DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Pharmacological screening of essential oils for identification of lead compounds to target Inflammatory Bowel Disease

Isabel Cristina do Vale Ferreira

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Alexandrina Mendes (Faculdade de Farmácia da Universidade de Coimbra) e da Professora Doutora Emília Duarte (Faculdade de Ciências e Tecnologia da Universidade de Coimbra)

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À memória de minha Mãe

Ao meu Pai

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TABLE OF CONTENTS

ABBREVIATIONS

AP-1	Activator protein 1
APC	Antigen-presenting cells
ASA	aminosalicylic acid
AZA	Azathioprine
BGM-CSF	granulocyte–macrophage-colony-stimulating factor
BSA	Bovine serum albumin
CD	Chron's disease
DMEM	Dulbeco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DOC	Deoxycholic acid
EGTA	Ethylene glycol tetraacetic acid
FBS	Fetal Bovine Serum
GC	Glucocorticoids
GR	Glucocorticoid Receptor
GI	Gastrointestinal tract
IBD	Inflammatory Bowel Disease
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon-gamma
IgG	Immunoglobulin G
IKK	IκB kinase
IL	Interleukin
IκB	Inhibitor of NF-κB
iNOS	Inducible isoform of the nitric oxide syntase
IRAK	IL-IR–associated kinase
LPS	Lipopolysaccharide
LT	Lymphotoxin
MCP-1	Monocyte chemotactic protein-1
MIP-1a	Macrophage inflammatory protein-1a
MMP	Matrix metalloproteinase
MyD88	Myeloid differentiation factor 88
NIK	NF-kB-inducing kinase

NF-κB	Nuclear Factor kappaB
NO	Nitric oxide
PBS	Phosphate buffered saline
PLA2	Phospholipase 2
PVDF	Polyvinylidene Difluoride
ROM	Reactive oxygen metabolites
ROS	Reactive oxygen species
SDF-1	Stromal cell-derived factor-1a
SDS	Sodium dodecyl sulfate
SLC	Secondary lymphoid tissue chemokine
TBS	Tris-buffered saline
TGF	Transforming growth factor
TIR	Toll/interleukin-1 receptor
TNF	Tumor necrosis facto
TLR	Toll like receptors
T_H	T helper cell
UBQ	Ubiquitination
UC	Ulcerative colitis
VCAM-1	Vascular cell adhesion molecule-1
6-MP	6-mercaptopurin

LIST OF FIGURES

LIST OF TABLES

RESUMO

Doença Inflamatória Intestinal (DII) é o termo utilizado para descrever a inflamação crónica do trato gastrointestinal. As formas principais da DII são a Colite Ulcerosa e a Doença de Chron. Embora a etiologia da DII não seja totalmente compreendida, três componentes principais estarão envolvidos no aparecimento da doença em pessoas susceptíveis: alterações na flora intestinal, na função de barreira do epitélio e uma resposta imunitária exacerbada.

Esta resposta pode seguir um de dois caminhos: uma resposta T_H1 exacerbada, que é mais associada à doença de Chron, ou uma resposta T_H2 exacerbada, mais relacionada com a colite ulcerosa. A activação desta resposta e a libertação de citocinas correspondente está associada à inflamação crónica, envolvendo a síntese de mediadores de destruição tecidular como metaloproteases e espécies reactivas de oxigénio e nitrogénio, nomeadamente óxido nítrico. O factor de transcrição, factor nuclear kappa B (NF-κB) desempenha um papel fundamental na elaboração da resposta inflamatória através da regulação da expressão de muitos dos genes envolvidos nessa resposta, nomeadamente metaloproteases (Megías et al., 2007) e a isoforma indutível da sintetase do óxido nítrico (iNOS), que produz grandes quantidades de óxido nítrico (Aktan, 2004).

Dado o papel central da inflamação na patogénese da DII e a função crucial do NF-κB nesse processo, este trabalho teve como principal objectivo o *screening* de um conjunto de óleos essenciais para actividade anti-inflamatória em células do epitélio intestinal. Com este fim, a linha celular de células humanas de adenocarcinoma colorectal, C2Bbe1, foi usada como modelo para investigar a capacidade de óleos essencias de inibir a activação do NF-κB e a expressão dos seus genes alvo, usando a iNOS como indicador, induzido por uma mistura de citocinas pró-inflamatórias, compostas de interferon-γ, factor de necrose tumoral-α e interleucina-1β. Para este estudo, quatro óleos essenciais, obtidos das plantas *Thapsia villosa*, *Otanthus maritimus, Lavandula luisieri* e *Laserpitium elasii*, foram seleccionados, baseando-se na sua composição, tal que, em conjunto, representam as principais famílias químicas encontrados em óleos essenciais. O óleo essencial de *Annona muricata* foi seleccionado dada a sua indicação etnofarmacológica como um agente anti-inflamatório. Finalmente, α-pineno, o principal componente do óleo essencial obtido das folhas de *Juniperus oxycedrus*, foi também testado, uma vez que foi anteriormente dado como inibidor da activação de NF-κB e produção de óxido nítrico induzidas por IL-1, em condrócitos humanos (Neves et al., 2009).

Os óleos essenciais que inibiram a expressão de iNOS foram então testados para a sua capacidade de reduzir a activação do NF-κB induzida pela mistura de citocinas, através da medição dos níveis citoplamáticos do seu inibidor, IκB-α, na forma total e fosforilada. De modo a avançar ainda mais o processo de identificação de compostos activos, depois de completa a fase de *screening*, o óleo essecial que exibiu a maior potência na inibição do NF-κB, foi fraccionado por cromatografia gasosa/espectrometria de massa e as fracções resultantes foram então testadas quanto à sua capacidade de inibir a expressão de iNOS induzida pela mistura de citocinas.

Os resultados obtidos mostraram que o óleo essencial de *Lavandula luisieri* numa concentração de 0,02% (v/v) suscitou a maior inibição da expressão da iNOS induzida pela mix (16,8 ± 12%) e activação de NF-κB, medido pelos níveis do seu inibidor, IκB-α, na sua forma total e fosforilada (37 ± 11,5%). Para além disso, as fracções C e D do mesmo óleo foram eficazes na redução da expressão de iNOS, enquanto as fracções A e B não tiveram qualquer efeito. Ainda assim, a fracção C mostrou ser mais potente que a fracção D, uma vez que ambas as concentrações testadas reduziram a expressão de iNOS (78,8 ± 5,5% e 28,6 ± 14,2%, respectivamente), enquanto que apenas a concentração mais elevada da fracção D provocou uma diminuição significativa (50,3 ± 13,3%).

Por outro lado, os resultados obtidos não suportaram, no modelo usado, uma acção anti-inflamatória para o óleo essencial de *Annona muricata*, embora tal acção possa também ocorrer por um mecanismo distinto da inibição de NF-κB.

Tendo em conta todos os resultados obtidos, estes mostraram que o óleo essencial de *Lavandula luisieri* contém composto(s) capazes de inibir a activação de NF-κB e a expressão dos seus genes alvo nas células do epitélio intestinal humanas. No nosso conhecimento, este é o primeiro estudo que associa os óleos essenciais de *Lavandula luisieri* com actividade anti-inflamatória. Além disso, este estudo permitiu a identificação de duas fracções desse óleo que muito provavelmente contêm o(s) composto(s) responsáveis pela inibição da expressão da iNOS e da activação do NF-κB. Deste modo, o fraccionamento e *screening* adicionais são mandatários para a identificação do(s) composto(s) activo(s) e caracterização, em maior detalhe, do(s) mecanismo(s) correpondente(s), através dos quais exercem a sua actividade anti-inflamatória. Estes estudos irão abrir caminho para optimizações químicas e farmacológicas dos compostos activos identificados podendo conduzir ao desenvolvimento de novas moléculas, esperançosamente mais eficazes para o tratamento de DII.

Palavras-chave: Doença Inflamatória Intestinal, inflamação, NF-κB, citocinas óleo essencial

SUMMARY

Inflammatory bowel disease (IBD) is a collective term for chronic inflammation of the gastrointestinal tract. The major forms of IBD are Ulcerative colitis (UC) and Chron's disease (CD). Although the etiology of IBD is not fully understood, three major components are likely involved in susceptible hosts: altered intestinal flora and barrier function of the epithelium and exacerbated immune response. This response may follow one of two pathways: an exacerbated T_H1 response that is more associated with CD or an exacerbated T_H2 response that is more linked to UC. Activation of this response and corresponding cytokine release is associated with chronic inflammation, involving the generation of tissue destruction mediators, like matrix metalloproteinases (MMPs), and reactive oxygen (ROS) and nitrogen (RNS) species, namely nitric oxide (NO). The transcription factor, Nuclear Factor-kappaB (NF-κB), plays a key role in mounting the inflammatory response, by regulating the expression of many genes involved in that response, namely MMPs (Megías et al., 2007) and the inducible isoform of the NO Synthase (iNOS), which produces large amounts of NO (Aktan, 2004).

Given the central role of inflammation in the pathogenesis of IBD and the crucial function of NF-kB in that process, this work aimed at screening a set of essential oils for anti-inflammatory properties towards intestinal epithelial cells. For this, the human colorectal adenocarcinoma cell line, C2Bbe1, was used as a model to investigate the ability of the essential oils to inhibit the activation of NF-κB and the expression of its target genes, using iNOS as an indicator, induced by a mixture of pro-inflammatory cytokines, composed of interferon-γ, tumour necrosis factor-α and interleukin-1β. For this study, four essential oils, obtained from *Thapsia villosa, Otanthus maritimus*, *Lavandula luisieri* and *Laserpitium elasii*, were selected, based on their composition, so that, taken together, they represented the major chemical families usually found in essential oils. The essential oil from *Annona muricata* was selected due to its etnopharmacological indication as an anti-inflammatory agent. Finally, α-pinene, the major component of the essential oil obtained from the leaves of *Juniperus oxycedrus*, was also screened, since it was previously found to inhibit IL-1-induced NF-κB activation and NO production in human chondrocytes (Neves et al., 2009).

The essential oils found to inibit iNOS expression were then tested as to their ability to reduce the cytokine mixture-induced NF-kB activation by evaluating the cytoplasmic levels of total and phosphorylated forms of its inhibitor, IκB-α. To advance further the process of identification of active compounds, upon completion of the screening phase, the essential oil found to have the highest potency towards inhibition

of NF-κB, was fractionated by Gas Chromatography/Mass Spectrometry and the resulting fractions were then screened for their ability to inhibit the cytokine mixture-induced iNOS expression.

The results obtained showed that the essential oil from *Lavandula luisieri* in a concentration of 0.02% (v/v) elicited the greatest inhibition of the mix-induced iNOS expression (16.8 ± 12%) and NF-κB activation, evaluated as the cytoplasmic levels of the total and phosphorylated (37 ± 11.5%) forms of its inhibitor, IκB-α. Moreover, fractions C and D of the same oil, were effective in reducing iNOS expression, while fractions A and B had no effect. Nonetheless, fraction C was found to be more potent than fraction D, since both concentrations tested reduced iNOS expression (78.8 ± 5.5% and 28.6 ± 14.2%, respectively), whereas only the highest concentration of fraction D elicited a significant decrease (50.3 ± 13.3%).

On the other hand, the results obtained did not support, in the model used, an anti-inflammatory action for the essential oil of *Annona muricata*, although such an action can also occur through mechanisms other than the inhibition of NF-κB.

Taken together, the results obtained show that the essential oil from *Lavandula luisieri* contains compound(s) capable of inhibiting NF-κB activation and the expression of its target genes in human intestinal epithelial cells. To our knowledge, this is the first study that associates the essential oil from *Lavandula luisieri* with anti-inflammatory activity. Moreover, this study allowed the identification of two fractions of that oil that likely contain the compound(s) responsible for inhibition of iNOS expression and NF-kB activation. Further fractionation and screening is, thus, mandatory to identify the active compound(s) and characterize in more detail the corresponding mechanism(s) of anti-inflammatory action. These studies will pave the way for further chemical and pharmacological optimization of the active compounds identified and may ultimately lead to the development of novel and hopefully more effective molecules for the treatment of IBD.

Keywords: IBD, inflammation, NF-κB, cytokine, essential oil

CHAPTER I INTRODUCTION

1.1 The gastrointestinal tract

The human gastrointestinal tract includes the oral cavity, esophagus, stomach, small and large intestine, rectum and anus. Its primary function is the fractioning and chemical digestion of food, so that nutrient molecules can become available for the maintenance, growth and energetic necessities of the organism.

The large and small intestines comprise different regions with different characteristics and functions.

1.1.1 Small Intestine

The small intestine is the terminal site for the digestion of food, nutrient and mineral absorption and endocrine secretion.

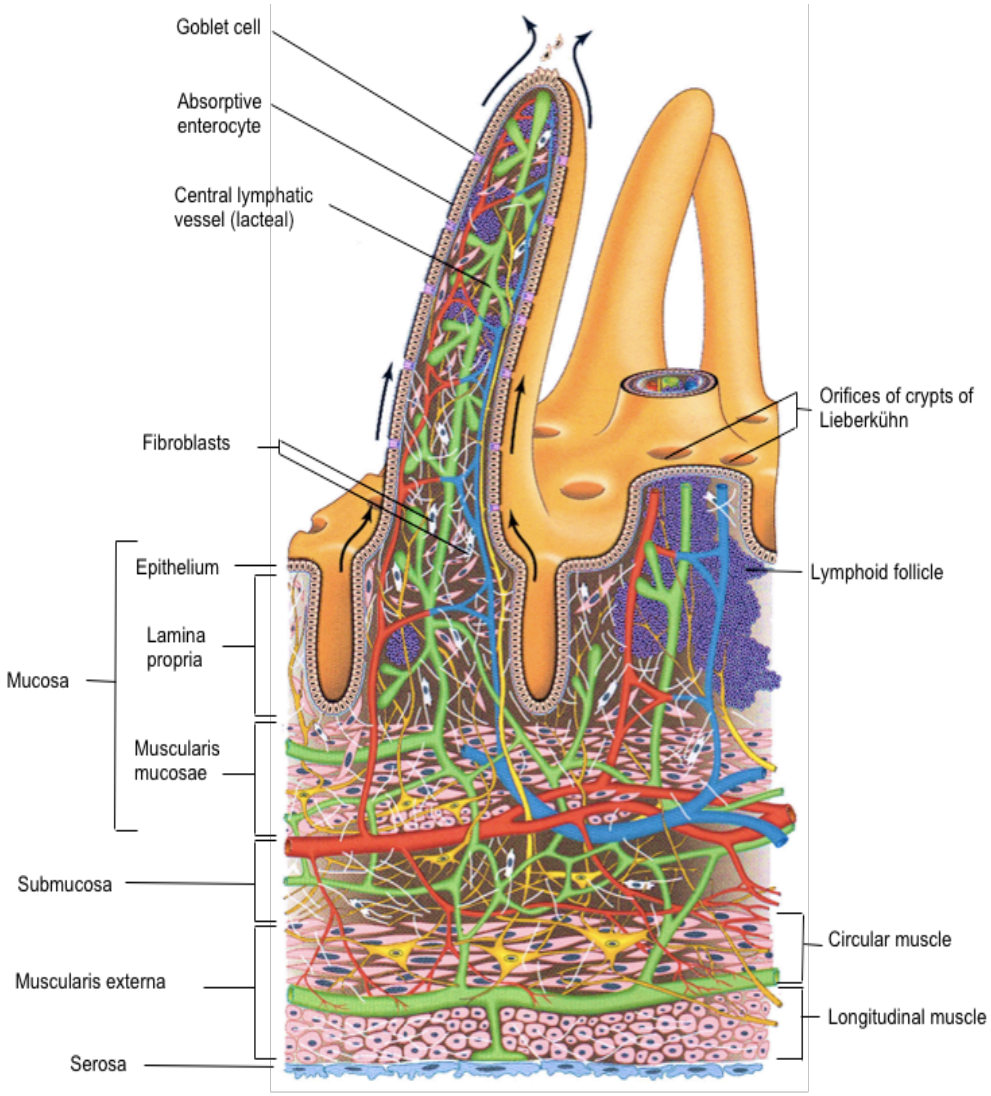

Figure 1: Microstructure of an intestinal villus and the layers of the small intestinal wall. Reprinted from Standring, 2008.

It can be divided in duodenum, jejunum and ileum, which share some characteristics that will be discussed together.

The intestinal wall is composed of mucosa (epithelium, *lamina propria* and *muscularis mucosae*), submucosa, *muscularis externa* (circular and longitudinal muscle) and serosa or adventitia.

The intestinal villi and glands are covered by a single layer epithelium composed of enterocytes, goblet cells, paneth cells, microfold cells, stem cells and neuroendocrine cells. Enterocytes, the most abundant type of cell in small intestinal epithelium, are columnar absorptive cells that are responsible for nutrient absorption (Barbara Young and Heath, 1993; Gonçalves and Bairos, 2006; Junqueira and Carneiro, 2004; Standring, 2008) (Figure 1).

1.1.2 Large Intestine

The functions of the large intestine comprise water absorption, fecal mass formation and mucus production. It can be divided in three major regions: cecum, colon (ascending colon, transverse colon, descent colon and sigmoid flexure) and rectum.

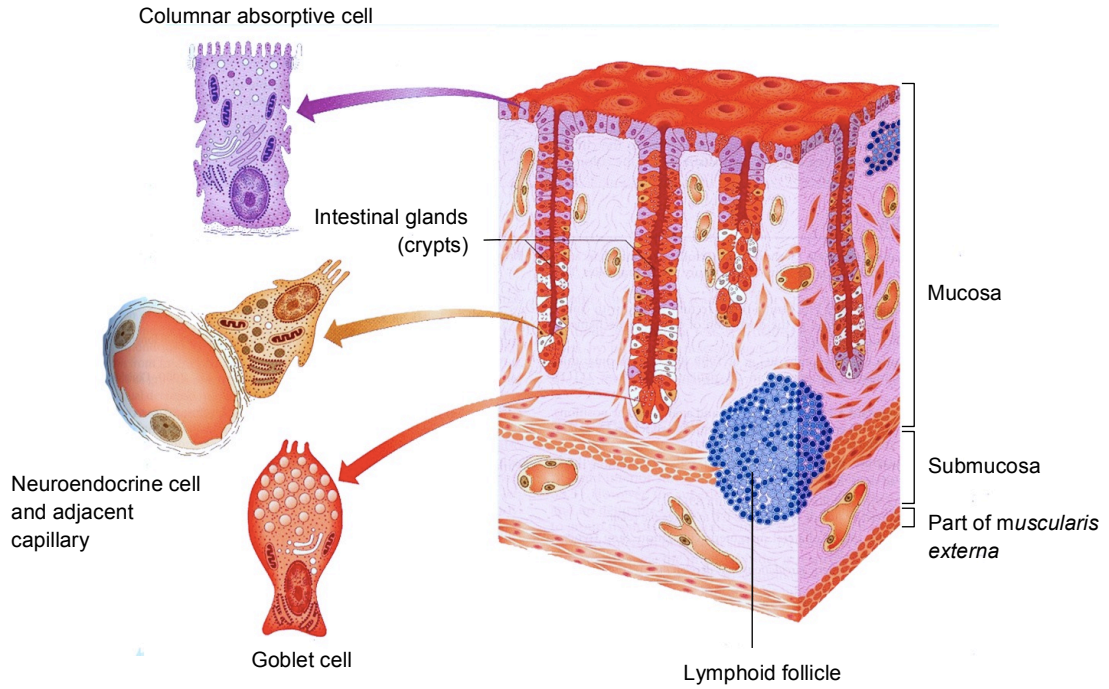

Figure 2: Microstructure of colonic wall and respective cells. Reprinted from Standring, 2008.

Except for the absence of circular folds and the longer glands (crypts), the tissue layers of the large intestine resemble those of the small intestine. The intestinal wall is composed of mucosa, submucosa and *muscularis externa*. The epithelium that layers the intestine wall is composed of enterocytes, mucous cells, microfold cells, stem cells and neuroendocrine cells (Figure 2). Like in the small intestine, enterocytes are the most numerous of epithelial cell types, being responsible for ion exchange and other transepithelium transport functions.

Both the large and small intestine play important roles and their malfunction is associated with impaired growth, malnutrition and several diseases among which we emphasize the inflammatory bowel disease.

1.2 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic condition without a medical cure, requiring a lifetime of care. It can cause significant morbidity, decreased quality of life and more than 2-fold increase in the mortality rate (Irvine and Marshall, 2000). IBD consists of two main disorders, ulcerative colitis (UC) and Chron's disease (CD), and has become a topic of major interest over the past 20 years. These disorders were firstly referenced in the medical literature in the mid 1800s. In his review of the historical aspects of IBD (1988), Kirsner referred to the work of Wilks and Noxon who in 1859 reported a case of a man with colitis, manifested by bloody, mucous diarrhea not caused by any dysenteric organism. The same review (Kirsner, 1988) mentions a later work by Hale-While (1888) that reported a case of a man with colonic ulcerations and bloody diarrhea not accompanied by infection or malignancy and refers to it as "ulcerative colitis". In 1932, Burrill Chron described an intestinal disorder with symptoms similar to those of UC, namely fever, diarrhea and emaciation, which he called "regional ileites" (Crohn et al., 1932). Because the symptoms of these diseases are similar, sometimes it is difficult to classify some cases as either UC or CD and in those circumstances they are called "indeterminate colitis" (Bouma and Strober, 2003; Hanauer, 2006; Kim and Ferry, 2002).

IBD is characterized by relapsing inflammation of the gastrointestinal tract and subsequent tissue damage due to unbalanced activation of the mucosal immune system in response to luminal antigens in genetically predisposed individuals (Bouma and Strober, 2003; Geier et al., 2007; Kim and Ferry, 2002). It has terrific effects on quality of life, imposing a substantial personal burden. The lack of understanding of the disease and the intimate nature of its symptoms generally lead to a lack of support

both by the community, family and coworkers. People with IBD can live productive lives calling upon the use of medication and surgery, but unfortunately there is no cure for IBD, yet.

As mentioned earlier, CD and UC share many similarities, but they also present clinical and pathological differences, namely as to the areas of the intestine involved. In CD, any part of the gastrointestinal tract (GI) can be affected, but most commonly the lower end of the small intestine (ileum), beginning of the large intestine (colon) and peri-anal area are involved (Blumberg and Strober, 2001; Bouma and Strober, 2003; Kim and Ferry, 2002; Podolsky, 2002). All layers of the intestine may be involved which results in deep ulcers that ultimately can lead to abscesses in the abdomen and the development of fistulas (connections between the bowel and other organs). CD is often discontinuous, alternating normal bowel between affected regions, known as “skip” lesions (Bouma and Strober, 2003; Kim and Ferry, 2002). In contrast, the inflammatory process in UC starts at the level of the anus and extends through the colon, affecting only the superficial layers of the intestine (mucosa and serosa) (Blumberg and Strober, 2001; Bouma and Strober, 2003). Despite the several clinical and pathological differences, there is no single characteristic that is exclusive of one or other disease.

1.2.1 Epidemiology

The incidence of IBD has risen over the past decades in North America and Europe and is increasing in industrialized countries (Borm and Bouma, 2004; Bousvaros et al., 2008; Kim and Kim, 2010; Kim and Ferry, 2002). The prevalence of IBD shows a north-south variation, with a higher incidence in the northern than in the southern regions of the globe, which suggests that environmental factors play a role in its pathogenesis. Indeed, factors such as “westernization”, sanitation and exposure to infection, age and gender, diet, familial aggregation genetics, cigarette smoking, oral contraceptives and appendectomy were proven to affect IBD pathogenesis (Table 1).

IBD is called “a disease of cleanliness”, since increased incidence is associated with better sanitation. It is assumed that improved hygiene alters the intestinal flora by decreasing the exposure to certain critical bacteria (Bosani et al., 2009; Hanauer, 2006; Lee and Buchman, 2009). Accordingly, IBD tends to occur more frequently in higher socioeconomic groups (Farrell and LaMont, 2002; Krishnan and Korzenik, 2002).

Some studies demonstrate differences in the incidence of IBD between genders, where ulcerative colitis is slightly more common in males (Hanauer, 2006) and CD marginally more frequent in women.

Table 1: Epidemiology of Inflammatory Bowel Disease.

	UC	CD	Reference
Incidence/ 100,000	2–15	3–15	(Molodecky and Kaplan, 2010)
Age of Onset	20-30 & 60-80		(Sonnenberg, 2010)
Ethnicy	> Jewish		(Baumgart and Carding, 2007)
Male/Female ratio	1:1	1.8:1	(Hanauer, 2006)
Smoking	↓	↑	(Harries et al., 1982; Somerville et al., 1984)
Oral contraceptives	↑	↑↑	(Cornish et al., 2008)
Appendectomy	Protective	Non-protective ?	(Reif et al., 2001)
Monozygotic twins	6.3%	58.3%	(Tysk et al., 1988)
Dizygotic twins	0.53%	1%	(Tysk et al., 1988)

1.2.2 Etiology and Pathogenesis

Despite incompletely understood, there are several theories concerning the etiology of IBD.

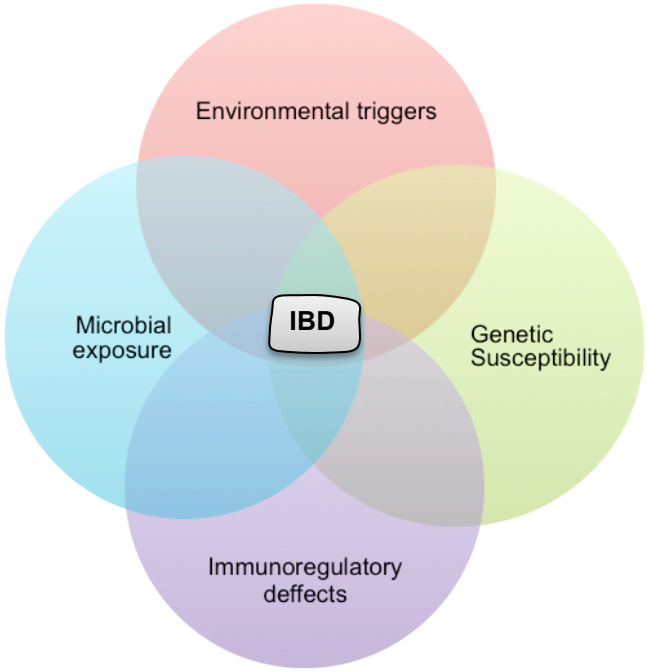

Figure 3: Ethiopathogenesis of IBD. The host luminal microbiota provides antigens that stimulate immune responses. In genetically susceptible hosts, there is an exacerbated immune response to those antigens. Environmental factors also play an important role in the initiation of the disease.

The prevailing theory states that IBD is a result of an abnormal immune response to the host microbiota in genetically susceptible individuals and that environmental triggers are necessary to initiate or reactivate the disease (Borm and Bouma, 2004; Fiocchi, 1998; Hanauer, 2006; Lee and Buchman, 2009; Sartor, 2006) (Figure 3). In fact, many pathogenic etiologies may result in a common immunological response by the gut and its accessory organs (Figures 3 and 4).

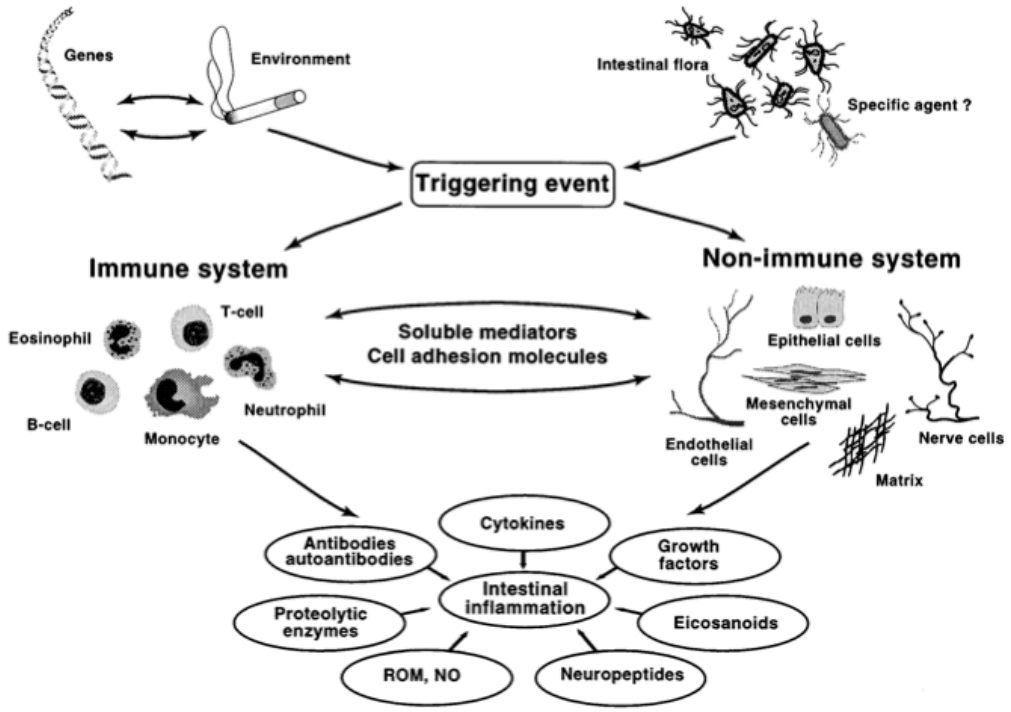

Figure 4: Interaction between the components involved in the etiopathogenesis of IBD. The combination of genetic susceptibility, environment factors and intestinal flora, trigger an event that activates the intestinal immune and non-immune systems. These two systems communicate through the production of soluble mediators and cell adhesion molecules, leading to further activation and amplification of the production of cytokines, growth factors, eicosanoids, neuropeptides, reactive oxygen metabolites (ROM), nitric oxide (NO), proteolytic enzymes, antibodies and autoantibodies which culminates in inflammation and tissue damage. Reprinted from Fiocchi,1998..

1.2.2.1 Importance of the flora in the development of IBD in susceptible hosts

The actual understanding of the interactions between the microbial flora and the host is quite incomplete because of the limited knowledge on the diversity and complexity of the microbial flora and the limited tools available to delineate those characteristics. Despite that, there is accumulating evidence that the initiation and pathogenesis of IBD lay in a dynamic balance between commensal flora and host defense responses at the mucosal frontier (Xavier and Podolsky, 2007).

The first reference to this postulate was made by Shorter and co-workers who in 1972 proposed that "IBD results from the establishment of a state of hypersensivity to antigen(s) of bacteria normaly present in the individual's gastroentestinal tract and the pathologic and clinical features of IBD then result from a predominant cell-mediated hypersensivity reaction in the bowel wall" (Shorter et al., 1972).

IBD pattients typicaly have greater numbers of adherent bacteria compared to normal subsejcts (Swidsinski et al., 2002) and as such, inflammation and lesions generally occur in intestinal regions with the highest bacterial concentration, like the ileum and colon (Thompson-Chagoyan et al., 2005).

The importance of the flora is supported by studies in mouse models demonstrating that mutant murine strains maintained in germ-free conditions do not develop colitis, but when they are reconstituted with single comensal ou mixed gut bacteria, colitis rapidly emerges and tretatment with antibiotics reduces intestinal inflammation (Blumberg and Strober, 2001; Elson et al., 2005; Farrell and LaMont, 2002; Krishnan and Korzenik, 2002; Onderdonk et al., 1977). To date, however, there hasn't been a conclusive association of a single organism with the development of IBD (Hanauer, 2006; Hendrickson et al., 2002; Kim and Ferry, 2002; Rath et al., 2001).

1.2.2.2 Epithelial barrier function

The mucosal of a monolayer epithelium consists of columnar cells that are held together by circumferential intercellular junctions to form a selective barrier, defending the integrity of the mucosal frontier and being involved in nutrient and fluid absorption and secretion (Xavier and Podolsky, 2007). The physical separation of potentially stimulating microflora and the reactive cells of the mucosal immune system is achieved by the existence of the mucosa's epithelium (Bouma and Strober, 2003). Those epithelial cells are active producers of chemokines that are responsible for the recruitment of leukocytes within the intestinal mucosa (Bamias et al., 2005).

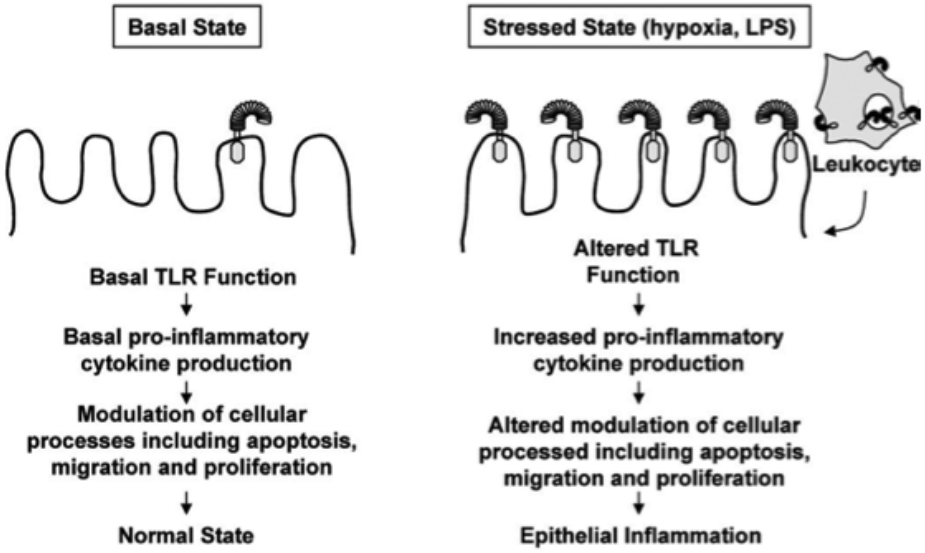

Figure 5: Model of TLR-mediated inflammation in the mucosa. Basal conditions: TLR signaling is necessary for the maintenance of the mucosal homeostasis. Stress: There is an increase in TLR expression leading to an impaired epithelial function, increased injury and decreased repair. Reprinted from (Gribar et al., 2008).

Through the expression of TLRs, the mucosal epithelial cells are able to: i) recognize microorganisms and/or substances that they produce in the mucosal lumen (Cario et al., 2000), ii) induce anti-microbial genes and iii) control the innate and adaptive immune responses (Cario and Podolsky, 2005; Gribar et al., 2008). Thus, the mucosal epithelium is responsible for the detection of antigens at the mucosal surface and the subsequent induction of a tolerant response induced by innocuous commensal bacteria and/or of an active immune response elicited by pathogens that can invade the epithelium (Xavier and Podolsky, 2007) (Figure 5).

In the healty gut, the relationship between comensal bacteria and the host is symbiotic (Abreu, 2002) and the exposure to comensal bacteria inhibits the inflammatory response by down-regulating the inflammatory genes and blocking the activation of the NF-kB pathway (Figure 6). In IBD, however, genetically predisposed individuals appear to loose that tolerance and the exposure to luminal microflora triggers the inflammatory response by the cells lining the mucosa, leading to tissue damage.

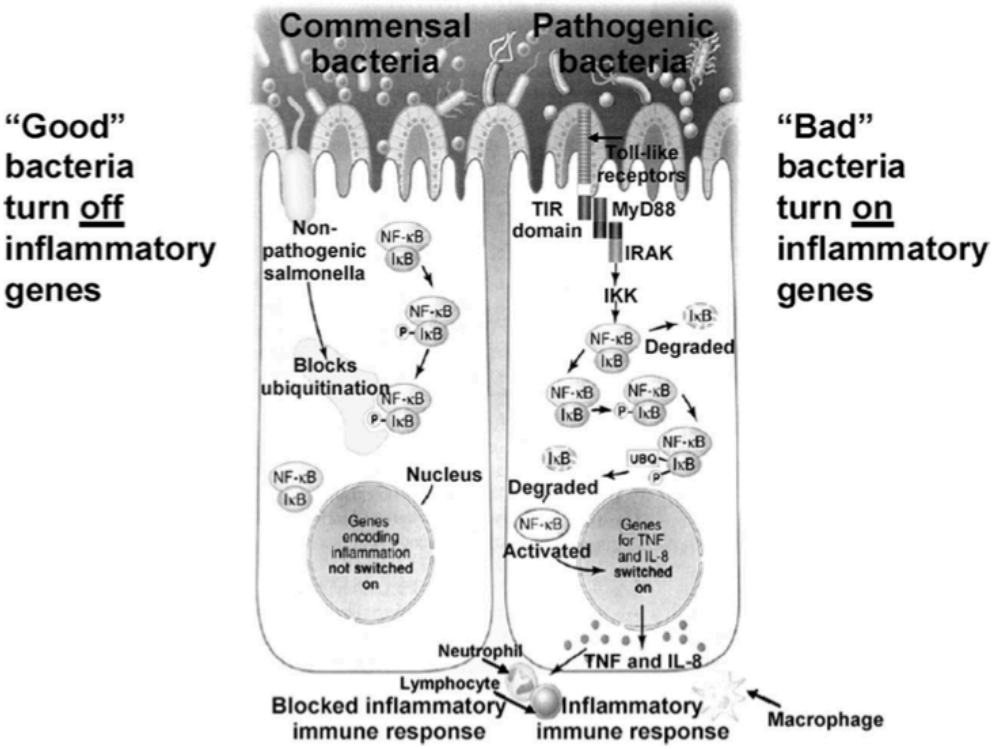

Figure 6: Differential activation of the inflammatory immune response by comensal and pathogenic bacteria. Commensal bacteria block the inflammatory immune response by blocking the ubiquitination required for regulated IκB (inhibitor of NF-κB)-α degradation. This prevents subsequent translocation of the active NF-κB dimers to the nucleus. Pathogenic bacteria activate the inflammatory response through activation of the NF-κB pathway. The bacteria bind to TLRs leading to the subsequent stimulation of the IkB kinase (IKK) and degradation of phosphorylated IkB-a. This allows the dissociation of NF-κB, which migrates to the nucleus and activates inflammatory gene expression. Abreviations: IRAK, IL-IR–associated kinase; MyD88, myeloid differentiation factor 88; TIR domain, toll/interleukin-1 receptor; UBQ, ubiquitination. Reprinted from Hanauer, 2006.

In the healthy gut, the epithelial cells are able to reside in the high bacterial concentration due to their usually low levels of toll like receptors (TLRs) (Cario and Podolsky, 2005) (Figure 5 and 6). Dysfunctions in the TLR signaling pathway (e g. mutations or polymorphisms) can break microbial tolerance and may lead to inflammatory responses.

The epithelial barrier is compromised in some first-degree relatives of patients with Crohn's disease (Irvine and Marshall, 2000; May et al., 1993; Soderholm et al., 2002). Intestinal biopsies of IBD patients show a downregulation of junctional

complexes, namely of E-cadherin and β-cathenin which are major components of tight junctions, necessary for a normal barrier function. The tight junctions that seal the space between adjacent epithelial cells are the rate-limiting step that defines the overall epithelial permeability (Weber and Turner, 2007). The junctions are regulated in response to cytokines and chemokines that promote the establishment of the subsequent immune cell network (Xavier and Podolsky, 2007). In addition, TNF-a, IL-8 and IL-6, which are considered essential to the pathogenesis of IBD, are expressed in the intestinal epithelium (Hyun and Mayer, 2006).

1.2.2.3 Regulation of cytokine gene transcription in IBD

The dysregulated cytokine production by mucosal lymphocytes in CD and UC suggests an abnormal regulation of cytokine gene transcription (Neurath, 1998). Therefore, the identification of signaling pathways and transcription factors that govern cytokine gene transcription is of extreme importance.

Several data show increased expression of the p65 subunit of the transcription factor NF-κB in lamina propria macrophages and epithelial cells (Neurath et al., 1996; Rogler et al., 1998). NF-κB is a central coordinator of innate and adaptive immunity (Bonizzi and Karin, 2004; Hayden and Ghosh, 2011; Ruland and Mak, 2003).

In mammals the NF-κB family is consists of five members: Rel A (p65), Rel B, c-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100). These proteins form homo- and hetero-dimers and it is likely that the different forms of NF-κB may activate different sets of target genes. The activated form of NF-kB usually consists of two proteins, a p65 subunit and a p50 subunit. In unstimulated cells, NF-κB dimers are found retained in the cytoplasm bounded to IκBs (IκBα, IκBγ and IκBβ). In response to multiple extracellular stimuli such as pro-inflammatory cytokines, viruses, TLRs and antigen receptors, the IκBα cinase complex (IKK) is activated. The activated IKK complex catalysis IκBs phosphorilation (Ser-32 and Ser-36 for IκBα and Ser-19 and Ser-23 for IκBβ), polyubiquitination (Lys21 and Lys22 for IκBα) and subsequent degradation by 26S proteasome. Once IκBα is degraded, the nuclear localization of NF-κB is exposed, allowing translocation to the nucleus and activation of gene transcription (Bonizzi and Karin, 2004; Ruland and Mak, 2003; Schottelius and Baldwin Jr, 1999). Once in the nucleus NF-κB, activates the expression of the genes for many cytokines, chemokines, enzymes and adhesion molecules characteristics of chronic inflammatory diseases. One example is the gene for inducible nitric oxide synthase (Cavicchi and Whittle, 1999; Xie et al., 1994), (Figure 7).

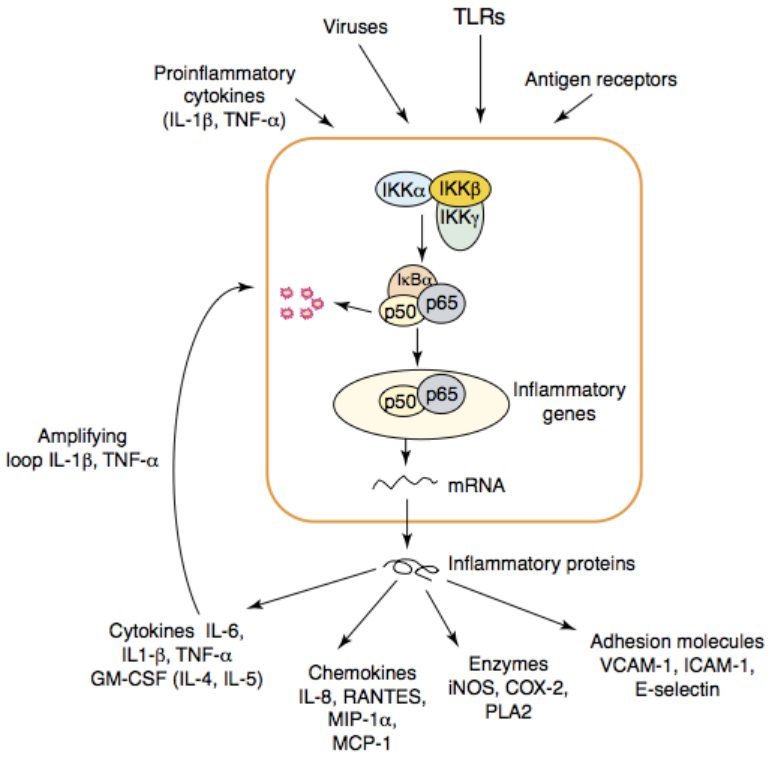

Figure 7: Activation of NF-κB Pathway. NF-κB pathway is activated by a variety of inflammatory signals (pro-inflammatory cytokines, viruses, TLRs and antigen receptors). Those signals stimulate the IκB kinase (IKK) thus promoting the proteolytic degradation of the inhibitory protein IκB-α. The free NF-κB (heterodimer of p50 and p65) then migrates to the nucleus, where it binds to promoter regions of genes for inflammatory proteins such as cytokines, chemokines, enzymes and adhesion mollecules. IL-1β and TNF-α expression is induced and induce NF-κB activation thus performing an amplifying loop. Abbreviations: BGM-CSF, granulocyte–macrophage-colony-stimulating factor; ICAM-1, intercellular adhesion molecule 1; IKK, IκB kinase; IL-1b, interleukin-1b; iNOS, inducible nitric oxide synthase; LT, lymphotoxin; MCP-1, monocyte chemotactic protein-1; MIP-1a, macrophage inflammatory protein-1a; NIK, NF-κB-inducing kinase; PLA2, phospholipase 2; SDF-1, stromal cell-derived factor-1a; SLC, secondary lymphoid tissue chemokine; TLRs, Toll-like receptors; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule-1. Reprinted from Bonizzi and Karin, 2004.

Emerging studies state, that the p65 subunit of NF-κB is not the only transcription factor involved in the pathogenesis of IBD. Several other regulatory proteins, such as AP-1, NFAT and STAT, are very likely to play a key role as well (Neurath, 1998; Schreiber et al., 2002).

1.2.2.4 Immune response

Over the past years, it has become more evident that one of the multiple factors underlying the development of IDB is a dysregulated immune response to the normal enteric microbiota, leading to relapses manifested by chronic inflammation and subsequent tissue damage (Geier et al., 2007; Kim and Ferry, 2002; Podolsky, 2002; Xavier and Podolsky, 2007). Disease pathogenesis and perpetuation also involves a dysregulated respoponde to initial antigen stimulation, leading to up-regulation of pro-inflammatory cytokines (TNF, IFNγ, IL-1, IL-6, IL-8 and IL-18). This creates an detrimental unbalanced mucosal expression of pro-inflammatory versus anti-inflammatory cytokines (TGF-β, IL-10, IL-4 and IL-13) (Kim and Ferry, 2002) (Figure 8).

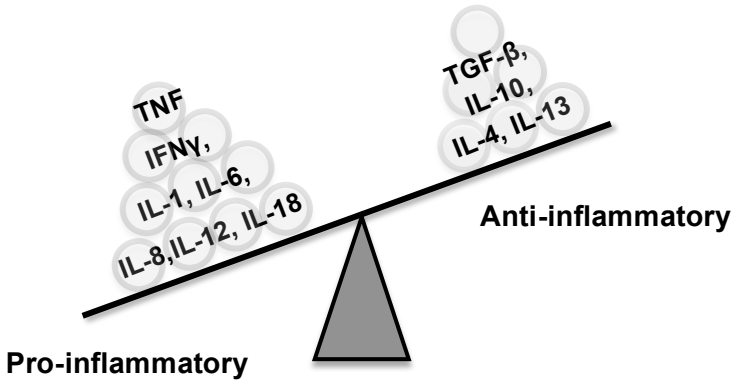

Figure 8: Unbalanced pro-inflammatory and anti-inflammatory cytokines in IBD. Abbreviations: TNF, tumor necrosis factor; IFN-γ, interferon-gamma; IL, interleukin; TGF-β, transforming growth factor.

1.2.2.4.1 Innate immunity

Innate immune response seems to be a prerequisite for excessive activation of adaptive immunity, the main driver of tissue damage characteristic of IBD patients (Xavier and Podolsky, 2007). It is a non-specific defense against pathogens that responds immediately or within the first few hours after infection/insults. It is considered the first line of defense and includes epithelial surfaces as physical barriers (skin, intestinal mucosa) as well as immune cells that identify and remove unfamiliar substances. In contrast to acquired immune system, it reacts to the chemical properties of the antigen rather than to the specific antigen itself. The early response is driven by neutrophils, which move from the circulation through gaps in the vascular endothelium to the tissue, releasing antimicrobial peptides and reactive oxygen species (ROS) that help killing the invading microorganisms, but also cause tissue damage. Neutrophils also secrete chemokines and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) that recruit and activate other white blood cells (e g. macrophages) (Hanauer, 2006; Hyun and Mayer, 2006) (Table 2).

Table 2: Cytokines produced by cells involved in innate immune responses to IBD.

Innate imune response		
Cytokine	**CD**	**UC**
IL-1β	↑	↑
TNF	↑↑	↑
IL-6	↑	↑
IL-8[a]	↑	↑
IL-12	↑	N
IL-18	↑	↑
IL-23	↑	N
IL-27	↑	N

[a] Representative of a large number of chemokines;
IL, interleukin;
N, normal.

Reprinted from from Sartor, 2006

1.2.2.4.2 Adaptative immunity

In IBD there is a loss of tolerance against antigens derived from food and autologous bacterial flora of the intestine (Neuman, 2007; Sartor, 2006). According to models in which essentially pro-inflammatory cytokines are overproduced, the inflammation process occurs as a result of either excessive effector T-cell function or deficient regulatory T-cell function (Bouma and Strober, 2003). As mentioned earlier, the integrity of the barrier is compromised in genetically susceptible individuals, which leads to a stimulation of the immune system by products of commensal bacteria and/or dietary sources. They can penetrate the mucosal barrier, interacting directly with immune cells (dendritic cells and lymphocytes) promoting a classic adaptive response or may stimulate the surface epithelium though receptors that are components of the innate immune system (like TLRs) leading to the production of cytokines and chemokines that recruit and activate mucosal immune cells.

Despite the broad variety of causes, mucosal inflammation normally follows one of two pathways: an exacerbated T_H1 response which is more associated with CD or an exacerbated T_H2 response which is more linked to UC (Bouma and Strober, 2003; Hanauer, 2006; Kim and Ferry, 2002; Neuman, 2007; Xavier and Podolsky, 2007). T_H1 cells orchestrate cell-mediated responses and, in contrast, T_H2 cells mediate humoral responses (Ardizzone and Porro, 2002) (Figure 9).

T_H1 activation is associated with the release of IL-2, IL-6, IL-12, IL-23, INFγ, TNFα and IL-1β and the generation of metalloproteinases (MMPs), which are essential mediators of tissue damage. In contrast, T_H2 activation is associated with the release of IL-4, IL-5, IL-10 and IL-13 (Ardizzone and Porro, 2002; Blumberg and Strober, 2001; Bouma and Strober, 2003; Bousvaros et al., 2008; Hyun and Mayer, 2006) (Figures 9 and 10 and Table 3). Some authors advocate that UC doesn't fit clearly into the T_H1/T_H2 dichotomy, showing a mixed response (Hyun and Mayer, 2006) or a modified T_H2 response (Ardizzone and Porro, 2002; Podolsky, 2002).

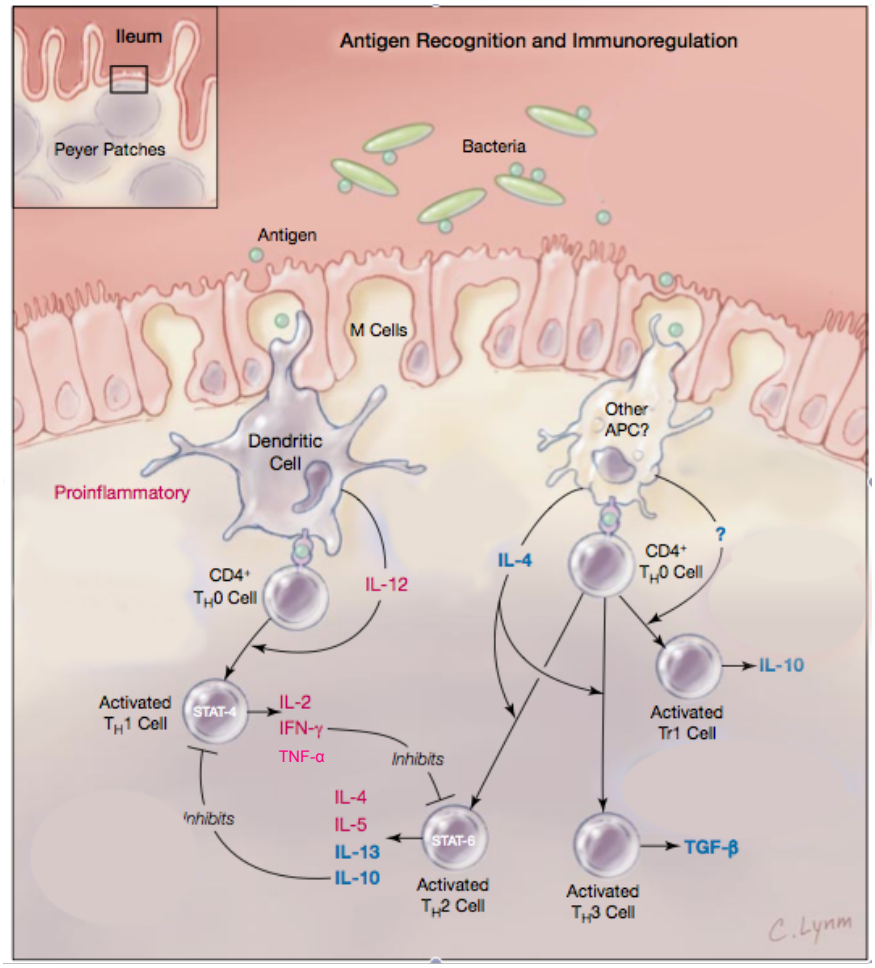

Figure 9: T-lymphocyte activation pathways. Either by activating the classic antigen-presenting cells (APC) (macrophages, dendritic cells and certain activated epithelial cells) or by direct stimulation of pattern-recognition receptors, the products of commensal bacteria promote the differentiation of T_H1 or T_H2 cells. Adapted from Blumberg and Strober, 2001.

It is unclear if the pathogenesis of UC and CD is primarily regulated by one specific cytokine, but in the case of CD, interest has been focused on IL-23 and -12. IL-12 and IL-23 are heterodimeric molecules that share a common subunit, IL-12p40. Since both these cytokines are associated with inflammation, IL-12p40 is considered a potential target for the treatment of CD (Bouma and Strober, 2003; Hyun and Mayer, 2006).

Table 3: Differential production of T cell-derived cytokines in CD and UC.

T-cell response		
Cytokine	**CD**	**UC**
IFN-γ	↑	N
IL-5	N	↑
IL-13	N	↑
IL-17	↑	N
IL-21	↑	N

IL, interleukin;
N, normal.

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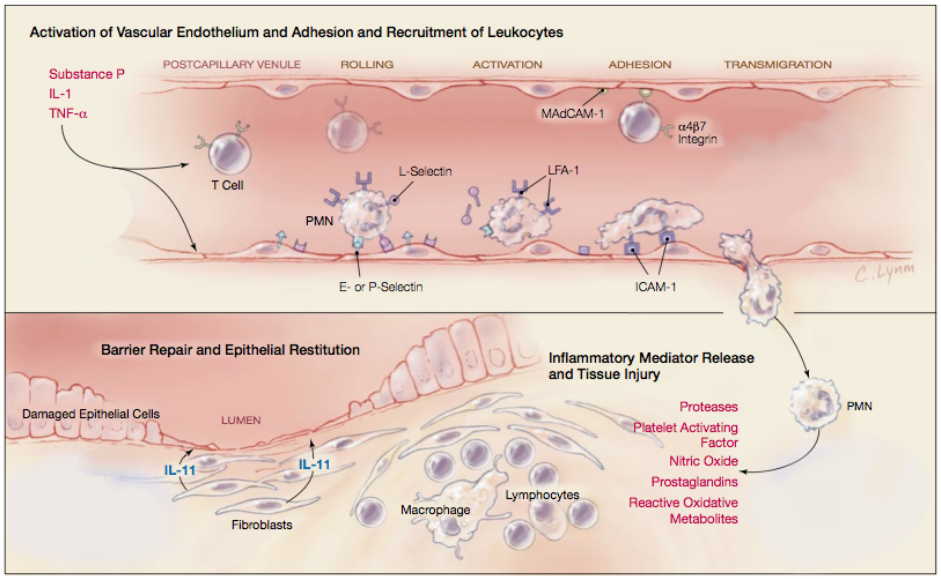

Figure 10: Inflammatory cascade and repair. After the triggering event, there is an activation of the humoral and cell-mediated immune response that stimulates the production of antibodies by B cells and the activation of macrophages, neutrophils and other leukocytes, respectively. The pro-inflammatory cytokines secreted lead to the recruitment of more leukocytes to the lamina propria. The influx of these cells leads to the production of inflammatory mediators (Proteases, nitric oxide (NO), reactive oxygen species (ROS), prostaglandins) that amplify the inflammatory process. Adapted from Blumberg and Strober, 2001.

1.2.3 Pharmacologic Therapeutic approaches

Despite increasing investigation, still there isn't a cure for IBD and the treatment options focus mainly on maintaining the highest quality of life possible for the patient. The main goals of therapy are to alleviate the symptoms by decreasing the inflammatory response, to allow lesions to heal and to prevent relapse.

1.2.3.1 Aminosalicylates

5-aminosalicylic acid (5-ASA)-based compounds are used in the treatment of patients with mild to moderately active UC and CD (Podolsky, 2002). These agents are effective in inducing remission in both UC and CD. 5-ASAs are a class of anti-inflammatory drugs that inhibit the cycloxygenase and 5-lipoxygenase pathways of arachidonic acid metabolism (Grisham, 1994). Although their mechanism of action remains not fully understood, they may reduce inflammation by scavenging free radicals, (Grisham, 1994; Podolsky, 2002; Rang et al., 2007), by inhibiting prostaglandin and leukotriene production, and by decreasing neutrophil and macrophage chemotaxis (Molin and Stendahl, 1979; Nielsen et al., 1988; Rang et al., 2007). These drugs may also protect the intestinal epithelium by enhancing the expression of heat shock proteins (Burress et al., 1997) .The 5-ASAs may be divided into sulfa-free agents, which include mesalamine, olsalazine, and balsalazide, and their parent compound, sulfasalazine (combination of sulfapyridine with 5-aminosalicylic acid). All of them can produce significant side effects, like gastrointestinal manifestations (nausea, vomiting, anorexia), headaches, allergic reactions, pancreatitis, hepatitis and pulmonitis (Kim and Ferry, 2002; Sands, 2000).

1.2.3.2 Glucocorticoids

Glucocorticoids (GC) are used as second line therapy, when 5-ASA-based compounds are inadequate, in moderate to severe exacerbations of IBD (Podolsky, 2002). They are used to treat patients during periods of disease flare in order to reduce symptoms and induce remission. These drugs are not ideal for long term treatment because of the large number of adverse effects and failure to prevent clinical relapse (Podolsky, 2002; Rang et al., 2007). Corticosteroids are systemic immunosupressors and anti-inflammatory agents that enter the cell and binds to the cytoplasmic glucocorticoid receptor (GR). The complex GC-GR interacts with others transcription factors including NF-κB, which has shown to be upregulated in IBD, blocking them.

Another mechanism of action of glucocorticoids is the inhibition of phospholipase A2 (PLA2) a key enzyme in eicosanoid synthesis, which is upregulated in IBD (Barnes, 1998; Minami et al., 1993).

Corticosteroids act by inducing molecules that downregulate inflammation and by suppressing the production of inflammatory mediators (e.g. eicosanoids, ROS). Prednisolone is currently the corticosteroid most widely used in IBD due to its high anti-inflammatory activity without significant mineralocorticoid activity (Kim and Ferry, 2002; Podolsky, 2002; Rang et al., 2007).

The side effects of these agents are plenty and include fluid retention, hyperglycemia, myopathy, emotional disturbances, adrenal suppression, dyspepsia, dysphagia and imbalanced bone metabolism (Hoes et al., 2009; Kasper D.L. et al., 2008).

1.2.3.3 Antibiotics

Regarding the central role of microflora in the development of IBD, it is expected that antimicrobial drugs ameliorate IBD (Ardizzone and Porro, 2002; Podolsky, 2002). In fact, metronizadole, an antimicrobial drug targeting aerobic microganisms has demonstrated efficacy in the treatment of mild to moderate Crohn's disease and in postsurgical prophylaxis. Ciprofloxacin, a quinole with activity against gram-negative organisms (Kim and Ferry, 2002) has also been used to treat active disease and fistulas, as a single agent or in combination with metronidazole. Both drugs cause adverse effects that include peripheral neuropathy, gastrointestinal complications (metronidazole), like increased diarrhea, and rarely, a transient skin rash (ciprofloxacin) (Kim and Ferry, 2002; Sands, 2000).

1.2.3.4 Immune modifiers

Immunomodulators act by downregulation of T cell activation, blockade of DNA synthesis and antagonism of pro-inflammatory cytokine activity. Actually, there are three immunomodulators used in the therapy of IBD: thioguanine derivates [6-mercaptopurine (6-MP) and azathioprine (AZA)], cyclosporine and methotrexate. 6-MP and AZA exert antiproliferative effects on mitotically active lymphocyte populations by inhibiting *de novo* purine synthesis with subsequent decrease of pro-inflammatory cytokine production. Associated side effects include nausea, arthralgia, hepatitis and pancreatitis. Cyclosporine is a cyclic peptide derived from the fungus *Tolypocladium inflatum* that inhibits T-lymphocyte proliferation and subsequent pro-inflammatory

cytokine synthesis (IL-4, IL-12, TNF, INF-γ) through inhibition of a serine phosphatase (calcineurin) responsible for the activation of T-cell specific transcription factors. Associated side effects include nephrotoxicity, hypertension and neurological sequelae. Methotrexate possesses immune modulating and anti-inflammatory properties and has been used to treat other chronic inflammatory diseases (e. g. rheumatoid arthritis, psoriasis). It leads to impaired DNA synthesis trough inhibition of folate-dependent enzymes, downregulation of cytokine levels (IL-1, IL-2, IL-6 and IL-8) and neutrophil chemotaxis. Associated side effects include nausea, stomatitis, diarrhea, anemia and neutropenia (Kim and Ferry, 2002; Sands, 2000).

1.2.3.5 Biological Agents

TNF-α is a cytokine that plays a central role in the induction of inflammation. The first antibody for the treatment of CD, infliximab, was approved by the Food and Drug Administration (FDA) in October 1998 (Sands, 2000). Infliximab is an IgG4 mouse/human (25%/75%) chimeric monoclonal antibody that binds to both soluble and membrane-bound TNF-α, inducing monocyte apoptosis and lysis of TNF-producing cells through antibody dependent cytotoxicity (Isaacs et al., 2005; Kim and Ferry, 2002; Neuman, 2007; Podolsky, 2002). Later, in February 2007, the FDA approved adalimumab, a humanized IgG1 monoclonal antibody that binds to soluble and membrane bound TNF-α, inducing T-cell apoptosis, for induction and maintenance of remission in moderate to severe CD, including the cases in which there is intolerance or loss of response to infliximab (Korzenik and Podolsky, 2006; Noble et al., 2008).

Adhesion molecules (e.g. α4-integrin) are very important in the inflammatory process because they mediate the leukocyte migration into tissues. α4-integrin is expressed on lymphocytes and mediates their migration via binding to the endothelial receptor, vascular cell adhesion molecule-1 (VCAM-1). In January 2008, a humanized IgG4 monoclonal antibody against α4-integrin, natalizumab, was approved by FDA to treat IBD.

Biological therapies are the most expensive drugs available targeting IBD and because of that they are reserved for later disease stages not responsive to other therapies.

1.2.3.6 Novel therapeutic approaches

Over the last decade there's been a development of novel therapeutic strategies that target specific immunological pathways in the intestinal mucosa, in contrast with traditional mainstay of IBD therapy, which consisted of non-specific, immunosuppressive and anti-inflammatory drugs. The emerging therapies focus on: i) inhibition of cytokines responsible for inducing T_{H1}/T_{H2} responses or the downstream cytokines; ii) blockade of cell-signalling pathways associated with the activation of lymphocytes and macrophages through inhibition of NF-κB; and iii) prevention of neutrophil and macrophage degranulation which would decrease the release of ROS, proteolytic enzymes and other molecules involved in tissue damage (Hanauer, 2006; Korzenik and Podolsky, 2006).

1.2.4 Surgery

At some point during their lives, two thirds to three quarters of patients with CD require surgery. Surgery is considered when medications are not working and/or complications like fistulae, abscesses or scarring and narrowing of the bowel, arise. The main goal of surgery in CD is to preserve the healthy bowel as much as possible, enabling the best possible qualify of life for the patient. The surgery consists in the removal (ressection) of the diseased segment of the bowel and associated abscess, followed by the union of the two ends (anastomosis). Despite not being a cure for CD because of the frequent recurrence at or near the site of anastomosis, it may allow the patient to have some years without symptoms.

In the case of UC, due to not completely successful therapy or arising complications, one quarter to one third of the patients require surgery. The "cure" of UC can be achieved by ablation of the diseased colon (colonectomy).

1.3 Aims

IBD can develop from many different mechanisms, which strongly suggests that CD and UC are heterogeneous diseases that share a common final pathway. Considerable progress has been made in both defining the mechanisms underlying the development of IBD and the therapies that effectively target those mechanisms, but there is still a lack of knowledge concerning the precise mechanisms involved. Although several therapies have been discovered and continue to arise, they focus mainly in controlling the symptoms of the disease and have demonstrated a wide range of side effects. Therefore, it is important to continue the search for novel therapies that

target central mechanism(s) underlying the development of IBD to, ultimately, achieve the medical cure for this illness.

Considering the crucial role of inflammation in IBD, the search for compounds capable of targeting the mechanisms involved in that response is a promising strategy for the development of new drugs with potential disease-modifying effects for therapeutic intervention in IBD. Plant extracts offer a huge diversity of compounds, most of them commercially unavailable and structurally difficult to synthesize. Therefore, essential oils, which are plant extracts composed of hydrophobic molecules small enough to diffuse across cell membranes, are valuable for the screening of pharmacologically active compounds.

In this context, the aim of this study was to screen a set of essential oils for anti-inflammatory properties towards intestinal epithelial cells. Given the central role of NF-kB in inflammation and, in particular, in IBD (Jobin and Sartor, 2000; Makarov, 2000; Schottelius and Baldwin Jr, 1999; Tak and Firestein, 2001), it is considered a potential and relevant pharmacological target. Therefore, to address the purpose of this study, we used the human colorectal adenocarcinoma cell line, C2Bbe1, to evaluate the ability of the essential oils under screen to inhibit the activation of the transcription factor, NF-κB, and the expression of its targets genes induced by pro-inflammatory cytokines. The cytokine mixture chosen for this study is composed of IFN-γ, TNF-α and IL-1β, which are known to be involved in the pathogenesis of IBD (Bouma and Strober, 2003; Ligumsky et al., 1990; MacDonald et al., 1990; Mahida et al., 1989; Murata Y et al., 1995; Reinecker et al., 1993) and are widely used in similar studies (Cavicchi and Whittle, 1999; Megías et al., 2007; Wang et al., 2008). The first screening step, aiming at discarding essential oils unlike to contain active compounds in the cell model used, consisted in evaluating the ability of the essential oils to inhibit the expression of a NF-κB-dependent gene, the inducible isoform of the nitric oxide synthase (iNOS). The essential oils found to be effective were then tested as to their ability to reduce the cytokine mixture-induced NF-κB activation by evaluating the cytoplasmic levels of total and phosphorylated forms of its inhibitory protein, IκB-α. To advance further the process of identification of active compounds, upon completion of the screening phase, the essential oil found to have the highest potency towards inhibition of NF-κB, was fractionated by Gas Chromatography/Mass Spectrometry and the resulting fractions were then screened for their ability to inhibit cytokine mixture-induced iNOS expression.

For this study, four essential oils, obtained from *Thapsia villosa, Otanthus maritimus*, *Lavandula luisieri* and *Laserpitium elasii*, were selected, based on their

composition, so that, taken together, they represented the major chemical families usually found in essential oils. The essential oil from *Annona muricata* was selected due to its etnopharmacological indication as an anti-inflammatory agent. Finally, α-pinene, the major component of the essential oil obtained from the leaves of *Juniperus oxycedrus*, was also screened, as it was previously found to inhibit IL-1-induced NF-κB activation and NO production in human chondrocytes (Neves et al., 2009).

CHAPTER II MATERIALS AND METHODS

2.1 Materials

Fetal bovine serum (FBS) was purchased from GIBCO (Invitrogen Life Technologies, California, USA). Cytokines used for cell stimulation were acquired from PeproTech (London, UK). The reagents used to prepare cell extracts, namely deoxycholic acid (DOC), sodium dodecyl sulfate (SDS) and ethylene glycol tetraacetic acid (EGTA) were purchased from Sigma-Aldrich Chemical Co. (Missouri, USA). The Polyvinylidene difluoride (PVDF) membrane and Enhanced Chemifluorescence (ECF) reagents for western blotting detection were purchased from GE Healthcare Life Sciences (Uppsala, Sweden). The detergent, Tween 20, was purchased form Fisher Scientific (Pittsburgh, PA, USA).

Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich Chemical Co. (Missouri, USA).

2.2 Cell Culture

The human colorectal adenocarcinoma cell line, C2Bbe1, a clone derived in 1988 from the Caco-2 cell line by limiting dilution (Peterson and Mooseker, 1992), was used to evaluate the ability of essential oils and their fractions to inhibit the activation of NF-κB and the expression of iNOS induced by treatment with a mixture of pro-inflammatory cytokines (Mix) composed of 1000 U/ml IFN- γ, 10 ng/ml IL-1β and 10 ng/ml TNF-α.

The cells were routinely grown at 37ºC, 5% CO_2/95% air in High glucose Dulbecco's Modified Eagle Medium (DMEM), supplemented with FBS (10% v/v), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were subcultured at a ratio of 1:3 upon reaching 80% confluence, which occurred approximately every 3-4 days.

2.3 Essential oils and respective fractions

Five essential oils obtained from the plants, *Annona muricata*, *Thapsia villosa*, *Otanthus maritimus*, *Lavandula luisieri* and *Laserpitium elasii*, were used in this study. Essential oil preparation, fractionation and chemical analysis was performed and kindly supplied by Prof. Carlos Cavaleiro from the Laboratory of Pharmacognosy, Faculty of Pharmacy/CEF, University of Coimbra (Coimbra,Portugal). Alpha-pinene (98% purity, Sigma) was also kindly supplied by Prof. Carlos Cavaleiro.

2.4 Dilution of the essential oils and their fractions

Essential oils and their fractions were successively diluted (first at 1:5 and then this solution was further diluted by 1:2 or 1:4) in DMSO (Merck) and then in culture medium to achieve the highest concentration of 0.02% (v/v) and the lowest of 0.005% (v/v), respectively. In all cases, the final DMSO concentration did not exceed 0.1%.

2.5 Stimulation and cell culture

For western blot analysis, C2Bbe1 cells were plated at 2.1 x 10^5 cell/mL in 24-well plates (1 mL/well) and cultured for at least 7 days to 9 days to allow the cells to reach a hyperconfluent state in which they become more differentiated as recommended by American Type Culture Collection. The culture medium was changed every two days. Cells were used between passage numbers 20 and 37. Upon completion of the required days in culture, the cells were left untreated for the duration of the experiment (Control) or treated with the pro-inflammatory cytokine mixture (Mix) whose composition was described under “Cell culture”, in the presence or absence of the essential oils, respective fractions or the specific NF-κB inhibitor, Bay 11-7082 (≥95% purity; Calbiochem), used as a positive control. The essential oils, fractions or control compound were added to the cell cultures 30 min before the pro-inflammatory stimulus and then further incubated for the time periods indicated in figure legends.

2.6 Assessment of cell viability

In order to select non cytotoxic concentrations of the essential oils and their fractions, the mitochondria-dependent reduction of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) salt to formazan was evaluated as a measure of cell viability (Mosmann, 1983). The absorbance of the solution resulting from the dissolution of the formazan crystals is directly proportional to the amount of reduced MTT salt, which depends on the reducing capacity of mitochondria, thus reflecting cell viability.

After culture under the relevant treatments for 18h, the cells were incubated for 1h, at 37ºC, with 0.5 mg/mL MTT solution in DMEM. The medium was then removed and the dark blue crystals of formazan were dissolved in acidified isopropanol. Formazan quantification was performed by measuring the absorbance of the corresponding solution, using an automatic plate reader (SLT Spectra) set at a test wavelength of 570 nm and a reference wavelength of 620 nm (Neves et al., 2009).

2.7 Preparation of cell extracts

After stimulation, as described above, the cells were washed twice with ice cold PBS [10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH7.4] and scraped off with ice-cold lysis buffer supplemented with protease and phosphatase inhibitors.

In order to obtain cytoplasmic and nuclear extracts, the cells were first lysed in 150 µL of buffer 1 [10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, Complete Mini (Roche Diagnostics, Indianapolis, IN, USA) diluted at 1:7, PhosStop (Roche Diagnostics, Indianapolis, IN, USA) diluted at 1:10, pH 7.5] for 15 minutes, on ice. Cell lysates were then centrifuged at 5000 rpm for 5 min at 4ºC. The supernatants – cytoplasmic extracts - were recovered and stored at -20ºC. The remaining pellets were ressuspended in 30 µL of buffer 2 [20 mM HEPES, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 300 mM NaCl, 20% (v/v) glycerol, Complete Mini (Roche) diluted at 1:7, PhosStop (Roche) diluted at 1:10, pH 7.5] and after 20 min of incubation on ice, were centrifuged at 14000 rpm during 20 min at 4ºC. The supernatants – nuclear extracts - were recovered and stored at -20ºC.

In order to obtain total cell extracts, the cells were lysed in 150 µL RIPA buffer [150 mM NaCl, 50 mM Tris-HCl, 5 mM EGTA, 1% Triton X100, 0.5% DOC, 0.1% SDS, Complete Mini (Roche) diluted at 1:7, PhosStop (Roche) diluted at 1:10, pH 7.5] and incubated on ice for 30 min. After centrifugation at 14000 rpm for 20 min at 4ºC, the supernatants - total cell extracts - were recovered and stored at -20ºC.

2.8 Protein Quantification

The protein concentration in cell lysates was measured using the bicinchoninic acid/cooper (II) sulphate protein assay kit (Sigma-Aldrich, Missouri, USA). Absorbance values were measured at 570 nm in a SLT microplate reader (SLT Spectra) and the protein concentration in the cell lysates was calculated by interpolation in a standard curve built from known concentrations of bovine serum albumin (BSA).

2.9 Western Blot Analysis

In order to detect and evaluate the expression levels of specific proteins, *immunoblot* or *western blot* assays were performed after separation of the proteins in cell extracts (total, cytoplasmic or nuclear) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The proteins in the cell extracts were denatured in 4x concentrated denaturing buffer [2.5% SDS, 0.00625 M Tris-HCl, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue, pH 6.8] for 5 min at 95ºC. For each sample, 30 μg of total protein were loaded and resolved on SDS-PAGE (10% w/v poliacrylamide gel) during approximately 1 h at 130 V [electrophoresis buffer: 100 mM Tris, 100 mM Bicine, 0.1% SDS]. Proteins were then transferred to PVDF membranes, previously activated in methanol, by electroblotting in a wet system [electroblotting buffer: 96 mM glycine, 12.5 mM Tris, 20% (v/v) methanol], at 350 mA, for 3 h at 4ºC. The protein transfer was verified by staining the PVDF membranes with Ponceau S 0.1 % (0.1 g Ponceau S, 5 mL glacial acetic acid, Milli-Q water up to 100 mL). Membranes were blocked in Tris-buffered saline [TBS (20 mM Tris, 137 mM NaCl, pH 7.6)] containing 0.1 % (v/v) Tween20 (TBS-T) and 5 % (w/v) non-fat dry milk for 2 h with constant agitation, at room temperature or overnight, without agitation, at 4ºC. Mouse monoclonal anti-human iNOS (R&D Systems, Minneapolis, MN) (1:1000), rabbit polyclonal anti-human COX-2 (AbCam, Cambridge, United Kingdom), mouse monoclonal anti-human Phospo-IκB-α (1:1000) and rabbit polyclonal anti-human IκB-α (Cell Signaling Technology, Danvers, MA, USA) (1:1000) antibodies were diluted in TBS-T containing 1% non-fat dry milk and the membranes were incubated overnight at 4ºC with constant agitation. The primary antibodies were detected by incubation of the membranes with an alkaline phosphatase-conjugated IgG secondary antibody raised against the host of the primary antibody, diluted in TBS-T with 1% non- fat dry milk, for 1 h, with constant agitation, at room temperature. After incubation with the relevant secondary antibody (anti-rabbit or anti mouse alkaline phosphatase-conjugated antibodies (1:20,000) (Amersham Biosciences)), the membranes were washed every 5 min for 30 minutes in TBS-T.

The proteins of interest were detected using the Enhanced ChemiFluorescence reagent (ECF) for alkaline phosphatase-based detection of protein blots. Alkaline phosphatase cleaves a phosphate group from the ECF substrate to yield a highly fluorescent product. The membranes were incubated with ECF for 5 minutes and the resulting fluorescent product was detected using the imager Typhoon™ FLA 9000 (GE Healthcare Life Sciences, Uppsala, Sweden).

For subsequent probing, the membranes were stripped of the antibodies using a 0.2 M NaOH solution for 5 min, at room temperature, washed several times with TBS-T, blocked with TBS-T with 5% non-fat dry milk and reprobed with primary and respective secondary antibodies. In order to control for the amount of protein loaded for each sample, the membranes were incubated, after stripping, with a mouse monoclonal anti-human actin, clone C4, antibody (Millipore Corp. Bedford, MA, USA)

(1:5000) for 1 h at room temperature and constant agitation. The intensity of the bands was evaluated using the ImageQuant software (GE Healthcare Life Sciences). For each sample of each membrane, the ratio between the intensity of the band corresponding to the protein of interest and that of the band corresponding to actin (housekeeping gene) was calculated.

2.10 Data Analysis

Statistical analysis was performed using GraphPad Prism 5 for Mac OS X (GraphPad Software, www.graphpad.com). For each experimental condition, the results are presented as the mean value ± standard deviation (SD) of, at least, 3 independent experiments. Unless stated otherwise, evaluation of the statistical significance between paired values was performed by the Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

CHAPTER III RESULTS

3.1 Effect of the essential oils on cell viability

To select non-toxic concentrations, the effect of the essential oils and α-pinene on cell viability was evaluated by the MTT assay. The results presented in figure 11 show that only the highest concentration (0.02% v/v) of the essential oil from *Annona muricata* significantly affected cell viability under the experimental conditions used. In that concentration, the essential oil from *Annona muricata* decreased MTT reduction by more than 70% relative to control cells.

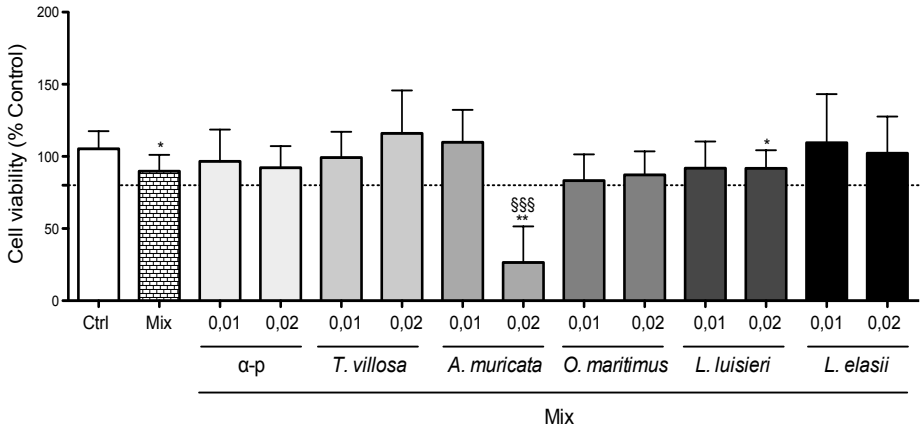

Figure 11: Effect of the essential oils on cell viability. C2Bbe1 cells were pre-incubated for 30 min with the indicated concentrations of each essential oil and then further incubated for 18h with a mixture of pro-inflammatory cytokines (Mix: 1000 U/mL INF-γ, 10 ng/mL IL-1β and 10 ng/mL TNF-α). Viability was assessed by the MTT assay, as described under "Materials and Methods", and the results expressed as the percentage relative to untreated cells (Ctrl). Each column represents the mean ± SD of, at least, 3 experiments. The dotted line represents 80% of the maximal MTT reduction. * $p < 0.05$ and ** $p < 0.01$ relative to Ctrl; §§§ $p < 0.001$ relative to Mix.

3.2 Effect of the essential oils on the Mix-induced iNOS protein levels

The results presented in figure 12 show that only three of the essential oils tested were effective in reducing the mix-induced expression of iNOS, although with different potencies. The greatest inhibition was achieved with the highest concentration (0.02%, v/v) of the essential oil from *Lavandula Luisieri*, which reduced iNOS levels to 16.8 ± 12.0% of those found in cells treated with the Mix alone. Although much less efficiently, the highest concentration (0.02%, v/v) of the oils from *Laserpitium elasii*

(54.3 ± 18.3%) and *Otanthus maritimus* (61.8 ± 9%) also reduced iNOS levels relative to those found in cells treated with the Mix alone. There is a statistically significant difference between the inhibitory effect of the lowest and the highest concentrations of *Lavandula luisieri* (p = 0,0037), which suggests a dose-response relationship.

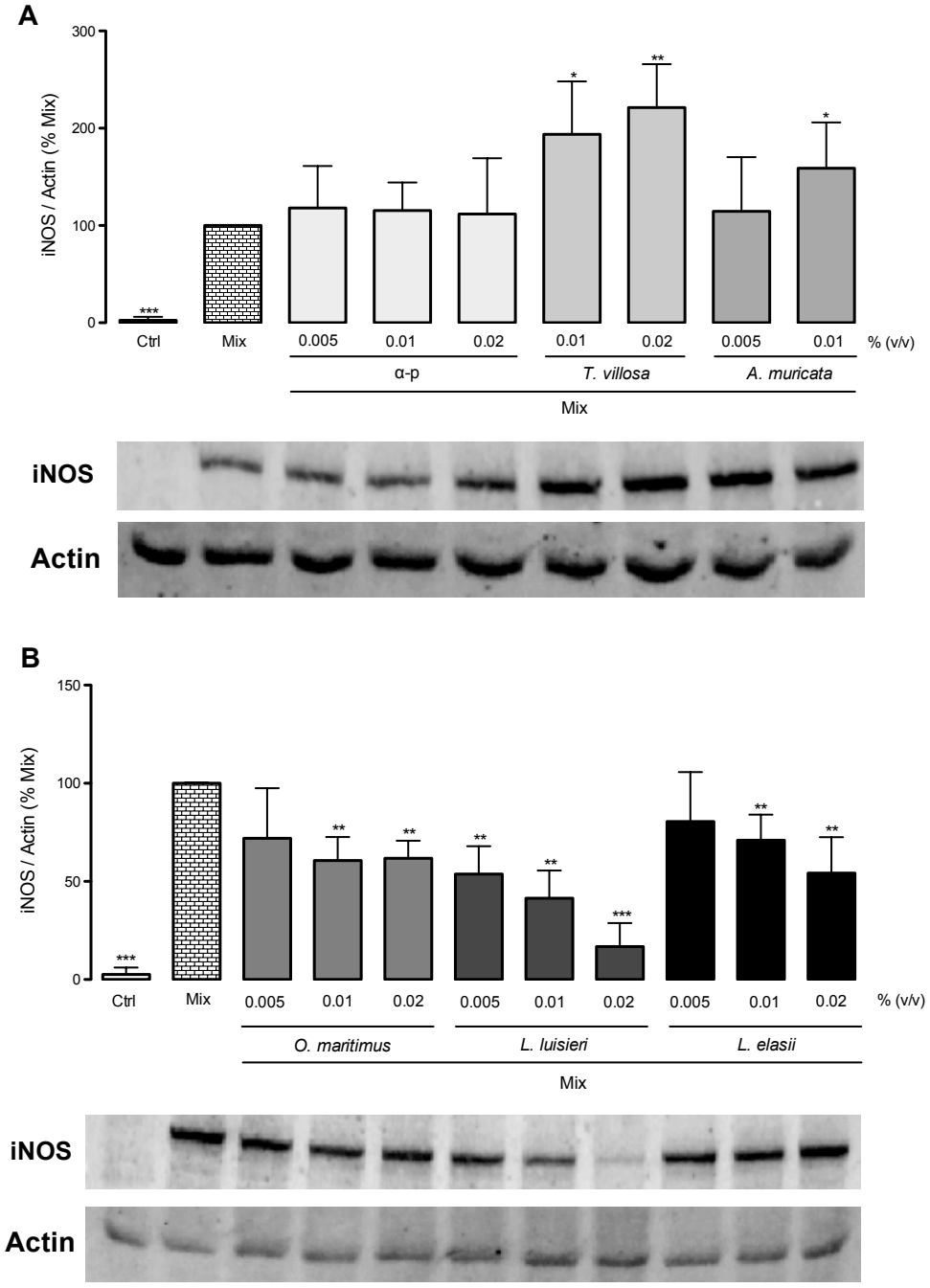

Figure 12: Effect of α-pinene and essential essential oils from *Thapsia villosa, Annona muricata* (A), *Otanthus maritimus*, *Lavandula luisieri* and *Laserpitium elasii* (B) on iNOS expression. C2Bbe1 cells were pre-incubated for 30 min with the indicated concentrations of each essential oil and then further incubated for 18 h with a mixture of pro-inflammatory

cytokines (Mix) composed of INF-γ (1000 U/ml), IL-1β (10 ng/ml) and TNF-α (10 ng/ml). iNOS expression was assessed by western blot analysis and the intensities of the iNOS bands were normalized to the intensity of the respective actin band. Results are expressed as the percentage relative to Mix-treated cells. Each column represents the mean ± SD of, at least, 4 experiments. The images shown are representative of the results obtained in all experiments. Ctrl: untreated cells. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ relative to Mix.

3.3 Effects of *Lavandula luisieri* and *Laserpitium elasii* on the phosphorylation and degradation of IκB-α

In order to determine if the inhibitory action of *Lavandula luisieri* and *Laserpitium elasii* on iNOS expression was due to effects on NF-κB activation, the phosphorylation of IκB-α was examined by western blot.

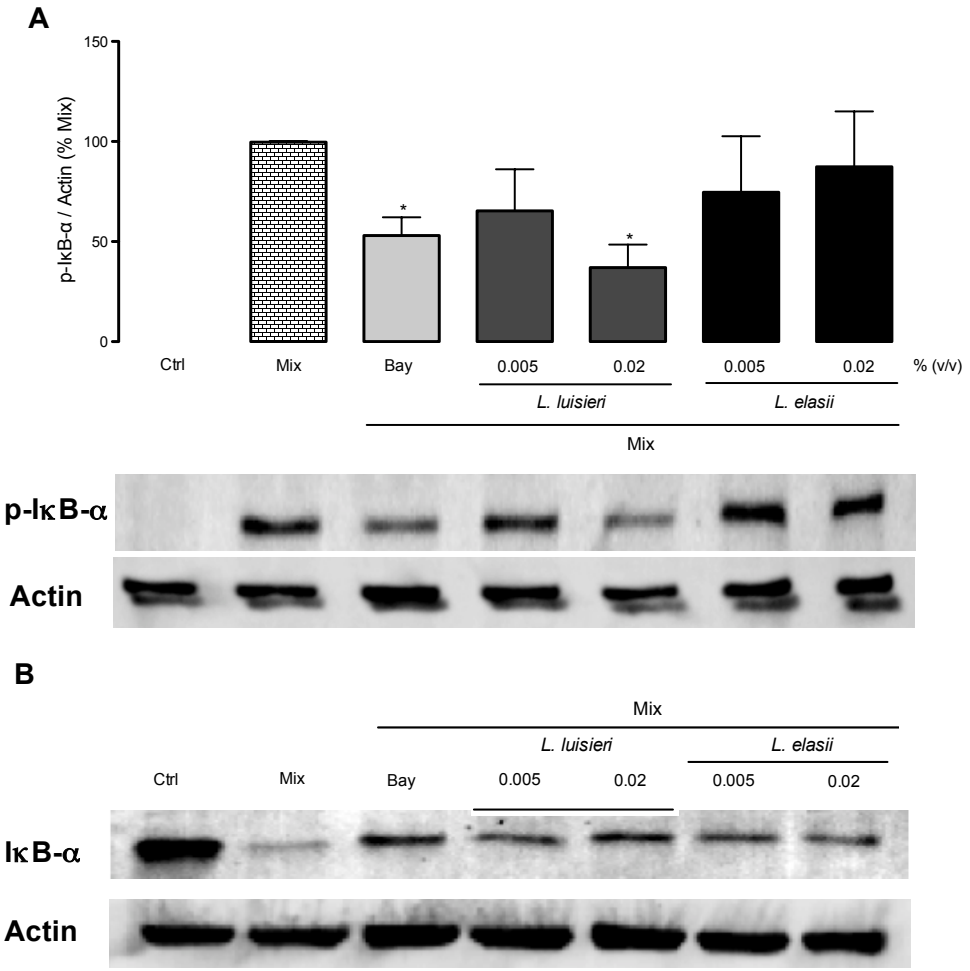

Figure 13: Effect of the essential oils from *Lavandula luisieri* and *Laserpitium elasii* on p-IκB-α protein levels. C2Bbe1 cells were preincubated for 30 min with or without the indicated concentrations of each

essential oil or the specific NF-κB inhibitor, Bay 11-7082, and then incubated for 5 min (A) or 30 min (B) with a mixture of pro-inflammatory cytokines (Mix) composed of INF-γ (1000 U/ml), IL-1β (10 ng/ml) and TNF-α (10 ng/ml). Phospho-IκB-α (p-IκB-α) levels were assessed by western blot and the intensities of the bands normalized to the intensity of the respective actin band. Results are expressed as the percentage relative to cells treated with Mix alone. Each column represents the mean ± SD of at least 3 experiments. The images shown are representative of the results obtained in all experiments. Ctrl: untreated cells. * $p < 0.05$ relative to Mix. B) Image showing the effect of the essential oils from *Lavandula luisieri* and *Laserpitium elasii* on total IkB-α protein levels (preliminary results, n=1).

After 5 min of stimulation with the Mix, as expected, there was an increase in the serine phosphorylated IκB-α protein (detected by a Ser32-phospho-specific IκB-α antibody). Both the NF-κB inhibitor, Bay 11-7082 (Bay), and the highest concentration of the essential oil from *Lavandula luisieri* showed a significant reduction of the Mix-induced IκB-α phosphorylation (47 ± 9.2% and 63 ± 11.5% respectively, relative to Mix-treated cells) (Figure 13A). To further confirm the ability of the essential oil from *Lavandula luisieri* to inhibit NF-κB activation, total IκB-α levels were also evaluated. Preliminary results (Figure 13B) suggest that, at least, the highest concentration of that oil was effective in increasing the levels of total IκB-α. This is further supported by the finding that under the same conditions, the specific NF-κB inhibitor, Bay, was also effective in increasing the amount of IκB-α, as expected.

3.4 Effects of *Lavandula luisieri* fractions on cell viability

Since the essential oil from *Lavandula luisieri* showed the highest inhibitory activity, it was fractionated and the resulting four fractions (named A to D) tested for cell viability in concentrations equal or below 0.02% (v/v).

None of the fractions tested significantly affected cell viability, either when combined with the Mix (Figure 14A) or alone (Figure 14B).

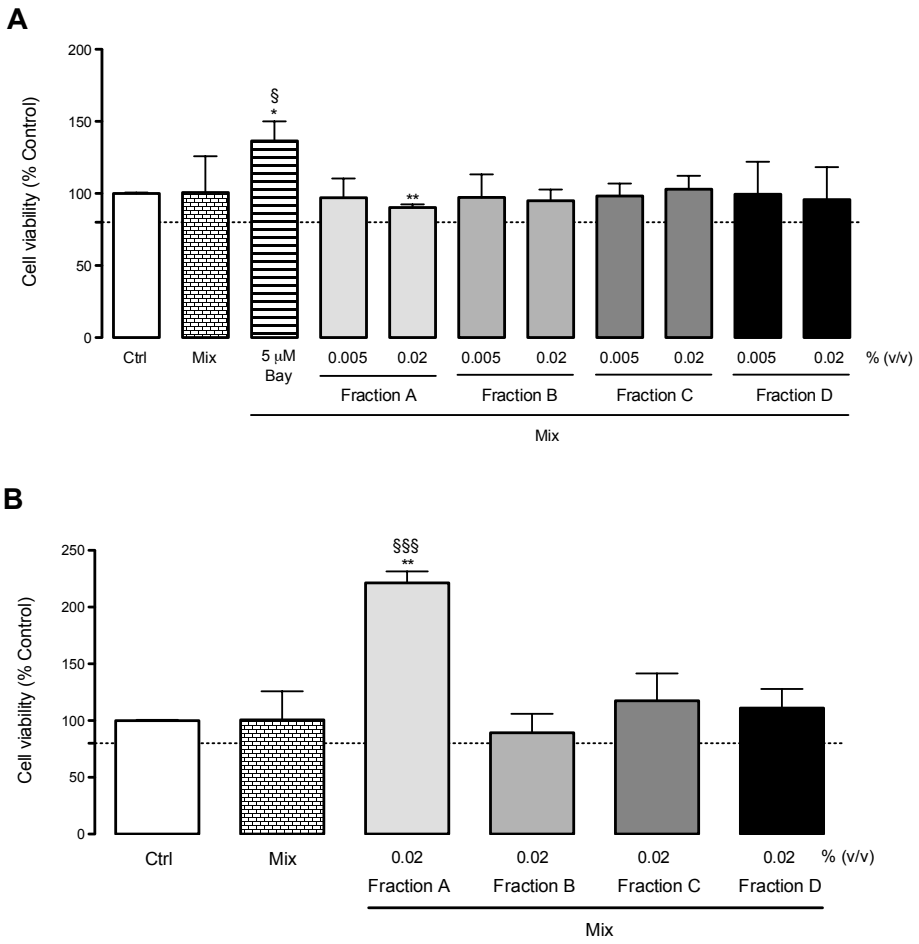

Figure 14: Effect of *Lavandula luisieri* fractions on cell viability. C2Bbe1 cells were preincubated for 30 min with the indicated concentrations of each fraction of the essential oil from *Lavandula luisieri* and then further incubated for 18 h in the presence (A) or absence (B) of a mixture of pro-inflammatory cytokines (Mix) composed of INF-γ (1000 U/ml), IL-1β (10 ng/ml) and TNF-α (10 ng/ml). Viability was assessed by the MTT assay and the results expressed as the percentage relative to untreated cells (Ctrl). Each column represents the mean ± SD of at least 4 experiments. * $p < 0,05$ and ** $p < 0.01$ relative to Ctrl. § $p< 0,05$ and §§§ $p < 0.001$ relative to Mix.

3.5 Effects of *Lavandula luisieri* fractions on the Mix-induced iNOS protein expression

Using the strategy described for the whole essential oils, western blot analysis was performed to determine the ability of the fractions to inhibit iNOS expression. The results obtained (Figure 15B) show that only fractions C and D were effective in reducing iNOS expression. In its highest concentration, fraction D reduced the Mix-induced iNOS levels to 50.3 ± 13.3% of those obtained in cells treated with the Mix

alone, whereas both fraction C concentrations elicited significant reductions of iNOS levels (78.7 ± 5.5% and 28.6 ± 14.2% respectively, relative to the Mix alone). The statistically significant difference (p = 0.0006) between the inhibitory effects of the lower and the highest concentrations of fraction C, suggests a dose-response relationship.

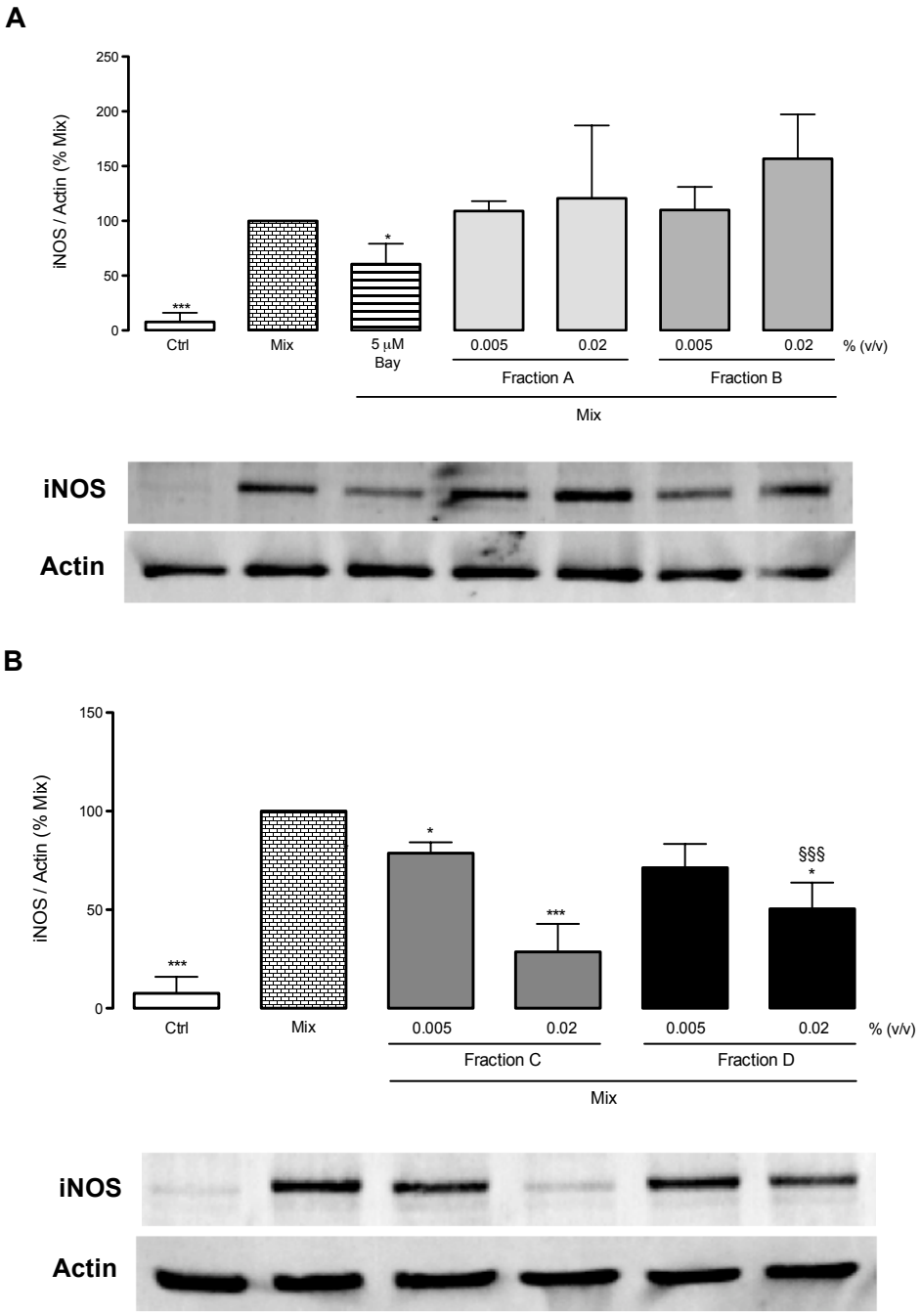

Figure 15: Effect of *Lavandula luisieri* fractions on iNOS expression. C2Bbe1 cells were preincubated for 30 min with the indicated concentrations of each of the four fractions obtained from the essential oil from *Lavandula luisieri* or with the specific NF-κB inhibitor, Bay 11-7082, and then incubated for 18 h with a mixture of pro-inflammatory cytokines (Mix), composed of

INF-γ (1000 U/ml), IL-1β (10 ng/ml) and TNF-α (10 ng/ml). iNOS expression was assessed by western blot and the intensities of the bands normalized to the intensity of the respective actin band. Results are expressed as the percentage relative to cells treated with Mix.. Each column represents the mean ± SD of at least 3 experiments. The images shown are representative of the results obtained in all experiments. Ctrl: untreated cells. * $p < 0.05$ and *** $p < 0.001$ relative to Mix.

CHAPTER IV DISCUSSION

4.1 Discussion

As previously stated under “Aims”, the main goal of this work was to screen essential oils, representing the major chemical families found in such plant extracts, for anti-inflammatory properties, namely inhibition of NF-κB activation, towards intestinal epithelial cells.

To ensure that any apparent reduction of the effects induced by the pro-inflammatory stimulus used (Mix) was due to a specific inhibitory action and not to possible cytotoxic effects of the essential oils, the MTT reduction assay was used to assess cell viability. As mentioned in the results section, only the highest concentration of the essential oil from *Annona muricata* (0.02%, v/v) was cytotoxic to the cell line used. Nonetheless, the Mix (89.7 ± 11.5) alone and the highest concentration (0.02%, v/v) of the oil from *Lavandula luisieri* (91.8 ± 12.6) showed a small, but statistically significant decrease of the MTT reduction capacity ($p<0.05$) in comparison with untreated cells. Since the decrease observed in the cells treated with the Mix alone was identical to that found in cells treated with the Mix in the presence of 0.02% of the essential oil from *Lavandula luisieri* it seems likely that such decrease was due to the Mix or any of its components and not to the essential oil. It can be argued, however, that in such case, a similar diminution should be visible with all the other essential oils tested, as the Mix was always present. This possibility was ruled out for the essential oil from *Lavandula luisieri* by testing the effect of its fractions on cell viability in the absence of the Mix (Figure 14B). The results obtained show that none of the fractions significantly decreased the amount of MTT salt reduced, while fraction A actually elicited a significant increase. On the other hand, when the cells were treated with that fraction in the presence of the Mix, the increase was completely reversed and a small but significant decrease, similar to that obtained with the whole essential oil, was found (Figure 14A). Furthermore, the decrease observed with the Mix in figure 11 is not apparent in the results presented in figure 14. Thus, it seems likely that the decrease apparent in figure 11 is indeed due to the Mix, but is so small that it would likely be overridden by increasing the number of experiments.

Having identified non-cytotoxic concentrations for each essential oil, the screening proceeded by evaluating their ability to inhibit the Mix-induced iNOS expression. Despite, in human chondrocytes, α-pinene was shown to decrease IL-1-induced NF-κB activation and NO production (Neves et al., 2009), it did not reduce iNOS expression induced by the cytokine mix in the human colorectal adenocarcinoma cell line, C2Bbe1, used as a model in this study (Figure 12). The use of different stimuli in the two studies may have contributed to the apparent discrepancy. Moreover, cell

differences may also account for the lack of effect of α-pinene on the intestinal cell line. Accordingly, the possibility that higher concentrations of α-pinene are required to inhibit iNOS expression in intestinal epithelial cells cannot be ruled out. Nonetheless, since the purpose of the first screening step was to discard the essential oils/compound most unlike to be effective in IBD, the actions of α-pinene were not further investigated.

The oils from *Thapsia villosa* (in both concentrations used) and *Annona muricata* (in the highest concentration used) were also discarded since they significantly increased the expression of iNOS induced by the Mix. Even though this effect can be due to an intrinsic ability of the essential oil or its components to activate signal transduction pathways that culminate on induction of iNOS expression, as found for other compounds of natural origin (Nakamura et al., 2006) the possibility that those essential oils were contaminated with even trace amounts of endotoxin cannot be discarded. Indeed, in such cases, it is important to test the essential oils for contamination with endotoxins, which was out of the scope of this study and also impossible due to time constraints.

In contrast, other oils, namely those from *Otanthus maritimus*, *Laserpitium elasii* and *Lavandula luisieri*, showed a significant ability to inhibit the Mix-induced expression of iNOS (Figure 12). That effect was more evident and statistically significant with the essential oil from *Lavandula luisieri*, which was the only one effective in the lowest concentration (0.005%, v/v) tested, showing also the largest inhibitory effect in the highest concentration used (0.02%, v/v). Thus, this oil, as well as that obtained from *Laserpitium elasii* which was the second more effective, were further tested to evaluate their ability to inhibit NF-kB activation induced by the cytokine mix.

Since the specific NF-kB inhibitor, Bay, effectively decreased the mix-induced iNOS expression as well as the phosphorylation of IκB-α, it strongly suggests that the Mix-induced iNOS expression in the human intestinal cell line, C2Bbe1, is mediated by the NF-κB pathway. This is in agreement with other studies in this as well as in the parent cell line, Caco-2 (Cavicchi and Whittle, 1999; Megías et al., 2007; Romier et al., 2008). Accordingly, the highest concentration of the essential oil from *Lavandula luisieri* which decreased iNOS expression, also reduced the Mix-induced NF-κB phosphorylation (Fig. 13A) and degradation (Fig. 13B). Although the lowest concentration of the oil from *Lavandula luisieri* showed a significant decrease in iNOS expression, it wasn't accompanied by a similar reduction in the levels of phosphorylated IκB-α, which suggests that other mechanisms besides inhibition of NF-κB activation, may account for its ability to inhibit the Mix-induced iNOS expression. Besides containing binding sites for binding sites for NF-κB, the promoter region of the

iNOS gene, also contains binding sites for other transcription factors, namely AP-1 (Kuo et al., 1997; Rachmilewitz et al., 1995; Xie et al., 1994); .Activation of AP-1 is known to be downstream of the mitogen-activated protein kinase signalling pathway and p38MAPK was shown to increased in Caco-2 cells treated with the same citokines used in this study (Wang et al., 2008). Thus, the lowest concentration of the essential oil from *Lavandula luisieri*, could be exerting its anti-inflammatory activity via inhibition of AP-1.

Finally, screening of the fractions obtained from the oil of *Lavandula luisieri* identified fractions C (0.02 and 0.05%, v/v) and D (0.02%, v/v) as being effective in inhibiting iNOS expression (Figure 15). The statistically significant difference ($p < 0.001$) between the inhibitory effects of the lower and the highest concentrations of fraction C, suggests a dose-response relationship. The inihibitory activity of more than one fraction of the oil from *Lavandula luisieri* suggests that the activity of the whole oil may represent the sum of the effects of different compounds acting on the same target and/or the synergistic effect elicited by the action of different compounds on distinct targets that converge on the same response.

On the other hand, the results obtained did not support, in the model used, an anti-inflammatory action for the essential oil of *Annona muricata*, as indicated etnopharmacologically. Nonetheless, the possibility that such an action occurs through mechanisms other than the inhibition of NF-κB cannot be exclude by the present study.

4.2 Conclusions

In summary, this study shows that among the essential oils from *Thapsia villosa, Annona muricata*, *Otanthus maritimus*, *Lavandula luisieri* and *Laserpitium elasii,* only the one from *Lavandula luisieri* has anti-inflammatory activity potentially relevant in IBD. To our knowledge, this is the first study that associates the essential oil from *Lavandula luisieri* with anti-inflammatory activity. Moreover, this study allowed the identification of two fractions of that oil that likely contain the compound(s) responsible for inhibition of iNOS expression and NF-κB activation. Further fractionation and screening is, thus, mandatory to identify the active compound(s) and characterize in more detail the corresponding mechanism(s) of anti-inflammatory action. Such studies will pave the way for further chemical and pharmacological optimization of the active compounds identified and may ultimately lead to the development of novel and hopefully more effective molecules for the treatment of IBD.

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