are expressed as means ± SEM. *P<0.005 *vs.* untreated cells. The experiments were performed three times, which each individual treatment being run in triplicate.

### 3.3. Antiradical activity of the extracts

The extracts of *L. dentata* and *L. stoechas* showed similar antiradical effect in the DPPH assay, being their IC50 0.07 ± 0.008 and 0.06 ± 0.006 mg/ml, respectively.

### 3.4. Intestinal anti-inflammatory effect of the extracts in the TNBS model of rat colitis

In the colitic control group, and accordingly to that described above, the intrarectal instillation of TNBS induced severe necrosis and inflammation of the colonic mucosa, typically extending 4 - 5 cm along the colon (Table 9). This was accompanied by bowel wall thickening, hyperemia and focal adhesions to adjacent organs. The treatment of colitic rats with the extracts of *L. dentata* or *L. stoechas* significantly ameliorated the colonic damage when compared with the colitic control group. This beneficial effect was evidenced macroscopically by a significant reduction, of approximately 1-2 cm, in the extension of inflamed/necrotic tissue of the colonic segments, thus being assigned a lower macroscopic score than in untreated control rats (Table 9). In addition, this intestinal anti-inflammatory effect was associated, in some of the treated groups (*L. dentata* at 10 mg/kg and *L. stoechas* at 25 mg/kg), with a significant reduction in the colonic weight/length ratio in comparison with untreated colitic control rats (Table 9). The treatment of colitic rats with dexamethasone (1.2 mg/kg) resulted in a significant reduction of the colonic damage score as well as the weight/length ratio when compared with control colitic rats, thus revealing the intestinal anti-inflammatory effect exerted by the glucocorticoid. The beneficial effects observed macroscopically with the different treatments were also corroborated biochemically. In this sense, TNBS promoted an intense neutrophil infiltration, as revealed by the higher colonic MPO activity observed in control colitic rats in comparison with the healthy control group. This enzyme activity was significantly reduced by 60 – 70 % after treatment with the lower doses of both extracts, which revealed a significant decrease of the massive neutrophil infiltration that takes place into the inflamed tissue in untreated colitic rats (Table 9).

**Table 9.** Effects of different doses of *Lavandula dentata* and *Lavandula stoechas* extracts on macroscopic damage score, weight/length ratio, myeloperoxidase (MPO) activity and glutathione (GSH) content in TNBS rats experimental colitis.

| Group (n=10) | Damage Score (0-10) | Weight/Length ratio (mg/cm) | MPO (Um/g tissue) | GSH (nmol/g tissue) |
|---|---|---|---|---|
| Non-colitic | 0 | 68.2 ± 1.8 | 31.7 ± 3.3 | 2071 ± 140.9 |
| TNBS control | 7.6 ± 0.3 | 221.7 ± 17.9 | 1017 ± 145.4 | 500.5 ± 47.9 |
| *L. dentata* (10 mg/kg) | 5.2 ± 0.5* | 115.4 ± 10.2* | 300.1 ± 106* | 1598 ± 205.8* |
| *L. dentata* (25 mg/kg) | 6.5 ± 0.4* | 181.8 ± 28.3 | 670 ± 125.1 | 893.3 ± 240.2* |
| *L. stoechas* (10 mg/kg) | 6.7 ± 0.4 | 168.3 ± 14.1 | 381.2 ± 120* | 676.5 ± 175.1 |
| *L. stoechas* (25 mg/kg) | 5.3 ± 0.4* | 125.3 ± 23.3* | 970 ± 180.6 | 1073 ± 300.5* |
| Dexamethasone (1.2 mg/kg) | 5.5 ± 0.6* | 90 ± 9.1* | 237.7 ± 68.4* | 1559 ± 153.2* |

Data are expressed as mean ± SEM. *$P<0.05$ *vs.* TNBS control group

Furthermore, the depletion of colonic glutathione content observed in control colitic rats was partially prevented after treatment with either *L. dentata* or *L. stoechas*, at both doses assayed, which may result in the protection of the intestine tissue from the oxidative insult that occurs in TNBS-rat colitis (Table 9). The beneficial effects observed after dexamethasone treatment to colitic rats were also associated with significant decreased of colonic MPO activity and increased GSH content (Table 9).

The intestine anti-inflammatory activity observed with both extracts was also corroborated when the pro-inflammatory inducible enzyme iNOS was evaluated by western blotting. In comparison with non-colitic animals, TNBS-induced colitis promoted an increased expression of this enzyme in the inflamed colonic tissue, which has been associated with the deleterious effects that NO over-production has on intestinal function [17].

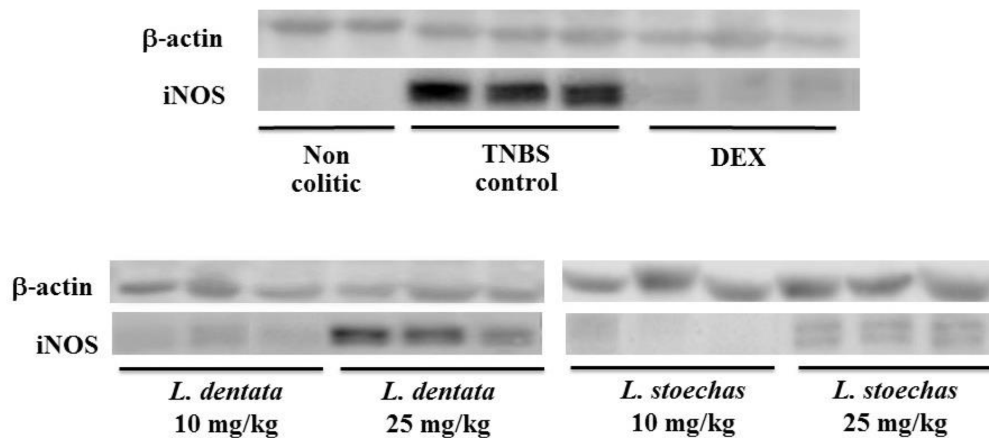


**Figure 33.** Effects of *L. dentata* (10 and 25 mg/kg) and *L. stoechas* (10 and 25 mg/kg) extracts, and dexamethasone (DEX) (1.2 mg/kg), on colonic iNOS expression in trinitrobenzenesulphonic acid (TNBS) rat colitis determined by western blot.

The treatments with *L. dentata* (at both doses) and *L. stoechas* (at 10 mg/kg) were able to down-regulate the colonic iNOS expression, similarly to that achieved with the administration of dexamethasone (Figure 33). The qPCR analysis of the mRNA expression of different inflammatory markers in the colonic segments revealed that the colonic inflammation induced by TNBS in rats was characterized by an increased expression of the pro-inflammatory cytokines IL-1β, IL-17 and IL-6, the chemokine MCP-1, the adhesion molecule ICAM-1 (Figure 34), as well as by decreased expression of some biochemical markers of the epithelial integrity, including TFF-3 and MUC-3 (Figure 35). The administration to colitic rats of both extracts, *L. dentata* and *L. stoechas*, resulted in the amelioration in most of these markers, confirming the intestinal anti-inflammatory effect evidenced in this experimental model of rat colitis, although they did no display the same profile. Thus, IL-1β expression was significantly reduced with both extracts (at both doses assayed) (Figure 34); however, whereas *L. dentata* reduced the expression of IL-6 and ICAM-1, *L. stoechas* was able to inhibit the expression of IL-17 and MCP-1 (Figures 34).

It is interesting to note that, in most of the cases, when the inhibitory effect on the expression was obtained, this was similar to the effect obtained with the glucocorticoid dexamethasone (Figures 34).

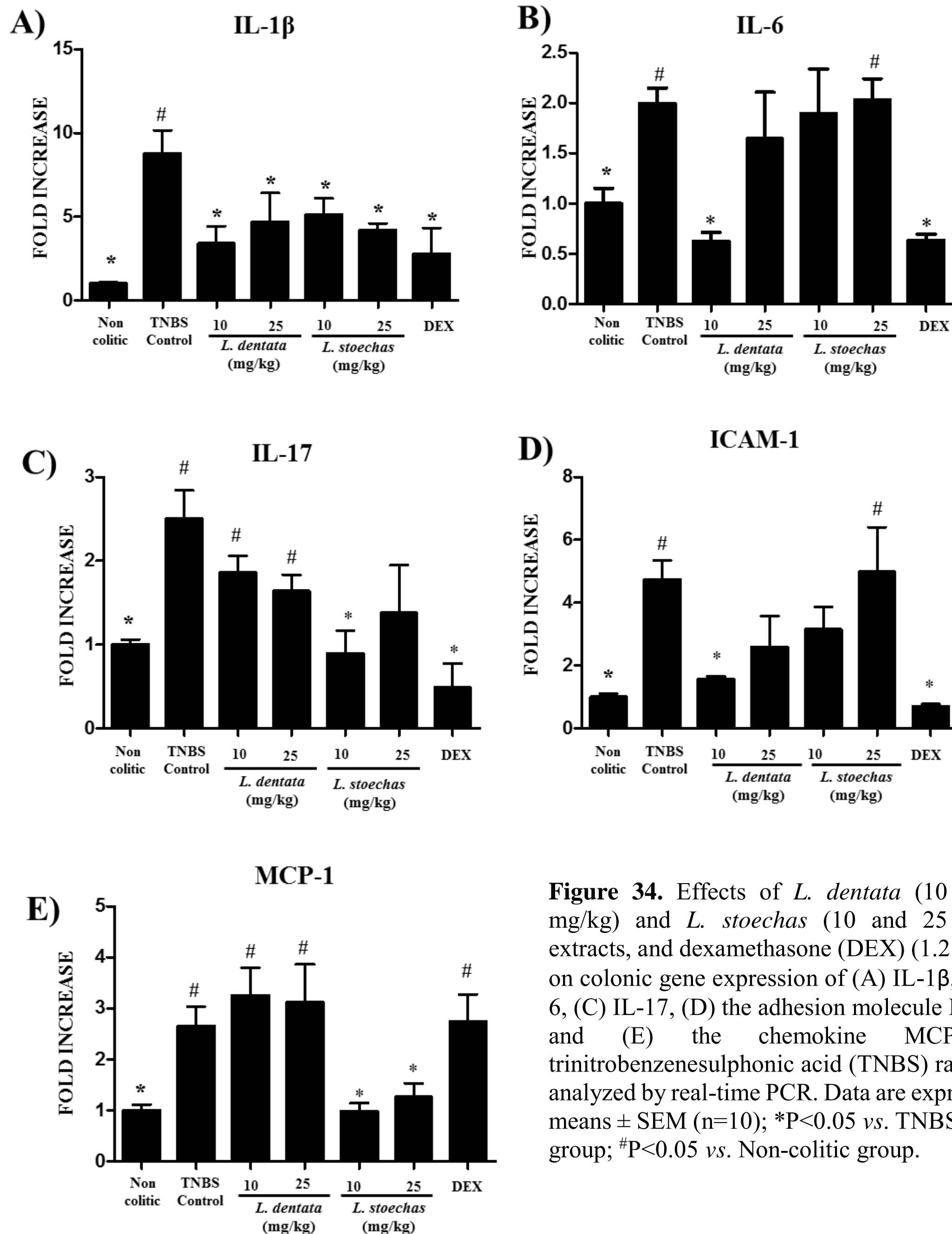


**Figure 34.** Effects of *L. dentata* (10 and 25 mg/kg) and *L. stoechas* (10 and 25 mg/kg) extracts, and dexamethasone (DEX) (1.2 mg/kg), on colonic gene expression of (A) IL-1β, (B) IL-6, (C) IL-17, (D) the adhesion molecule ICAM-1 and (E) the chemokine MCP-1 in trinitrobenzenesulphonic acid (TNBS) rat colitis, analyzed by real-time PCR. Data are expressed as means ± SEM (n=10); *P<0.05 *vs*. TNBS control group; #P<0.05 *vs*. Non-colitic group.

Furthermore, the biochemical markers of colonic barrier function were up-regulated in the colitic rats treated with the extracts: the mucin MUC-3 (both extracts at 10 mg/kg) and TFF-3 (*L. dentata* at both doses); this effect was also observed after treatment of colitic rats with dexamethasone (Figure 35).

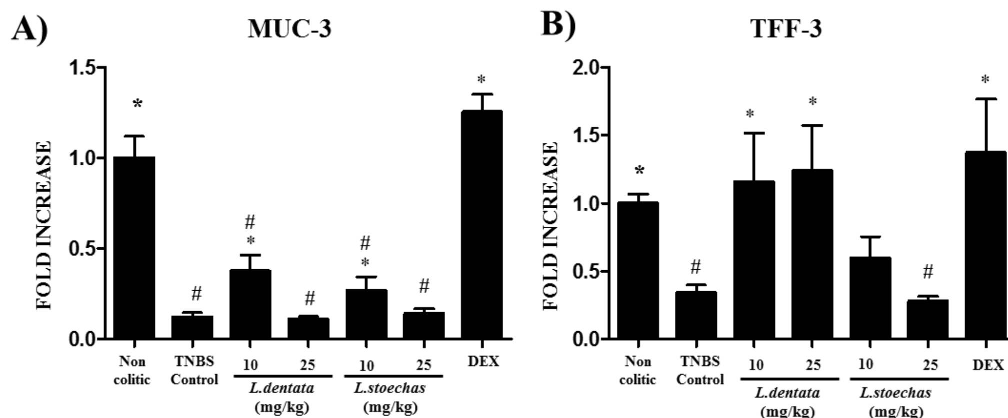


**Figure 35.** Effects of *L. dentata* (10 and 25 mg/kg) and *L. stoechas* (10 and 25 mg/kg) extracts, and dexamethasone (DEX) (1.2 mg/kg), on colonic gene expression of the colonic barrier function mediators (A) MUC-3 and (B) TFF-3 in trinitrobenzenesulphonic acid (TNBS) rat colitis**,** analyzed by real-time PCR. Data are expressed as means ± SEM (n=10); *P<0.05 *vs*. TNBS control group; $^{\#}$P<0.05 vs. Non-colitic group.

## 3.5. Effect of *Lavandula dentata* and *Lavandula stoechas* extracts administration on DSS induced mouse colitis

Additional experiments were performed in order to study in more detail the intestinal anti-inflammatory effect of both *L. dentata* and *L. stoechas* observed in the TNBS model of rat colitis. With this aim, these extracts were evaluated in the acute phase of DSS colitis in mice, a well established model with resemblance to human UC [404]. The extracts were administered to colitic mice for one week after the removal of DSS from drinking water. Although any of the extracts did not reduce DAI index (Figure 36A), the macroscopic evaluation of the colonic segments showed their beneficial effects, since *L. dentata* and *L. stoechas* significantly reduced the colonic weight/length ratio in comparison with untreated control colitic mice (Figure 36B). As commented before in the TNBS model of rat colitis, a correlation has been reported to exist between this ratio and the severity of the colonic damage in this experimental model. [376] (Figure 36B).

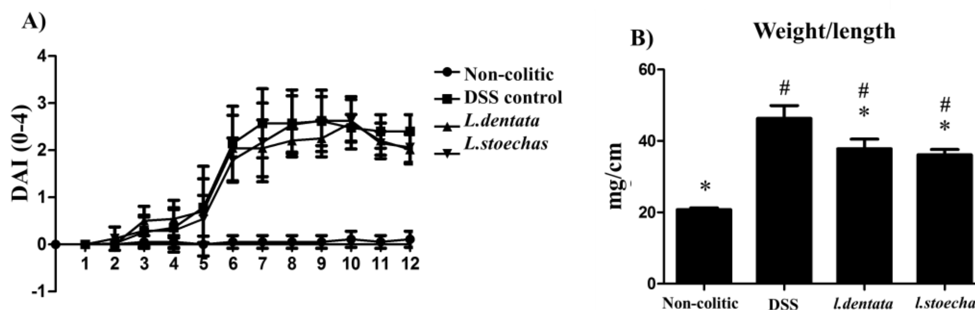


**Figure 36.** Effect of *L. dentata* and L. stoechas on dextrane sodium sulfate (DSS) mice colitis. A) Disease activity index (DAI) value over 12-day of experimental period, based on criteria proposed previously [17]. B) Colonic weight/length ratio, expressed as mean ±SEM, *P<0.05 *vs*. DSS control colitic group. $^{\#}$P<0.05 *vs*. Non-colitic group.

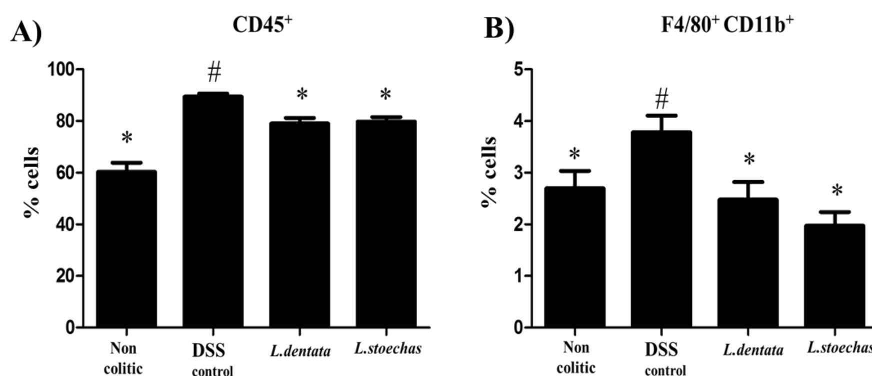


**Figure 37.** Effect of *L. dentata* and *L. stoechas* treatment on proportion of $CD45^+$ and $CD11b^+F4/80^+$ cells in colon of mice in dextran sodium sulfate (DSS) model of colitis. Data are expressed as means ± SEM (n-8); *P<0.05 *vs*. Control group; $^{\#}$P<0.05 *vs.* Healthy group

The analysis of the cells from the *lamina propria* by flow cytometry with the following gating strategy revelaed that the inflamed colon was associated with an intense infiltration of leukocytes ($CD45^+$ cells) in comparison with a non-colitic one.

This infiltration was reduced with both treatments significantly (Figure 36B) and was characterized by marked increase in proportion of $CD45^+$ (Figure37A) cells and in particular $CD11b^+$ $F4/80^+$cells (figure 37B) that was reduced in mice treated with *L. dentata* or *L. stoechas*.

$CD45^+CX3CR1^+$ cells were selected from *lamina propria* live cells. DCs ($CD11c^+$ $F4/80^-$) were excluded from this population. This population represent the pool of

monocyte and intestinal macrophages (Figure 38A). It is possible to distinguish two different populations: CD11b$^{+}$ F4/80$^{+}$ and CD11b$^{+}$F4/80$^{low}$ (Figure 38B). The first one is a macrophage population characterized by Ly6C$^{-}$ MHCII$^{+}$ phenotype. The second one is a heterogeneous population that represents stages in a single differentiation continuum from Ly6C$^{+}$ monocytes to mature LyC6$^{-}$ MHCII$^{+}$ macrophages (figure 38B), these cells are similar to Ly6C$^{+}$ inflammatory monocytes in the blood.

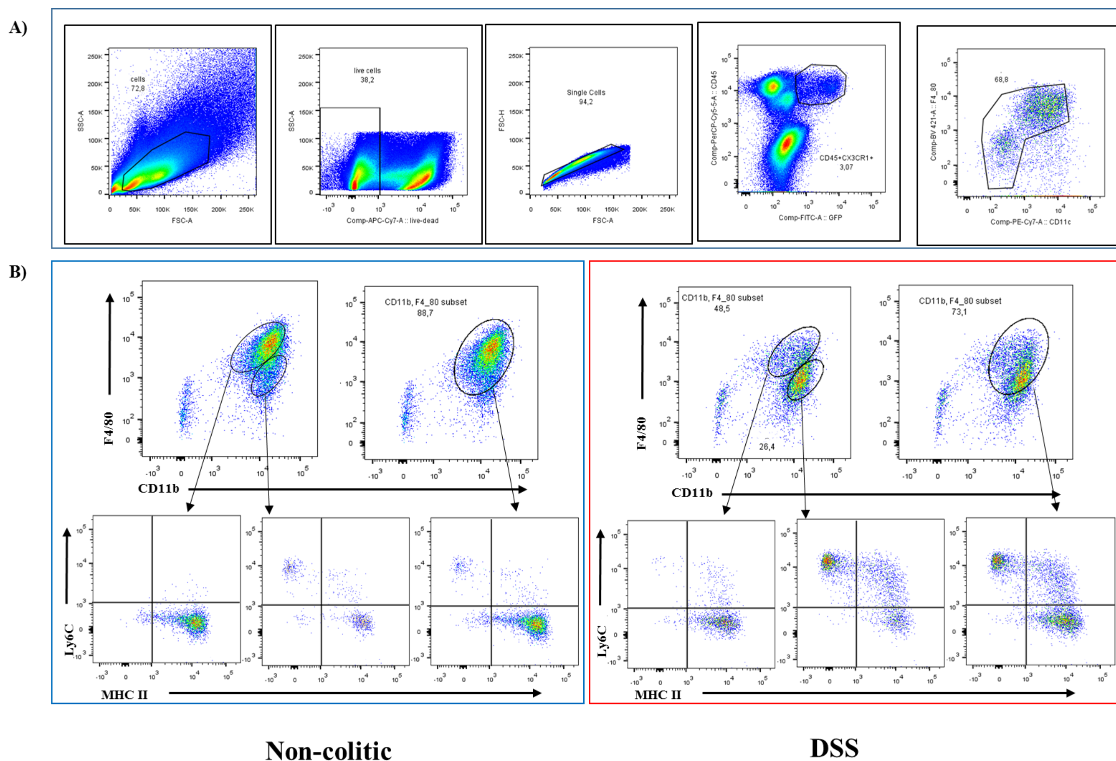


**Figure 38.** Effect of inflammation on composition of colonic myeloid cells. A) Gating strategy used to identify CD11b$^{+}$ F4/80$^{+/low}$ cells. B) Representative expression of Ly6C and MHCII on CD11$^{+}$F4/80$^{+/low}$ cells in no-colitic and colitic mice.

When these populations were analyzed together, on the basis of their expression of LyC6 and MHCII, it was possible to observe that a high proportion of mature macrophages in non-colitic mice was present, whereas in colitic group there was an accumulation of cell expressing Ly6C in different phases of differentiation and obviously a reduced number of Ly6C$^{-}$ MHCII$^{+}$ (Figure 38B). Therefore, the inflammatory process

interferes with the differentiation of intestinal macrophages that play a key role in maintenance of homeostasis.

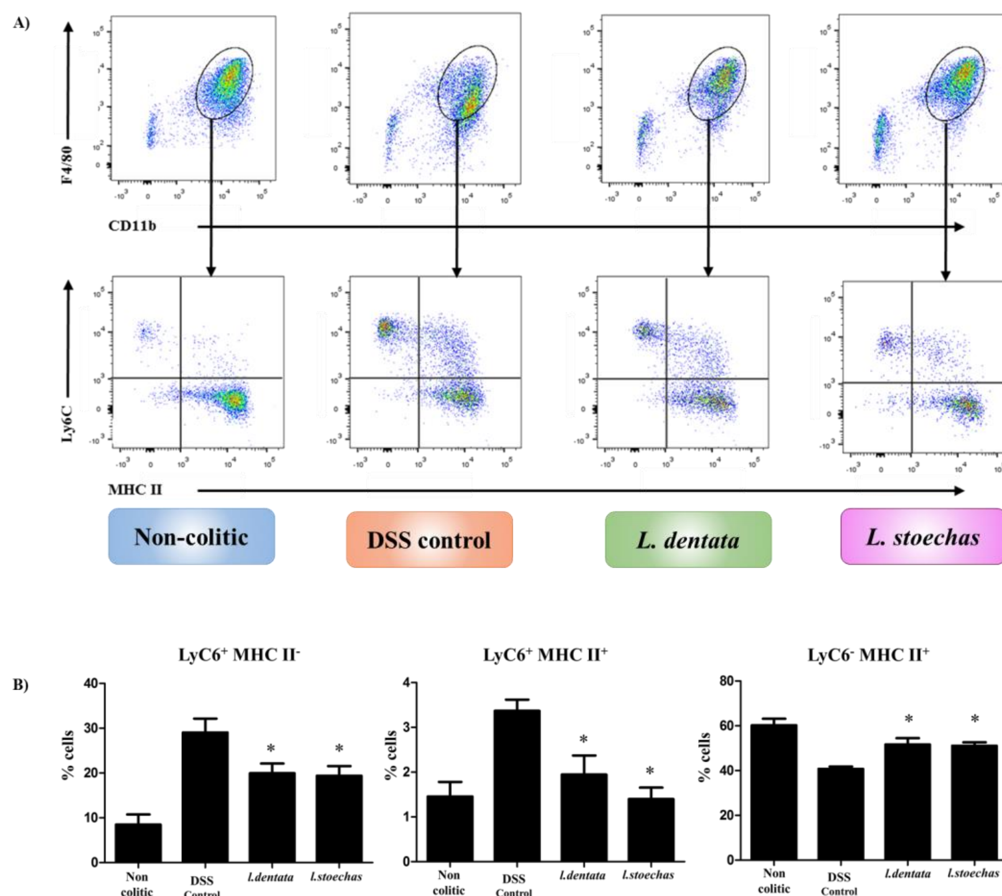


**Figure 39:** A) Representative expression of Ly6C and MHCII on $CD11^{+}$ $F4/80^{+/low}$ cells in different experimental groups: no-colitic, colitic mice, colitic mice treated with *L. dentata* and *L. stoechas*. B) *L. dentata* and *L. stoechas* effect on proportion of $Ly6C^{+}MHCII^{-}$, $Ly6C^{+}MHCII^{+}$ and $Ly6C^{-}$ $MHCII^{+}$ populations in *lamina propria* of inflamed colon. Data are expressed as means ± SEM (n=8); *P<0.05 *vs.* Control group; #P<0.05 *vs.* non-colitic mice.

Colitic mice treated with *L. dentata* or *L. stoechas* showed a significant reduction in proportion of monocytes $Ly6C^{+}$ $MHCII^{-}$ and $Ly6C^{+}$ $MHCII^{+}$ with consequent increased proportion of mature $Ly6C^{-}$ $MHCII^{+}$ macrophages in comparis on with colitic group (Figure 39A, B). Thus, *L. dentata* and *L. stoechas* treatments were able to favour

the differentiation, by increasing the proportion of Ly6C$^-$ MHCII$^+$ cells in similar manner (Figure 39A, B)

### 3.6. Anti-inflammatory effect of the extracts in the carrageenan-induced hind paw edema in mice

The subplantar injection of carrageenan into the hind paw of the mice generated an inflammatory response characterized by the higher volume of the extremity than that obtained after the injection of saline, which was maintained for at least the 6 hours that the experiment lasted.

The macroscopic evaluation of the inflammatory process revealed that only the extract of *L. stoechas* exhibited anti-inflammatory effect, since it significantly inhibited the carrageenan-induced paw edema in mice (Table 10). After 1h and 2h post carrageenan, no significant anti-inflammatory activity was observed at any of the doses assayed. However, 3h after injection of carrageenan, the dose of 100 mg/kg showed a significant anti-inflammatory effect, being this maintained 6 h after, and at that time, the dose of 25 mg/kg *L. stoechas* was also able to inhibit the hind paw edema. Diclofenac-treated mice also showed a significant decrease in paw thickness at all time points evaluated (Table 10).

Biochemically, the inflammatory response induced 6 h after carrageenan administration was associated with the increased expression of the inducible enzymes COX-2 and iNOS when evaluated by qPCR (Figure 40). The treatment with either *L. dentata* or *L. stoechas* extract, at 100 mg/kg, significantly reduced the RNA expression of both enzymes, showing a similar efficacy to that obtained with diclofenac (Figure 40). Finally, when the expression of other pro-inflammatory mediators, like the pro-inflammatory cytokines IL-6, IL-1β, TNF-α, as well as the protease of the matrix metalloproteinase 9 (MMP-9), were analysed, both extracts showed different results.

**Table 10.** Effects of *Lavandula dentata* and *Lavandula stoechas* hydroalcholic extracts and diclofenac in carrageenan-induced paw edema in mice.

| Group | Dose mg/kg | % increase of paw thickness | | | |
|---|---|---|---|---|---|
| | | 1h | 2h | 4h | 6h |
| Non-colitic | - | 6.17 ± 4.9 | 5.3 ± 5.9 | 6.62 ± 5.1 | 4.40 ± 4.1 |
| TNBS control | - | 35.66 ± 12.8 | 38.46 ± 10.5 | 41.35 ± 15.7 | 34.22 ± 11.1 |
| Diclofenac | - | 20.85 ± 9.2* | 24.59 ± 13.1* | 19.76 ± 7.9* | 20.21 ± 5.2* |
| *L. dentata* | 10 | 43.40 ± 11.8 | 40.23 ± 9.1 | 36.69 ± 19.4 | 30.88 ± 10.9 |
| *L. dentata* | 25 | 43.26 ± 6.3 | 44.61 ± 10.8 | 28.08 ± 12.4 | 35.21 ± 8.5 |
| *L. dentata* | 100 | 33.85 ± 9.9 | 44.98 ± 12.5 | 30.23 ± 15.8 | 30.72 ± 14.2 |
| *L. stoechas* | 10 | 27.58 ± 13.1 | 34.53 ± 14.8 | 30.39 ± 11.7 | 30.7 ± 2.9 |
| *L. stoechas* | 25 | 39.65 ± 13.2 | 38.20 ± 12.8 | 32.66 ± 4.9 | 23.22 ± 8.7* |
| *L. stoechas* | 100 | 36.59 ± 16.2 | 39.31 ± 16.9 | 22.62 ± 7.7* | 14.08 ± 6.1* |

Data are expressed as mean ± SEM. *$P<0.05$ *vs.* TNBS control group.

Whereas the extract of *L. stoechas*, at the highest dose studied (100 mg/kg), was able to significantly reduce the expression of all the markers, similarly to that observed after diclofenac treatment, *L. dentata* extract was only able to reduce the expression of IL-1β (25 and 100 mg/kg) (Figure 40).

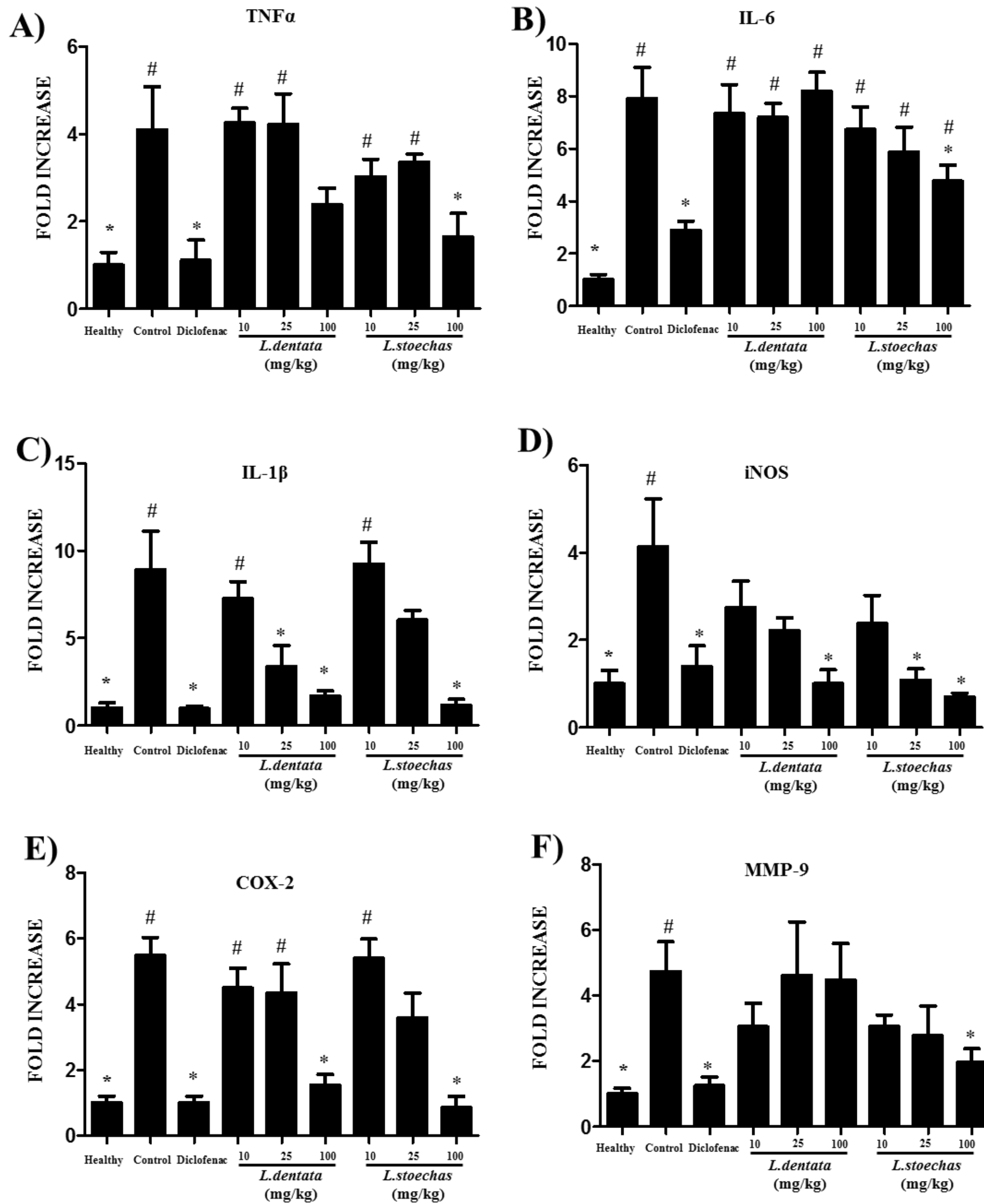


**Figure 40.** Effects of *L. dentata* (10, 25 and 100 mg/kg) and *L. stoechas* (10, 25 and 100 mg/kg) extracts, and diclofenac (10 mg/kg), on the hind paw gene expression of (A) TNF-α, (B) IL-6, (C) IL-1β, (D) iNOS, (E) COX-2 and (F) MMP-9 in carrageenan-induced paw edema in mice. Data are expressed as means ± SEM (n=10); *P<0.05 *vs.* Control group; $^{\#}$P<0.05 *vs*. Healthy group.

# DISCUSSION

# DISCUSSION

Different drug treatments are currently used in the treatment of human IBD with the main goal of inducing and then maintaining remission of mucosal inflammation and associated symptoms, thus theoretically providing an improved quality of life to patients. Although the personalized selection of the pharmacological treatment results in efficacy in most of the cases, these compounds are usually associated with the onset of different side effects that may strongly affect patient compliance and quality of life, thus limiting their use for prolonged periods of time in a significant proportion of IBD patients. This has prompted the use of alternative and complementary treatments by people suffering these intestinal conditions and, among these, the use of herbal remedies is continuously increasing [312]. Despite of this, there have been only a limited number of controlled clinical trials to prove their efficacy and safety. While some herbal preparations may be qualified for controlled patient studies based on their traditional use, since they are well-described and reasonably safe in the target population. However, it is reasonable to develop a pharmacological rationale first that allows, as well, a targeted approach and the appropriate quality assurance measures.

Medicinal plant-based traditional medicine has long provided pharmacotherapy for many people worldwide, including that for the treatment of chronic inflammatory conditions. The rationale for their use is mainly related to safety, since they have been consumed from ancient times, as well as to the presence of different active components that can act simultaneously on different targets in the inflammatory response, including the oxidative stress. Among these active components, polyphenols have been extensively characterized by their wide spectrum of bioactivity, particularly their potent antioxidant activity. In fact, different studies have previously reported the efficacy of isolated polyphenols in experimental models of colitis, including flavonoids (quercitrin, rutin, morin among others), coumarins (paopalantine, 4-mehtylesculetin), resveratrol or curcumin, among many others [405]. However, the evidence for the effectiveness of polyphenols for IBD in humans remains very limited. One of the main concerns with their use may lie in the wide range of doses of polyphenols that have been applied in the

different rodent models. This should be given careful consideration because infeasible human equivalent doses may not be warranted. Future consideration of targeted delivery of polyphenols may improve their ability to ameliorate colitis. In this context, the evaluation of plant extracts can be considered as an important approach for the development of future treatments in IBD. In this context, the preclinical studies reporting the beneficial effects of plant extracts on experimental models of colitis are numerous, only a few plant extracts have been shown efficacy in different clinical assays. This would justify the search of new plant extracts with potential application in intestinal inflammation based on their consideration as suitable remedies for digestive disorders in Traditional Medicine. With this aim we have focused our study in five plant extracts: *Serpylli herba* extract from wild thyme (*Thymus serpyllum*), which is marketed by the Martin Bauer Group (MB-Holding GmbH & Co. KG, Vestenbergsgreuth, Germany), and four extracts obtained from different medicinal plants (*Phlomis lychnitis*, *Phlomis purpurea, Lavandula stoechas* and *Lavandula dentata*) used in Traditional Medicine in Andalucía for inflammatory and/or gastrointestinal complaints.

## 1. *Serpylli herba*

*Serpylli herba* is an officinal drug in the European Pharmacopeia composed by the aerial parts of wild thyme (*Thymus serpyllum*) (Figure 41). This plant belongs to the mint family (Lamiaceae), and is characterized as an evergreen, creeping shrub which is native to most of Europe and North Africa. The scented flowering shoots appear from July to September. *Serpylli herba* is a good seasoning for many meat and vegetable dishes and cabbage or green salads, and throughout Europe and the United States the dried leaves are used for an herbal tea, thus indicating its safety in humans. Traditionally, its medicinal interest has been focused on its essential oil, which can be obtained by steam distillation (oil of Serpolet); in fact, this drug is usually used in folk medicine as an infusion to treat upper respiratory tract infections, in which their active components, the monoterpenes carvacrol and thymol, have been proposed to play a role. Of note, these compounds have been recently reported to exert anti-inflammatory and antioxidant properties, both in vivo

and in vitro [406]. Among the different non-volatile compounds of *Serpylli herba*, which comprise triterpenic acids, hydroxycinnamic acid derivatives and flavonoids, rosmarinic acid is the most abundant phenolic compound [407], which has been described to possess anti-inflammatory, radical scavenging and cancer chemopreventive properties in the intestinal tract in mice[408, 409].

**Figure 41.** *Thymus serpyllum* (https://en.wikipedia.org/wiki/Thymus_serpyllum)

In the present study, we reported for the first time that a standardized aqueous extract from *Serpylli herba* possesses anti-inflammatory effects in two different experimental models of IBD: the TNBS model in rats and the DSS-induced colitis in mice, being probably the most commonly used models to initially evaluate the potential application of a given treatment in intestinal inflammation. They show some resemblance to the main manifestations of IBD. Whereas the TNBS model of rat colitis induces a transmural lesion with pathological characteristics similar to Crohn's disease, the DSS model in mice produces inflammation limited to the colonic mucosa that is more closely related to human ulcerative colitis [410]. The beneficial effects exerted by this extract were evidenced both histologically, by a recovery of the inflammatory process and a reduction in the inflammatory infiltrate, and biochemically, by an amelioration in most of the inflammatory markers evaluated, that have been involved in the pathogenesis of IBD. Of note, the favorable effect was exerted when the extract was administered either before induction of the colonic damage (TNBS model) or following a curative treatment

protocol (DSS model), promoting the recovery of the inflamed tissue in both cases with time. In consequence, the fact that *Serpylli herba* extract has shown beneficial effects in two different models of experimental colitis, makes it a good candidate to be developed for human IBD therapy, even more so when considering its low toxicity which is supported by its long standing and common use in traditional medicine and food. Of note, the efficacy obtained with this extract was similar to that of sulphasalazine, an aminosalicylate currently used in the treatment of human IBD [411].

Furthermore, the present study gives some clues about the mechanisms involved in the intestinal anti-inflammatory effect of the *Serpylli herba* extract. One of these mechanisms could be related to the well-known antioxidant properties ascribed to the majority of the plant extracts, due to their content in polyphenolic compounds, as it happens with the present extract that contains phenolic acids, mainly rosmarinic acid, but also flavonoids, which has been reported to exert antioxidant properties [412, 413]. For example, apigenin is prominently present in *Serpylli herba* extract [414] and a likely mediator for exerting immunomodulatory effects [415]. This can result in the attenuation of the situation of intense oxidative insult that characterizes human IBD [172, 416], which has been also described to occur in different experimental models of colitis, including the TNBS and the DSS models [17, 417], being considered an important mechanism for tissue damage during chronic intestinal inflammation. In this regard, in the present study, the treatment of TNBS colitic rats with *Serpylli herba* extract partially counteracted the depletion of colonic glutathione levels that took place in control colitic animals. Since free radical generation has been proposed to play an important role early on in the pathogenesis of IBD [172, 416], and could contribute to the initial neutrophil infiltration in the inflamed colonic mucosa, the effect exerted by this extract in preserving the colonic mucosa from oxidative insult may collaborate to decrease the neutrophil infiltration that occurs in response to TNBS, as evidenced histologically and biochemically, by a significant reduction in colonic MPO activity. The ability of the extract to reduce neutrophil infiltration in the inflamed tissue was confirmed in the DSS model of mouse colitis, as shown by the histological assessment of the colonic samples.

It is important to note that, in addition to the antioxidant properties that may be ascribed to this extract, other mechanisms can also collaborate in the attenuation of the neutrophil infiltration, like the inhibition in the production and release of other mediators with chemotactic activity, given the ability of this extract to modulate the altered immune response that characterizes both experimental models of colitis. Thus, an inhibitory effect was observed on colonic $LTB_4$ production, an eicosanoid with chemotactic properties, whose production has been reported to be increased in these intestinal conditions [418]. Similarly, the colitic rats treated with *Serpylli herba* extract showed a reduced expression of the inducible enzyme COX-2, which has been reported to play a key role in the regulation of the inflammatory response upon stimulation by luminal pathogens via Toll-like receptors [419], and whose downregulation has been involved in the mechanism of action of drugs used in the treatment of human IBD, including aminosalicylates [420]. In addition to COX-2, another inflammatory protein, the inducible enzyme iNOS is also predominantly expressed at sites of inflammation. In fact, iNOS acts in synergy with COX-2 to produce excessive inflammatory mediators which may be detrimental to the integrity of the colon and contribute to the development of intestinal damage that characterizes the inflammatory reaction [421]. As expected, the results obtained in the present study reveal that colonic inflammation was also associated with an increased iNOS expression in comparison with non-colitic samples, which was effectively inhibited by treatment to colitic rats with the extract.

Moreover, the results obtained reveal that *Serpylli herba* extract was able to ameliorate the deregulated immune response that takes place in experimental colitis; and in particular, analysis of specific cytokine changes related to various T-cell subsets could provide key mechanisms for the beneficial effects of this plant extract treatment. In the present study, both experimental models of colitis were associated to the upregulation pro-inflammatory cytokines, as well as Th1- and Th17-related cytokines in inflamed colonic tissue, in accordance with previous studies [417, 422, 423]. The treatment of colitic animals with *Serpylli herba* extract resulted in the inhibition of representative Th1 (IFN-γ) and Th17 (IL-17) cytokines, and the Th1/Th17 response-related pro-inflammatory cytokines like IL-1β, TNF-α and IL-6. Of note, IL-17 has been reported to

be one of the most deleterious mediators that actively participate in tissue inflammation since it contributes to neutrophil migration or other Th1 cells to the target tissue and their subsequent activation, enhances dendritic cell maturation and the induction of inflammatory cytokines, chemokines and matrix metalloproteases, thus facilitating the development of chronic intestinal inflammation in the intestine [3, 424, 425]. Thus, the severity of histological damage in colitis might be closely related to the activation of the Th1/Th17 pathway, and in consequence, the ability of the extract to down-regulate the Th1/Th17 cytokines IFN-γ and IL-17 seems then to be significant for its intestinal anti-inflammatory effect. Furthermore, the ability of the extract to modulate the immune response was reinforced by its remarkable effect in restoring, in both experimental models of colitis, the expression of the anti-inflammatory cytokine IL-10, which has been described to be of crucial importance for immunoregulation; in fact, mice deficient in this cytokine develop spontaneous severe inflammatory bowel disease, and it has been previously reported its therapeutic efficacy in several animal models of colitis [426]. For this reason, the increased expression of IL-10 in colitic animals treated with the extract may also account for its beneficial effect in promoting the recovery of the damaged intestinal tissue. The immunomodulatory properties of this extract were also confirmed when assayed in LPS-stimulated BMDM, since it was able to inhibit the production of nitrites and IL-6 in a dose dependent way.

In conclusion, *Serpylli herba* extract displays intestinal anti-inflammatory effects in two different experimental models of colitis in rats (TNBS) and mice (DSS), thus revealing the potential application for the treatment of human IBD. Herbal extracts have a good oral bioavailability since their components have been shown to be absorbed and found in plasma within 1 hour [427]. Moreover, it is also interesting that the flavonoids can also exert a local intestinal anti-inflammatory activity specially those found as glycosides that reach the distal portions of the bowel unaltered and, once there, are cleaved by the microbiota releasing the aglycone form of the flavonoid responsible for the beneficial effect [394]. Several mechanisms appear to be involved in this beneficial effect, among them we highlight the antioxidant properties, most probably due to the high polyphenol content, and the ability to ameliorate the altered immune response. The results

of this study support further investigations in the therapeutic potential of *Serpylli herba* extract in IBD treatment.

## 2. *Phlomis* sp. plants

The genus *Phlomis* is a characteristic element of open scrublands of the Mediterranean region. There are about 65 species distributed in Eurasia and North Africa, 4 of them native of the Iberian Peninsula*: P. crinita, P. herba-venti, P. lychnitis* and *P. purpurea*. There are different species from this genus that could be considered as interesting candidates for the treatment of IBD, since they have been used for many decades in folk medicine as stimulants, tonics, wound healers, pain relievers in gastrointestinal distress and anti-inflammatory, among others [428]. This is supported when considering their chemical composition; more than 150 different compounds have been identified as chemical components of *Phlomis*, including sesquiterpenoids, diterpenoids, triterpenoids, triterpene saponins and flavonoids [428, 429]. Of note, the antioxidant properties ascribed to many of these components are well known, being this of special relevance in the management of intestinal inflammation, given the key role attributed to the generation of free radicals in its pathogenesis [416]. In fact, the antioxidant properties of aminosalicylates, one of the most common drugs used in IBD therapy, have been proposed to definitively participate in their mechanism of action [430].

*P. lychnitis L.* (Figure 42A) and *P. purpurea L.* (Figure 42B), the most common species of the genus in Southern Spain, growing, the first one, in the Iberian Peninsula and Southwest of France, and the latter in North Africa. According to the ethnobotanic studies, previously performed by the department of Botany at the University of Granada, these species have been long used in the folk medicine as anti-inflammatory in genitourinary conditions and for digestive complaints like hemorrhoids, among others [431-434]. The chemical constituents reported for these plants include flavonoids, like chrysoeriol, apigenin and luteolin glycosides [428, 429], as well as phenylpropanoids [388], which are known to display antioxidant properties. However, there are only a few

studies describing their biological activities that could confirm their traditional use in folk medicine.

**Figure 42.** A) *Phlomis lychnitis* L.; B) *Phlomis purpurea* L. (Photographer J. Molero Mesa)

In this sense, Lopez *et al.* (2010) recently reported the potential use of *P. lychnitis* as neuroprotective due to the presence of antioxidant compounds, like verbascoside [388]. For that reason, the potential of these two species for their future use in the treatment of IBD was evaluated in the present study. With this aim, the hydroethanolic extracts from *P. lychnitis* or *P. purpurea* were obtained, and their in vivo anti-inflammatory effects were assayed in the TNBS model of experimental rat colitis. The chemical analysis of these plant extracts confirmed the presence of polyphenolic compounds, being their content quite similar in both of them. Correspondingly, their antioxidant ability, when evaluated in vitro by the DPPH method, was also comparable since no statistical differences between their IC50 values were observed.

When the two plant extracts were tested in vivo, at the doses of 10 and 25 mg/kg, they showed intestinal anti-inflammatory effects in the TNBS model of experimental colitis. Histologically, they were able to promote colonic recovery as evidenced by the significant reduction in the extension of the ulcerated and/or inflamed area observed in colons from treated animals. The colonic weight/length ratio was also decreased as a consequence of the extracts administration, revealing an amelioration of the tissue edema associated to the inflammatory process induced after TNBS colonic instillation.

Both *P. lychnitis* and *P. purpurea* extracts significantly reduced, at both doses, the colonic MPO activity, thus indicating a lower neutrophil infiltration in the inflamed colon in comparison with untreated colitic rats, which can clearly contribute to the beneficial effects displayed by these extracts. Different studies have attributed a pathogenic role to neutrophils in IBD, which increase their cellular activity once they have gained access to the inflamed areas of the colon and promote tissue destruction by the release of reactive oxygen metabolites and proteolytic enzymes. For this reason, a reduction in this enzyme activity is considered as a reliable marker of tissue recovery and it is associated with the intestinal anti-inflammatory effect of a given treatment. This effect was associated with amelioration of the colonic glutathione content depletion that takes place in colitic animals as a consequence of the intense oxidative stress that characterizes this experimental model of colitis. However, this improvement was only observed with the lowest dose of each extract. The loss of beneficial effect on this parameter when increasing the dose administered has been previously reported to occur with polyphenolic compounds, like the flavonoids quercitrin or rutin [435, 436]. To explain this, it has been proposed that polyphenolic compounds may display a dual activity, antioxidant or pro-oxidant, depending on the concentration considered. Something similar may have happened in our study, since both extracts contain flavonoids that may have exerted this dual effect. Furthermore, and in addition to the antioxidant properties of the extracts, mostly ascribed to their polyphenolic content, other mechanisms and chemical components might be involved in their intestinal anti-inflammatory effect, thus explaining the different profile shown by the two plant extracts when different biochemical markers were studied in colonic tissue.

In this regard, when the deregulated immune response was evaluated in colitic rats by determining the colonic expression of the pro-inflammatory cytokines IL-1β, TNF-α and IL-17, only *P. purpurea* showed a significant effect in reducing the expression of these cytokines; however, the inhibitory effect on IL-1β and TNF-α was only obtained with the highest dose administered. Of note, the cytokine IL-17 has been described to play a key role in the development of chronic intestinal inflammation [425, 437], since it contributes to neutrophil migration and their subsequent activation, enhances dendritic cell maturation, T cell priming and the production of inflammatory mediators from different cell types [424]. Furthermore, and as commented above, IL-17 can synergize with other cytokines to stimulate the release of additional pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6, thus being essential for the maintenance of the inflammatory response in the intestine [438]. Similarly, experimental colitis has also been reported to be associated with an increased IL-17 production [417, 423], as confirmed in the present study by its amplified expression in colonic specimens from colitic rats. The ability of *P. purpurea* extract to down-regulate IL-17, whereas *P. lychnitis* showed no effect, seems then to be significant for its intestinal anti-inflammatory effect.

The amelioration of the altered immune response exerted by the extracts was also evidenced when the chemokines CINC-1 and MCP-1 and the adhesion molecule ICAM-1 were evaluated. Thus, and similarly to what is commented above, the extract of *P. purpurea* was able to significantly reduce the expression of these mediators, whereas *P. lychnitis* showed no significant effect. All these mediators have been reported to increase inflammatory cell migration from the blood stream into the mucosa and submucosa during chronic IBD, enhancing intestinal tissue destruction [439]. In consequence, the lower expression of these mediators obtained after the treatment of colitic rats, especially with *P. purpurea*, may promote a reduced leukocyte infiltration, including neutrophils (according to the lower colonic MPO activity observed in treated colitic rats) or monocytes/macrophages. The latter could have also contributed to the reduction in colonic iNOS expression observed in treated colitic rats, since macrophages constitute one of the main sources of NO in inflammatory conditions [17]. Therefore, the deleterious effect that NO overproduction exerts on the colonic tissue in these intestinal conditions

[416] may be avoided, and this will facilitate the recovery of the damaged colon, as observed after the treatment of colitic rats with these two plant extracts.

Finally, an impairment of the epithelial barrier function has been proposed to be one of the first events that occurs in intestinal inflammation, facilitating the access of antigens from the intestinal lumen and triggering the exacerbated immune response [440, 441]. Among the different factors that contribute to the maintenance of epithelial integrity, colonic mucus layer has been reported to play a key role; in fact, human IBD has been associated with a defect in mucus production and a reduced number of goblet cells [442]. These observations have been also reported in experimental models of rodent colitis [443], and confirmed in the present study, since TNBS colitis was associated with a reduction in the expression of MUC-2 (the primary constituent of the mucus layer in the colon), MUC-3 (a membrane-bound mucin), and TFF-3 (a bioactive peptide that is involved in epithelial protection and repair) [444]. The treatment of colitic rats with either *P. lychnitis* or *P. purpurea* extracts significantly reversed the reduced expression of MUC-2 and MUC-3, thus preserving the mucus-secreting layer that covers the epithelium and acts as a physical barrier protecting its integrity. It is interesting to note that sulphasalazine was also able to increase the expression of both mucins. When TFF-3 was considered, only the extract of *P. purpurea*, at the dose of 25 mg/kg, was able to increase its expression in comparison with the colitic control group. In consequence, these effects may contribute to the restoration of the epithelial barrier, thus promoting TNBS colonic damage recovery. In addition, the administration of 25 mg/kg of *P. purpurea* to colitic rats also resulted in an increased expression of villin, a tissue-specific actin modifying protein that actively participates in cell morphology, epithelial-to-mesenchymal transition, cell migration and cell survival [445]. TNBS colonic inflammation was associated with a decreased expression of this protein, thus causing a delay in epithelial recovery, which seems to be counteracted after the administration of *P. purpurea* extract.

In conclusion, both extracts of *P. lychnitis* and *P. purpurea* display intestinal anti-inflammatory effect in experimental colitis in rats, thus confirming the traditional use ascribed to these plants in folk medicine in Southern Spain. Different mechanisms might

be involved in this beneficial effect, in which their antioxidant properties, most probably due to their polyphenolic content, may play a key role. Additional mechanisms might include the downregulation of the altered immune response, especially with *P. purpurea* extract, or the improvement of the compromised epithelial integrity. The different profiles shown by both extracts suggest that other active principles, different from polyphenols, could also participate in/contribute to their in vivo anti-inflammatory activity. Further research is therefore required to finally elucidate the putative compounds, as well as their mechanism of action, responsible for the intestinal anti-inflammatory effect shown by these extracts.

## 3. *Lavandula* sp. plants

*Lavandula dentata* L. (Figure 43A) and *Lavandula stoechas* L. (Figure 43B) are common in the Spanish heliophilous scrubs. The first one is typically located in coastal areas on basic substrates, reaching up to 700 m, whereas the second is preferably found on acid soils from sea level to 1700 m. Both species are usually used in traditional medicine for the treatment of various pathologies associated with inflammatory conditions such as arthritis [431], rheumatism [431, 434, 446], haemorrhoids [446], bumps and bruises (and more frequently in digestive disorders, such as liver and intestinal inflammatory conditions [431]). The medicinal importance of these plants is well documented; in fact, different Pharmacopeias include the medicines prepared with them [447]. The in vivo experiments performed in the present Thesis revealed that *L. dentata* and *L. stoechas* extracts, at doses of 10 and 25 mg/kg, exerted intestinal anti-inflammatory effects in the TNBS model of rat colitis, showing a similar efficacy to that observed with dexamethasone. This is a glucocorticoid that was used as a positive control in these assays, instead of sulphasalazine that was used in the other experiments performed with *Serpilly herba* and *Phlomis* sp.

**Figure 43.** A) *Lavandula dentata* L.; B) *Lavandula stoechas* L. (Photographer J. Molero Mesa)

Glucocorticoids constitute one of the most currently used pharmacological groups for the treatment of human IBD, mainly to induce remission in the acute flares of the disease that occurs in these patients [448]. The beneficial effects exerted by these plant extracts were evidenced macroscopically, with a reduction of the tissue damage and edema produced after TNBS instillation, and confirmed biochemically, with an amelioration of the different inflammatory mediators involved in the pathogenesis of IBD that were evaluated. In this regard, this experimental model is well characterized by the intense oxidative stress that causes glutathione content depletion in colitic animals, as previously reported [449]. However, it was significantly ameliorated in those colitic rats treated with both plant extracts. It has been reported that the initial generation of oxygen free radicals plays a key role in the early steps of the development of IBD, and this could contribute to the intense neutrophil infiltration that takes place in the inflamed colonic mucosa [416]. Activated neutrophils exhibit increased MPO activity, thus generating

reactive oxygen metabolites that, together with the release of proteolytic enzymes, promote tissue destruction at the inflammatory areas [450]. The treatment of colitic rats with *L. dentata* and *L. stoechas* extracts showed a reduction of colonic MPO activity, which indicates a lower neutrophil infiltration in the inflamed colon in comparison with untreated control rats. In this regard, the ability of both extracts to reduce the colonic expression of different mediators involved in chemotaxis, like ICAM-1 or MCP-1, can contribute to diminish neutrophil infiltration into the damaged tissue. This was supported by the in vitro experiments performed with these plant extracts in the epithelial cell lines CMT-93 and Caco-2. In CMT-93 cells, both extracts were able to downregulate the expression of MIP-2, a chemokine that attracts neutrophils and lymphocytes [451]. Moreover, in Caco-2 cells they significantly reduced the production and release of IL-8, a proinflammatory chemokine that increases inflammatory cell migration from the blood stream into the intestinal mucosa and submucosa during chronic IBD, enhancing intestinal tissue destruction [439].

The colonic inflammatory process was also characterized by increased colonic iNOS expression in comparison with non colitic animals, which, as commented before, is associated with NO overproduction, thus leading deleterious effects to intestinal function [452]. In fact, since macrophages have been considered as the main source of iNOS in intestinal inflammatory conditions, this would suggest an augmented macrophage infiltration in the inflamed mucosa [17]. Once again, both extracts were able to down-regulate iNOS expression in colitic rats. This beneficial effect was also corroborated in the in vitro studies, since both *L. dentata* and *L. stoechas* extracts reduced NO production in LPS-stimulated BMDM. In consequence, the beneficial effects showed by these extracts in the TNBS model of rat colitis could be related to an inhibitory effect on colonic NO production, which may prevent the detrimental effect ascribed to this mediator when it is locally produced in high proportions.

When considering the intestinal macrophages, it is noteworthy to note their key role in maintaining the homeostasis. In fact, they prevent the inappropriate response to commensal microbes that cause inflammatory bowel disease through the process known

as selective inertia [24]. Inflammation is associated with disrupted differentiation of the Ly6C$^{+}$ monocytes that replenish resident macrophages in resting intestine [119]. As consequence, monocytes and early stages of their differentiation dominate the inflammatory infiltrate [119], as it has been also shown in the present study in the DSS model of mouse colitis. When the colitic mice were treated with *L. dentata* or *L. stoechas* extracts, they showed an improvement in this differentiation process, thus reducing the proportion of monocytes with pro-inflammatory properties while increasing the proportion of intestinal resident macrophages. This would lead to the amelioration of the intestinal inflammatory status, although it is unclear the mechanism of action; thus, additional studies are necessary to establish the molecular pathways involved in this process.

Moreover, and as commented above, it is also well known the crucial involvement of different pro-inflammatory cytokines, including IL-1β, IL-12, IL-17, IL-6 and TNF-α, in the pathogenesis of the acute and chronic phases of intestinal inflammation. They have been reported to be up-regulated in both human IBD and in experimental colitis, thus revealing the existence of an excessive activation of adaptative immunity, which is affected by a combination of resident and recruited cell populations [453, 454]. The improvement of the colonic inflammatory process observed after treatment of colitic rats with both plant extracts, was associated with a decreased expression of the pro-inflammatory cytokines evaluated, thus showing an amelioration of the immune response in colonic inflammation. Of note, the immunomodulatory properties of both extracts were also shown in vitro, since they reduced the release and/or expression of different pro-inflammatory cytokines, including IL-1β, IL-6 and IL-12 in LPS-stimulated BMDM, or TNF-α and IL-6 in LPS-stimulated CMT-93 epithelial cells.

Furthermore, the experiments performed in LPS-stimulated human PBMCs support the beneficial effects exerted by these extracts on the immune response. In fact, the incubation of PBMCs with *L. dentata* and *L. stoechas* resulted in a reduced release of the pro-inflammatory mediators TNF-α and IL-12p40, in response to LPS. Moreover, and as a result of a bidirectional mixed lymphocyte reaction, these extracts promoted a

reduction in IFN-γ production, which would suggest a downregulation in the activation of adaptive immune response. Likewise, moDCs treated with *L. dentata* or *L. stoechas* released less amount of IL-12p70 in response to *Salmonella* in comparison with non-treated stimulated-cells. *Salmonella*-stimulated moDCs treated with the extracts also reduced the expression of costimulatory molecules, especially with *L. dentata* extract. This can be considered very interesting because DCs have a key role in antigen presentation. In fact, when *Salmonella*-stimulated moDCs were put in contact with Th0 naïve cells isolated from human blood, the differentiation into Th1 and Th17 subsets was observed (Figure 44). Preliminary experiments have revealed that the treatment of moDCs with the extracts resulted in a reduction of their inflammatory potential that lead to Th0 naïve differentiation switch: Th1 and Th17 subtypes were reduced, and no effect was observed in Treg differentiation (Data not shown). These results would suggest that probably the anti-inflammatory effects shown by *L. dentata* and *L. stoechas* could be related to the modulation of antigen presentation.

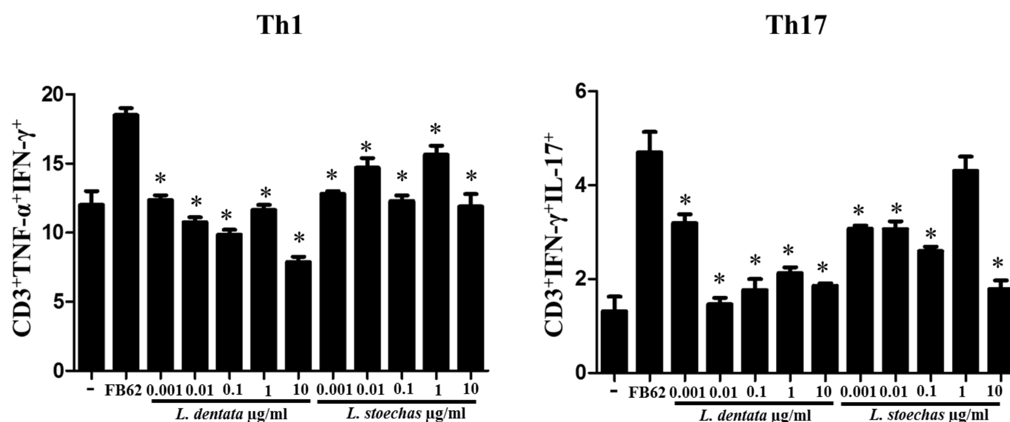


**Figure 44.** Proportion of Th1 and Th17 cells resulted from bidirectional mixed lymphocyte reaction: moDCs were treated with *Lavandula dentata* and *Lavandula stoechas* extracts and stimulated with *Salmonella thyphimurium* (FB62). After that, moDcs were co-cultured with Th naïve cells during five days. The experiments were performed two times, with each individual treatment being run in triplicate.

The impact of these extracts on IL-17 functions could also collaborate in their beneficial effects, which may be associated with the amelioration of the altered immune response that occurs in intestinal inflammation. In fact, there is an increasing interest in

elucidating the complex role exerted by IL-17 in these intestinal conditions, since this is a cytokine that has been reported to play an important role in the intestinal homeostasis as well as in the maintenance of the inflammatory response in the intestine [3, 438, 455]. Thus, IL-17, together with other cytokines, stimulates the production and release of pro-inflammatory cytokines that generate a vicious circle of inflammation that leads to mucosal damage. Furthermore, IL-17 participates in various pathways, such as maturation of DCs and infiltration of neutrophils and CD4 lymphocytes. The exacerbated activation of these functions contributes to the impairment of the immune response, which is one of the main features in IBD [455]. However, although a decrease in the deregulated expression of this cytokine was observed in colitic rats treated with both *Lavandula* extracts, only *L. stoechas* extract did it significantly, similarly to the effect obtained with dexamethasone.

All these effects on immune function exerted by the extracts can be attributed to their chemical composition. Classically, the beneficial effects of *Lavandula sp* in Traditional Medicine have been attributed to the essential oils. However, besides essential oils, there are other main constituents in these plants that can account for their beneficial effects, including polyphenols [456]. In fact, flavonoids are polyphenols that exhibit antioxidant and immunomodulatory properties that can clearly contribute to the antiinflammatory effects exerted by these extracts in these experimental models of colitis [393, 394]. Both extracts have shown similar anti-oxidant activity *in vitro* in the DPPH assay, closely related to the equivalent polyphenol content of both extracts. However, it is interesting to note that the differences in the chemical composition between both *Lavandula* extracts assayed could justify the distinctive effects observed when the different markers were evaluated in this inflammatory condition. In fact, the qualitative extracts profiles were obtained with reversed-phases HPLC coupled to DAD and MS, and after this analysis, it was possible to establish the main phenolic compounds present in either *L. dentata* or *L. stoechas*. There were clear differences when evaluating the main components in both species: the majority of compounds are present in both extracts, but salvianolic acid was present only in *L. stoechas* and isocutellarein 8-O-glucuronide was identified only in *L. dentata.*

In addition to the altered immune response in the intestinal tissue, the TNBS model is associated with an impairment of the epithelial barrier function caused by the disruption of the colon architecture, similarly to what has been reported to occur in human IBD, which facilitates the access of antigens from the intestinal lumen and promotes the exacerbated immune response [440, 441]. The colonic mucus layer clearly contributes to maintain the epithelial integrity, being composed predominantly of mucin glycoproteins and TFF3 that are synthesized and secreted by goblet cells. TFF3 plays a major role in wound healing and repair of the intestinal mucosa [457]. As previously reported, the TNBS-induced colonic damage was associated with a reduced expression of TFF-3 and MUC-3 in comparison with the non-colitic group [458]. The treatment with both extracts resulted in an increased expression of these markers, mainly *L. dentata* extract, which could contribute to restore intestinal epithelium integrity, thus facilitating colon damage recovery.

Finally, human IBD is often associated with extraintestinal symptoms, and up to 35% of patients show musculoskeletal complications that can be due to the course of disease as well as to side effects derived from the administration of corticosteroids, immunosuppressants or anti-TNF therapies [459]. It has been reported that, among others, arthralgia and peripheral arthropathy can worse even more the compromised quality of life in IBD patients [460, 461] For that reason, it could be interesting that the anti-inflammatory therapy not only takes effect in the intestine but also in these extra-intestinal manifestations with an important inflammatory component. In order to evaluate this possibility, the anti-inflammatory effects of *L. dentata* and *L. stoechas* extracts were assayed on the carrageenan-induced hind paw edema. The administration of *L. stoechas* extract showed a macroscopic anti-inflammatory effect by reducing paw thickness, from 3 to 6 h after injection of the offending agent. It has been proposed that edema formation after carrageenan injection in the mouse paw is the consequence of two events [23]: the initial phase begins instantly after the administration of carrageenan, lasting approximately one hour, and it is characterized by the release of histamine and serotonin; after this, the second phase starts and persists for other five hours in which a variety of inflammatory mediators are released: proteases, lysosomes, leukotrienes and

arachidonate metabolites, like prostaglandins, especially those of the E series, thus indicating the activation of COX [462, 463]. In fact, the inhibition of these enzymes is the basic mechanism of action of the NSAIDs, used in the treatment of pain and inflammation [464]. The biochemical analysis performed in this experimental model evidenced that both treatments reduced the expression of COX-2, which can contribute to their anti-inflammatory effect. In addition to COX-2, iNOS expression was also significantly increased, thus contributing to the inflammatory process in the joints. Treated groups showed a decrease of iNOS expression that could ameliorate the inflammatory process. Carrageenan injection also induced the release of some important pro-inflammatory cytokines, like IL-6, IL-1β and TNF-α, which, as commented previously, play a key role in the generation and in the maintenance of the inflammatory status. Moreover, the control group showed high expression of MMP-9; this protein secreted by macrophages regulates leukocyte migration in inflammatory diseases [23]. Treated groups evidenced a reduction in the expression of all these markers, confirming the down-regulation of the inflammatory response also in this experimental model.

In conclusion, *L. dentata* and *L. stoechas* extracts showed intestinal anti-inflammatory activity in the TNBS model of rat colitis and in the DSS model of mice colitis. The beneficial effects of these treatments could be explained due to their antioxidant properties, their capacity to down-regulate the immune response and to improve the intestinal epithelial barrier, which could validate their use in traditional medicine as anti-inflammatory drugs. Moreover, it is interesting that both extracts displayed anti-inflammatory effects also in joint inflammation, highlighting that their beneficial effects are manifested at systemic level. The results of this study are in support of further investigations into the therapeutic potential of *L. dentata* and *L. stoechas* extracts in IBD treatment as well as in extra-intestinal associated with IBD.

# CONCLUSIONS

# CONCLUSIONS

1. *Serpylli herba* showed intestinal anti-inflammatory effects in two experimental models of rodent colitis that could be attributed to its immunomodulatory properties.

2. *Phlomis lychnitis, Phlomis purpurea, Lavandula dentata* and *Lavandula stoechas* extracts showed intestinal anti-inflammatory activity in the TNBS model of rat colitis, being similar to that obtained with standardized extract *Serpylli herba*. This confirm their traditional use in digestive inflammatory disorders, and shows their potential development as intestinal anti-inflammatory agents for the treatment of human IBD.

3. In addition to their antioxidant properties, other mechanisms can contribute to this beneficial effect, like an improvement of the intestine epithelial barrier and a downregulation of the immune response. In fact, all tested extracts display a direct immunomodulatory effect in different cell types. In particular, *Lavandula dentata* and *Lavandula stoechas* were able to ameliorate the maturation of intestinal resident macrophages and could interfere with antigen presentation of DC to T cells, among others functions. This immunomodulation might lead to an improvment of the inflammatory process that characterizes IBD.

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# ABBREVIATIONS

| | |
|---|---|
| ·OH | Hydroxyl radical |
| AMP | Antimicrobial peptide |
| ANOVA | A one way analysis of variance |
| AOAH | Acyloxyacyl hydrolase |
| APC | Antigen-presenting cell |
| AJSESI | Agilent Jet Stream electrospray ionization |
| BMDM | Bone marrow-derived macrophages |
| BPC | Base peak chromatogram |
| Breg | Regulatory B cells |
| CARD15 | Caspase recruitment domain 15 gene |
| CBD | Cannabidiol |
| CD | Crohn's disease |
| CINC-1 | Cytokine-induced neutrophil chemoattractant 1 |
| CMC | Carboxymethyl cellulose |
| COX-2 | Cyclooxygenase-2 |
| CsA | Cyclosporine A |
| CX3CR1 | CX3C chemokine receptor 1 |
| Cx43 | Connexin 43 |
| DAD | Diode array detector |
| DAI | Disease activity index |
| DC | Dendritic cells |
| DEX | Dexamethasone |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | Deoxyribonucleic acid |
| DNBS | 2,4-dinitrobenzene sulfonic acid |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| DSS | Dextrane sodium sulfate |
| ECP | Eosinophilic cationic protein |
| ELISA | Enzyme-linked immunosorbent assay |

ABBREVIATIONS

| | |
|---|---|
| EPO | Eosinophil peroxidase |
| ER | Endoplasmic reticulum |
| FB62 | *Salmonella enterica serovar typhimurium strain SL1344* |
| FBS | Fetal bovine serum |
| Foxp3 | Forkhead box P3 |
| GALT | Gut-associated lymphoid tissue |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GSH | Total glutathione |
| $H_2O_2$ | Hydrogen peroxide |
| HBSS | Hank's Balanced Salt Solution |
| HPLC | High-performance liquid chromatography |
| IBD | Inflammatory bowel disease |
| ICAM | Intercellular adhesion molecule-1 |
| IDO | Indoleamine 2,3-dioxygenase |
| IEC | Epithelial cells |
| IFN-γ | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IL-6R | IL-6 receptor |
| iNOS | Inducible nitric oxide synthase |
| iTreg | Induced regulatory T cells |
| LOX | Lipoxygenase |
| LPS | Lipopolysaccharides |
| LT | Leukotrienes |
| MAPK | Mitogen-activated protein kinases |
| MCP-1 | Monocyte chemotactic protein-1 |
| M-CSF | Macrophage colony-stimulating factor |
| MIP | Macrophage inflammatory protein |

| | |
|---|---|
| MLR | Mixed lymphocyte reaction |
| MMP-9 | Matrix metalloproteinase 9 |
| moDCs | Monocyte derived dendritic cells |
| MPB | Major basic protein |
| mRNA | Messenger RNA |
| MS | Mass spectrometry |
| MUC-2 | Mucin |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NFAT | Transcription of nuclear factor of activated T cells |
| NF-κB | Nuclear factor- κB |
| NK | Natural killer |
| NOD2 | Intracellular nucleotide oligomeration domain 2 |
| NSAID | Nonsteroidal anti-inflammatory drugs |
| nTreg | Natural regulatory T cell |
| O2- | Superoxide radical |
| ONOO- | Peroxynitrite |
| p7F | 5, 6, 3',5'-tetramethoxy 7,4'-hydroxyflavone |
| PAF | Platelet activating factor |
| PAMP | Pathogens-associated molecular patterns |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase chain reaction |
| PGE2 | Prostaglandin E2 |
| PKC | Protein kinase C |
| PMN | Polymorphonuclear neutrophil |
| PP | Peyer's patches |
| PRR | Pattern recognition receptors |
| PTPN2 | Protein–tyrosine phosphatase non-receptor type |
| qPCR | Quantitative PCR |

| | |
|---|---|
| QTOF | Quadrupole-time-of-fligh |
| RA | Retinoic acid |
| RALDH | Retinaldehyde dehydrogenase |
| RNA | Ribonucleic acid |
| ROR | Retinoic acid orphan receptor |
| ROS | Reactive oxygen species |
| RPMI | Roswell Park Memorial Institute medium |
| RP | Reversed-phase |
| RT-PCR | Reverse Transcription-Polymerase Chain Reaction |
| SAZ | Sulfasalazine |
| SDS | Sodium dodecylsulfate |
| Ta | Annealing temperature |
| TBS-T | Tris buffered saline-Tween |
| TFF | Trefoil factor |
| TGF-β | Transforming growth factor-β |
| Th | T helper |
| THC | Δ-tetrahydrocannabinol |
| TJ | Tight junction |
| TLR | Toll-like receptors |
| TNBS | Trinitrobenzene sulfonic acid |
| TNF-α | Tumor necrosis factors |
| TSLP | Thymic stromal lymphopoietin |
| UC | Ulcerative colitis |
| UHD | Ultra-High-Definition |
| ZnSOD | Zinc superoxide dismutase |

# ANEXO

**PUBLICACIONES**

1. **Algieri F**, Rodríguez-Nogales A, Rodriguez-Cabezas ME, Risco S, Ocete MA, Gálvez J. Botanical Drugs as an Emerging Strategy in Inflammatory Bowel Disease. Med Inflamm. 2015 (en prensa).

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## CONTRIBUCIONES A CONGRESOS

### Comunicaciones orales

Autores: Toral Jiménez M, Romero Pérez M, Jiménez Moleón R, Rodríguez-Nogales A, **Algieri F**, Chueca N, Sánchez Santos M, Pérez Vizcaíno F, Gálvez J, Duarte Pérez JM. Titulo: *Lactobacillus fermentum* CECT5716 consumption improves hypertension and endothelial dysfunction induced by Tacrolimus: Role of T cells. Tipo de participación: comunicación oral. Congreso: 36 Congreso Sociedad Española de Farmacología. Publicación: Abstract book. Lugar celebración: Valencia (España). Fecha: 16-18/09/2015.

Autores: Garrido-Mesa J, Rodríguez-Nogales A, **Algieri F**, Vezza T, Utrilla MP, Rodríguez-Cabezas ME, León J, Salmerón J, Gálvez J. Titulo: The antidepressant agomelatine reduces intestinal inflammation in DNBS experimental colitis in mice. Tipo de participación: comunicación oral. Congreso: 36 Congreso Sociedad Española de Farmacología. Publicación: Abstract book. Lugar celebración: Valencia (España). Fecha: 16-18/09/2015.

ANEXO

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Autores: Rodríguez-Nogales A, **Algieri F,** Garrido-Mesa J, Garrido-Mesa N, Utrilla MP, Rodríguez-Cabezas ME, Chueca N, García F, Gálvez J. Titulo: Immuno-modulatory properties of *Lactobacillus fermentum* CECT5716 in DSS-colitis: impact on micro-RNA expression and bacterial diversity. Tipo de participación: comunicación oral. Congreso: VI Workshop Probioticos, prebioticos y salud: Evidencia científica. Publicación: Abstract book. Lugar celebración: Oviedo (España). Fecha: 5-6/02/2015.

Autores: Hidalgo-Cantabrana C, López P, Varela E, **Algieri F**, Rodriguez-Nogales A, Suárez A, Antolín M, Guarner F, Gálvez J, Margolles A, Ruas-Madiedo P. Titulo: Papel de los exopolisacáridos producidos por *Bifidobacterium animalis* subsp. *Lactis* en las interacciones bacteria-hospedador. Tipo de participación: comunicación oral. Congreso: VI Workshop Probioticos, prebioticos y salud: Evidencia científica. Publicación: Abstract book. Lugar celebración: Oviedo (España). Fecha: 5-6/02/2015.

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Autores: Rodríguez-Nogales A, Garrido-Mesa J, **Algieri F**, Garrido-Mesa N, Utrilla MP, Rodríguez- Cabezas ME, Chueca N, García F, Gálvez J. Título: Immunomodulatory properties of probiotics in DSS-colitis: impact on micro-RNA expression and bacterial

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Autores: Vezza T, **Algieri F**, Rodríguez-Nogales A, Garrido-Mesa J, Garrido-Mesa N, Utrilla MP, González-Tejero MR, Casares-Porcel M, Molero-Mesa J, Zarzuelo A, Rodríguez-Cabezas ME, Gálvez J. Título: Intestinal anti-inflammatory effect of *Satureja obovata* extract in the TNBS model of rat colitis. Tipo de participación: comunicación oral. Congreso: VI Reunión de Jóvenes Farmacólogos. Publicación: Abstract book. Lugar de celebración: Sevilla (España). Fecha: 12/06/2014.

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Autores: Rodríguez-Nogales A, **Algieri F**, Vezza T, Garrido-Mesa N**,** Rodríguez-Cabezas ME, Comalada M, Utrilla MP, Martin-Bueno M, Valdivia E, Maqueda M, Olivares M, Zarzuelo A, Gálvez J. Título: Comparative study of the in vitro immunomodulatory effects of the probiotics *Lactobacillus fermentum* CECT5716 and *Enterococcus faecalis* UGRA10. Tipo de participación: comunicación oral Congreso: Sociedad Española de Probióticos y Prebióticos. Publicación: Abstract book and Nutrición hospitalaria. Lugar de celebración: Madrid (España). Fecha: 31/01/2013.

Autores: **Algieri F**, Zorrilla P, Utrilla MP, Garrido-Mesa N, Rodríguez-Cabezas ME, Toral M, Rodríguez-Nogales A, Pérez L, Olivares M, Gonzales-Tejero MG, Casares M, Molero J, Zarzuelo A, Gálvez J. Título: Intestinal anti-inflammatory effects of *phlomis purpurea* extract in the TNBS model of rat colitis. Tipo de participación: comunicación oral. Congreso: IV Reunión de Jóvenes Farmacólogos. Publicación: Abstract book. Lugar de celebración: Granada (España). Fecha: 4/06/2012.

Autores: Rodríguez-Nogales A, Garrido-Mesa N, **Algieri F**, Zorrilla P, Zarzuelo A, Rodríguez-Cabezas ME, Gálvez J. Título: Minocycline attenuates piroxicam-induced colonic damage in IL-$10^{-/-}$KO mice. Tipo de participación: comunicación oral. Congreso: IV Reunión de Jóvenes Farmacólogos. Publicación: Abstract book. Lugar de celebración: Granada (España). Fecha: 4/06/2012.

**Poster**

Autores: Mediavilla C, Risco S, Rodríguez-Nogales A, **Algieri F**, Vezza T, Utrilla MP, Rodríguez-Cabezas ME, Gálvez J. Titulo: Evaluation of the *Lactobacillus fermentum* in the DCA experimental model of irritable bowel syndrome: Impact on anxiety behavior. Tipo de participación: póster. Congreso: 36 Congreso Sociedad Española de Farmacología. Publicación: Abstract book. Lugar celebración: Valencia (España). Fecha: 16-18/09/2015.

Autores: Vezza T, **Algieri F**, Rodríguez-Nogales A, Utrilla MP, Rubio LA, Peinado MJ, Ruiz R, Clemente A, Rodríguez-Cabezas ME, Gálvez J. Titulo: A pea (*Pisum sativum* L.) seed extract exhibits intestinal anti-inflammatory effect in the DSS model of mouse colitis. Tipo de participación: poster. Congreso: VI Workshop Probioticos, prebioticos y salud: Evidencia científica. Publicación: Abstract book. Lugar celebración: Oviedo (España). Fecha: 5-6/02/2015.

Autores: **Algieri F**, Rodríguez-Nogales A, Vezza T, Garrido-Mesa J, Garrido-Mesa N, Utrilla MP, Bañuelos O, González-Tejero MR, Casares-Porcel M, Molero-Mesa J,

Zarzuelo A, Rodríguez-Cabezas ME, Gálvez J. Titulo: Intestinal anti-inflammatory effect of Lavandula dentata and Lavandula stoechas extracts in the TNBS model of rat colitis.Tipo de participación: póster. Congreso: VIth International Congress of Ethnobotany (ICEB2014). Publicación: Abstract book. Lugar celebración: Córdoba (España). Fecha: 17-21/11/2014.

Autores: Vezza T, Rodríguez-Nogales A, **Algieri F**, Garrido-Mesa N, Zarzuelo A, Olivares M, Comalada M, Rodríguez-Cabezas ME, Gálvez J. Titulo: The viability of Lactobacillus fermentum CECT5716 is not required to exert its intestinal anti-inflammatory activity. Tipo de participación: póster. Congreso: The 9th European Mucosal Immunology Group Meeting (EMIG2014). Publicación: Abstract book. Lugar celebración: Glasgow (Escocia). Fecha: 9-12/10/2014.

Autores: **Algieri F**, Rodriguez-Nogales A, Garrido-Mesa J, Garrido-Mesa N, Utrilla MP, Rodriguez-Cabezas ME, Chueca N, García F, Gálvez J. Titulo: Immuno-modulatory properties of probiotics in DSS colitis: impact on micro-RNA expression and bacterial diversity. Tipo de participación: póster. Congreso: The 9th European Mucosal Immunology Group Meeting (EMIG2014). Publicación: Abstract book. Lugar celebración: Glasgow (Escocia). Fecha: 9-12/10/2014.

Autores: Rodríguez-Nogales A, Gálvez J, **Algieri F**, Garrido-Mesa J, Utrilla MP, Rodríguez-Cabezas ME, Thiemermann C. Titulo: The Inhibition of IκB kinase ameliorates experimental colitis in rats. Tipo de participación: póster. Congreso: The 9th European Mucosal Immunology Group Meeting (EMIG2014). Publicación: Abstract book. Lugar celebración: Glasgow (Escocia). Fecha: 9-12/10/2014.

Autores: Pawlukiewicz A, **Algieri F**, Vezza T, Rodríguez-Nogales A**,** González-Tejero MR, Casares-Porcel M, Molero-Mesa J, Zarzuelo A, Gálvez J. Título: In vitro anti-inflammatory and immunomodulatory effects of *Psoralea bituminosa* and E*chium flavum.* Tipo de participación: poster. Congreso: VI Reunión de Jóvenes Farmacólogos. Publicación: Abstract book. Lugar de celebración: Sevilla (España). Fecha: 12/06/2014.

Autores: Rodríguez-Nogales A, Garrido-Mesa J, **Algieri F**, Garrido-Mesa N, Utrilla MP, Rodríguez-Cabezas ME, Chueca N, García F, Gálvez J. Titulo: Immunomodulatory properties of probiotics in DSS-colitis: impact on micro-RNA expression and bacterial diversity. Tipo de participación: póster. Congreso: Falk Symposium 192. Publicación: Abstract book. Lugar de celebración: Paris (Francia). Fecha: 30-31/05/2014.

Autores: **Algieri F**, Rodríguez-Nogales A, Vezza T, Garrido-Mesa J, Garrido-Mesa N, Utrilla MP, Bañuelos O, González-Tejero MR, Casares-Porcel M, Molero-Mesa J, Zarzuelo A, Rodriguez-Cabezas ME, Gálvez J. Titulo: Intestinal anti-inflammatory effects of *Lavandula dentata* and *Lavandula stoechas* extracts in the TNBS model of rat colitis. Tipo de participación: póster. Congreso: Falk Symposium 192. Publicación: Abstract book. Lugar de celebración: Paris (Francia). Fecha: 30-31/05/2014.

Autores: Garrido-Mesa N, Garrido-Mesa J, Rodríguez-Nogales A, **Algieri F**, Utrilla MP, Toral M, Rodríguez-Cabezas ME, Gálvez J. Titulo: Beneficial effects of the association of *Saccharomyces boulardii* and doxycycline in an experimental model of reactivated colitis in mice. Tipo de participación: póster. Congreso: Falk Symposium 192. Publicación: Abstract book. Lugar de celebración: Paris (Francia). Fecha: 30-31/05/2014.

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Autores: Gálvez J, **Algieri F**, Rodríguez-Nogales A, Garrido-Mesa J, Utrilla MP, Rodríguez-Cabezas ME, Thiemermann C. Titulo: The Inhibition of IκB kinase ameliorates experimental colitis in rats. Tipo de participación: póster. Congreso: Falk Symposium 192. Publicación: Abstract book. Lugar de celebración: Paris (Francia). Fecha: 30-31/05/2014.

Autores: Lorente MD, Rodríguez-Nogales A, **Algieri F**, Vezza T, Garrido-Mesa N, Zarzuelo A, Olivares O, Comalada M, Rodríguez-Cabezas ME, Gálvez J. Titulo: The viability of *Lactobacillus fermentum* CECT5716 is not required to exert its intestinal anti-inflammatory activity. Tipo de participación: póster. Congreso: Falk Symposium 192. Publicación: Abstract book. Lugar de celebración: Paris (Francia). Fecha: 30-31/05/2014.

Autores: Toral M, Baú Betim Cazarin C, Rodríguez-Nogales A, **Algieri F**, Utrilla MP, Rodríguez-Cabezas ME, Garrido-Mesa J, Zarzuelo A, Maróstica MR, Gálvez J. Titulo: Preventative intestine anti-inflammatory effects of a prebiotic obtained from Passiflora edulis peel in the DSS model of mouse colitis. Tipo de participación: póster. Congreso: Falk Symposium 192. Publicación: Abstract book. Lugar de celebración: Paris (Francia). Fecha: 30-31/05/2014.

Autores: Vezza T, **Algieri F**, Rodríguez-Nogales A, Garrido-Mesa J, Utrilla MP, Montilla A, Cardelle-Cobas A, Olano A, Corzo N, Garrido-Mesa N, Zarzuelo A, Rodríguez-Cabezas ME, Gálvez J. Titulo: Intestine anti-inflammatory effects of oligosaccharides derived from lactulose in the TNBS model of rat colitis. Tipo de participación: póster. Congreso: Falk Symposium 192. Publicación: Abstract book. Lugar de celebración: Paris (Francia). Fecha: 30-31/05/2014.

Autores: Rodriguez-Cabezas ME, Lozano-Pérez AA, Rodríguez-Nogales A, Ortiz V, **Algieri F**, Garrido-Mesa J, Garrido-Mesa N, Utrilla MP, Matteis L, Martínez de la Fuente J, Cenis JL, Gálvez J. Titulo: Evaluation of resveratrol loaded silk fibroin nanoparticles in an experimental model of rat colitis. Tipo de participación: poster. Congreso: Falk Symposium 192. Publicación: Abstract book. Lugar de celebración: Paris (Francia). Fecha: 30-31/05/2014.

Autores: Chueca-Porcuna N, Rodríguez-Nogales A, Toral-Jiménez M, **Algieri, F**, Garrido-Mesa J, Duarte J, García F, Gálvez J. Título: Evaluación mediante pirosecuenciación de los efectos del probiótico *Lactobacillus fermentum* CECT5716 en la microbiota intestinal de ratones. Tipo de participación: poster. Congreso: XVIII Congreso Nacional: Sociedad Española de Enfermedades infecciosas y Microbiología clínica. Publicación: Abstract book. Lugar de celebración: Valencia (España). Fecha: 09-11/04/2014.

Autores: Baú Betim Cazarin C, Rodríguez-Nogales A, **Algieri F**, Utrilla MP, Rodríguez-Cabezas ME, Garrido-Mesa J, Zarzuelo A, Maróstica MR, Gálvez J. Título: Preventative intestine anti-inflammatory effects of a prebiotic obtained from *Passiflora edulis* peel in the DSS model of mouse colitis. Tipo de participación: poster. Congreso: V Workshop Probioticos, prebioticos y salud: Evidencia científica. Publicación: Abstract book. Lugar de celebración: Valencia (España). Fecha: 23-24/01/2014.

Autores: Garrido-Mesa J, Rodríguez-Nogales A**,** Garrido-Mesa N, **Algieri F**, Utrilla MP, Toral M, Zarzuelo A, Rodríguez-Cabezas ME, Gálvez J. Título: Beneficial effects of the association of *saccharomyces boulardii* and doxycycline in an experimental model of reactivated colitis in mice. Tipo de participación: poster. Congreso: V Workshop: Probióticos, prebióticos y salud: Evidencia científica. Publicación: Abstract book. Lugar de celebración: Valencia (España). Fecha: 23-24/01/2014.

Autores: Utrilla MP, **Algieri F**, Zorrilla P, Garrido-Mesa N, Rodríguez-Nogales A, Montilla A, Cardelle-Cobas A, Olano A, Corzo N, Zarzuelo A, Rodríguez-Cabezas ME, Gálvez J. Titulo: Intestine anti-inflammatory effects of oligosaccharides derived from

lactulose (OSLU) in the TNBS model of rat colitis. Tipo de participación: póster. Congreso: 7$^{th}$ Probiotics, Prebiotics & new foods Publicación: Abstract book. Lugar de celebración: Roma (Italia). Fecha: 08-10/09/2013.

Autores: Vezza T, **Algieri F**, Rodríguez Nogales A, Garrido Mesa N, Rodriguez Cabezas ME, Comalada M, Utrilla MP, Olivares M, Zarzuelo A, Gálvez J. Titulo: I*n vitro* immunomodulatory effects of the probiotic *Lactobacillus fermentum* CECT5716. Tipo de participación: poster. Congreso: 7$^{th}$ Probiotics, Prebiotics & new foods. Publicación: Abstract book. Lugar de celebración: Roma (Italia). Fecha: 08-10/09/2013.

Autores: Utrilla MP, Zorrilla P, Tchombe L, Garrido-Mesa N, Rodriguez-Cabezas ME, Toral M, **Algieri F**, Rodríguez-Nogales A, Gálvez J. Titulo: The polyphenolic-enriched extract AMANDA® exerts intestinal anti-inflammatory effect in the TNBS model of rat colitis. Tipo de participación: poster. Congreso: The 17$^{th}$ international congress Phytopharm 2013. Publicación: Abstract book. Lugar de celebración: Viena (Austria). Fecha: 08-10/07/2013.

Autores: Garrido-Mesa J, **Algieri F**, Zorrilla P, Rodríguez-Nogales A**,** Garrido-Mesa N, Utrilla MP, Bañuelos O, Gonzales-Tejero MR, Casares-Porcel M, Molero-Mesa J, Zarzuelo A, Rodriguez-Cabezas ME, Gálvez J. Titulo: Intestinal anti-inflammatory effects of *Lavandula dentata* and *Lavandula stoechas* in the TNBS model of rat colitis. Tipo de participación: Poster Congreso: The 17$^{th}$ international congress Phytopharm 2013. Publicación: Abstract book. Lugar de celebración: Viena (Austria). Fecha: 08-10/07/2013.

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Autores: Garrido-Mesa N, **Algieri F**, Rodriguez-Cabezas ME, Comalada M, Zarzuelo A, Rodríguez-Nogales A, Gálvez J. Titulo: Minocycline inhibits MAP Kinase Signaling pathways in stimulated Intestinal epithelial cells. Tipo de participación: poster. Congreso: 8$^{th}$ European Mucosal Immunology Group Meeting 2012 (EMIG). Publicación: Abstract book. Lugar de celebración: Dublín (Irlanda). Fecha: 10-13/10/2012.

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Autores: Rodríguez Nogales A, **Algieri F**, Vezza T, Ferraro E, Garrido Mesa N, Rodriguez Cabezas ME, Utrilla MP, Martin Bueno M, Valdivia E, Zarzuelo A, Comalada M, Maqueda M, Gálvez J. Titulo: Comparative study of the in vitro immunomodulatory effects of the probiotics *Escherichia coli* Nissle 1917 and *Enterococcus faecalis* UGRA10. Tipo de participación: poster. Congreso: 6th European Congress of Pharmacology, EPHAR 2012. Publicación: Abstract book. Lugar de celebración: Granada (España). Fecha: 17-20/07/2012.

Autores: Zorrilla P, Tchombe L, Utrilla MP, Garrido Mesa N, Rodriguez Cabezas ME, Toral M, **Algieri F,** Rodríguez Nogales A, Pérez L, Olivares M, Zarzuelo A, Gálvez J. Título: The intestinal anti-inflammatory effects of the extract Amanda in the TNBS model of rat colitis are associated with epithelial barrier function improvement. Tipo de participación: poster Congreso: 6th European Congress of Pharmacology, EPHAR 2012. Publicación: Abstract book. Lugar de celebración: Granada (España). Fecha: 17-20/07/2012.

## ESTANCIAS DE INVESTIGACIÓN EN OTROS CENTROS

- PhD International Mobility Programme. Centro: **European Institute of Oncology (IEO)**, Milan (Italia). Supervisor: Dr. Maria Rescigno. Tema: Aprendizaje de la metodología de inmunofluorecencia, estudio de células dendríticas humanas y murinas. 1 Abril-30 Junio 2015

- PhD International Mobility Programme. Centro: **Institute of Infection, Immunity and Inflammation, University of Glasgow** (Escocia). Supervisor: Dr. Allan Mowat (Profesor of Mucosal Immunology). Tema: Aprendizaje de la metodología empleada para el aislamiento de macrófagos intestinales, y de las bases de la citometría de flujo. 22 Julio-1 Noviembre 2014

- Centro: **Department of clinical and experimental pharmacology, toxicology and chemotherapy, University of study of Perugia, (Italia).** Supervisor: Dr. Carlo Riccardi

(Profesor Titular de la Universidad de Perugia). Tema: Aprendizaje de la metodología empleada para el aislamiento de linfocitos de la lámina propia del colon, y de las bases de la citometría de flujo. 18 Septiembre-7 Octubre 2012

## OTROS MÉRITOS

**Curso de escritura cientifica: "stategies for effective research publication"** (17-26/03/2014)

**Curso del descubrimiento a la innovación y a la valorización en el campo de las ciencias y técnicas de la salud: identificación de oportunidades** (6 7/02/2014)

**Actividad docente: Facultad de Farmacia. Universidad de Granada** (2013-2014)

**Participación en el proyecto de innovación docente**
Título: diseño de una propuesta de innovación en la evaluación de los seminarios impartidos por el departamento de farmacología. (25/09/2012-16/12/2013)

**Curso de histologia practica para el aprendizaje de los conceptos básicos de la tecnica histologica** (7-1/10/2013)

**Premio "mejor poster" presentado** en 6th European congress of pharmacology, EPHAR 2012
Titulo: Comparative study of the in vitro immunomodulatory effects of the probiotics Escherichia coli Nissle 1917 and Enterococcus faecalis UGRA10
Autores: Rodriguez Nogales A, **Algieri F**, Vezza T, Ferraro E, Garrido Mesa N, Rodriguez Cabezas ME, Utrilla MP, Martin Bueno M, Valdivia E, Zarzuelo A, Comalada M, Maqueda M, Galvez J

**Formación de postgrado en protección y experimentación animal para investigadores en ciencias biomédicas. FELASA categoría c.** Centro de enseñanzas virtuales de la universidad de granada (cevug). Fundación general universidad de granada-empresa (FGUGREM) (19/09/2011-19/11/2011).

**Formación del investigador responsable del diseño y dirección de los procedimientos. Categoría C**. Centro de enseñanzas virtuales de la universidad de granada (cevug). Fundación general Universidad de Granada-Empresa (FGUGREM) (22/11/2012-16/01/2013).

**Becaria formación personal investigador, fundación empresa - Universidad de Granada**. Universidad de granada. Facultad de Farmacia. Departamento de farmacología (1/04/2011 - 31/01/2012)

Universidad de Granada