



## DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA  
UNIVERSIDADE DE COIMBRA

# The Role of Polyphenols and Reactive Nitrogen Species in Inflammatory Conditions of the Gastrointestinal Tract

Elisabete Priscila Pinto Ferreira

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor João Laranjinha (Faculdade de Farmácia da Universidade de Coimbra)

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# Abbreviations

AJ- adherent junction  
AP-1-activator protein-1  
ARE-antioxidant response element  
BH<sub>4</sub>- (6R)-5,6,7,8-tetrahydrobiopterin  
BTB-broad complex tramtrack bric-a-brac  
bZIP- basic region-leucine zipper  
CaM-calmodulin  
CD- cluster of differentiation  
CTR- carbon terminal region  
DAPI - 4',6-diamidino-2-phenylindole  
DGR-Kelch domain/diglycine repeats  
DMSO – dimethyl sulfoxide  
DNA-deoxyribonucleic acid  
ECAMs- endothelial cell adhesion molecules  
EDRF- endothelium derived relaxing factor  
EDTA- ethylenediamine tetraacetic acid  
ELISA - enzyme-linked immunosorbent assay  
eNOS-endothelial NOS  
EpRE- electrophile response element  
ERK- extracellular signal-regulated kinases  
FAD- flavin adenine dinucleotide  
FMN- flavin mononucleotide  
GI – Gastrointestinal  
GSH-reduced glutathione  
GSK3 $\beta$ -glycogen synthase kinase 3 $\beta$   
GSSG-oxidized glutathione  
Hsp90- heat-shock protein 90  
IBD – Inflammatory Bowel Disease  
ICAM-1- intercellular adhesion molecule-1  
IEC- intestinal epithelial cell  
IFN- interferon  
IKK- I $\kappa$ B kinase  
IL- interleukin

iNOS-inducible NOS  
IVR-intervening/linker region  
I $\kappa$ B- inhibitor of NF- $\kappa$ B protein  
JNK- c-Jun N-terminal kinase  
Keap1 - Kelch-like ECH-associated protein 1  
MnSOD- manganese superoxide dismutase  
MTT - 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromid  
NADPH- nicotinamide adenine dinucleotide phosphate reduced  
Neh- Nrf2- ECH homology 2  
NEM- N-ethylmaleimide  
NEM- N-ethylmaleimide  
NF- $\kappa$ B-nuclear factor-k-light chain enhancer of activated B cells  
NLS- nuclear localization sequence  
nNOS- neuronal NOS  
NOD- nucleotide-binding oligomerization domain  
•NO - nitric oxide  
NOS- Nitric oxide synthase  
Nrf2-Nuclear factor erythroid 2- related factor 2  
NTR-N-terminal region  
ONOO<sup>-</sup> - peroxynitrite  
OPT- *o*-phthalaldehyde  
PAMP- pathogen-associated molecular patterns  
PBS - Phosphate-buffered saline  
PCR- polymerase chain reaction  
PIN-protein inhibitor of NOS  
PKC- protein kinase C PMNs- polymorphonuclear neutrophils  
PPR- pattern recognition receptors  
RHD- Rel homology domain  
RNS-reactive nitrogen species  
ROS-reactive oxygen species  
RWE-Red Wine Extract  
sGC-guanylate cyclase  
TFF- trefoil factors  
TJ- tight junction  
TLR- toll-like receptor

TNF- tumor necrosis factor

TNFR- tumor necrosis factor receptor

ZO- zonula occludens



# Abstract

Inflammatory Bowel Diseases (IBD) are a group of chronic inflammatory diseases of the gastrointestinal tract with a high degree of incidence worldwide. Nowadays, a specific treatment of IBD is still not available and the most currently drugs used in its treatment are associated with significant side effects that limit their use. The lack of effectiveness and the adverse effects of standard therapies have increased the need for searching new treatment strategies that combine efficacy and safety.

Dietary polyphenols have been shown to exert beneficial effects on human health but the underlying mechanism are still a matter of controversy. Initially though to be antioxidants *in vivo*, because of extensive biotransformation and poor bioavailability, it is now widely accepted that this is an unlike activity mediating their biological impact.

In fact, it has been shown that the anti-inflammatory effect of polyphenols (among others) cannot be merely explained on basis of their antioxidant capacity and it is now known that the redox regulation of several signal transduction pathways must be implied to explain their cellular effects.

Red wine is very rich in these phenolic compounds and in the last years numerous studies described health-promoting effects of this beverage, including anti-inflammatory proprieties, but the molecular mechanisms underlying its protective role remain largely unknown. A clear understanding of the molecular mechanisms of action of polyphenols is crucial in the valuation of these potent molecules as potential prophylactic and therapeutic agents in IBD.

Given the fact that the gastrointestinal tract is a compartment where the dietary polyphenols reach high concentrations in a non-modified structure this work pretends to evaluate the potential anti-inflammatory effect of a red wine polyphenolic extract (RWE) in gastrointestinal inflammation and investigate which are the mechanisms involved in its anti-inflammatory action. Particularly, to determine if RWE have the capacity to modulate nitric oxide fluxes, activate the Nrf2 pathway leading to the induced expression of cytoprotective genes and also affect the NF- $\kappa$ B pathway using cultured intestinal cell models.

Overall, results suport that RWE have a protective effect against inflammation not compromising cell viability. Mechanistically, this conclusion is supported by the interference with cellular inflammatory pathways, inhibiting the production of

inflammatory mediators. In fact, RWE inhibited I $\kappa$ B degradation induced by TNF- $\alpha$ , partially suppressed TNF- $\alpha$  -induced IL-8 overproduction and prevented the iNOS protein expression induced by cytokines, thus leading to a significant reduction in •NO overproduction. RWE also reduce the levels of tyrosine nitration and alter occludin expression and distribution. Furthermore RWE also have an effect in the Nrf2 pathway increasing its translocation to the nucleus and the expression of its target genes.

**Keywords:** Inflammatory Bowel Diseases; Red Wine polyphenolic extract; Anti-inflammatory effects; Nitric Oxide; Nrf2.

# Resumo

As Doenças Inflamatórias Intestinais (DII) são um grupo de doenças inflamatórias graves do trato gastrointestinal com um elevado grau de incidência na população mundial. Atualmente ainda não existe um tratamento específico para DII e os fármacos mais frequentemente usados no seu tratamento estão associados a efeitos adversos significativos que limitam o seu uso. A falta de efetividade e os efeitos adversos das terapias atuais têm aumentado a necessidade de procurar novas estratégias terapêuticas que combinem eficácia e segurança.

Os polifenóis da dieta têm vindo a demonstrar exercer efeitos benéficos para a saúde humana mas os mecanismos moleculares subjacentes são ainda tema de controvérsia. Inicialmente pensava-se que estes compostos atuassem como antioxidantes *in vivo* mas devido à sua extensa biotransformação e reduzida biodisponibilidade é agora aceite que o seu impacto biológico não se deve meramente a esta característica.

De facto, foi provado que os efeitos anti-inflamatórios (entre outros) dos polifenóis não podem ser somente explicados pela sua capacidade antioxidante e sabe-se agora que a regulação redox de várias vias de transdução de sinal devem estar implicadas na explicação dos seus efeitos celulares.

O vinho tinto é muito rico nestes compostos e nos últimos anos numerosos estudos descreveram efeitos benéficos para a saúde tais como propriedades anti-inflamatórias, no entanto os aspectos fundamentais dos mecanismos de ação moleculares subjacentes à sua ação protetora permanecem ainda por esclarecer. Uma clara compreensão dos mecanismos moleculares de ação dos polifenóis é crucial na validação destas promissoras moléculas como potenciais agentes profiláticos e terapêuticos em DII.

Dado que o trato gastrointestinal é um compartimento em que os polifenóis da dieta atingem concentrações elevadas numa estrutura não-modificada, este trabalho pretende avaliar o potencial efeito anti-inflamatório de um extrato polifenólico de vinho tinto (EVT) na inflamação gastrointestinal e investigar quais os mecanismos envolvidos neste efeito. Particularmente determinar se os EVT possuem a capacidade de modular fluxos de óxido nítrico, ativar a via do Nrf2 levando à indução da expressão de genes citoprotetivos e também afectar a via do NF- $\kappa$ B usando um modelo de células intestinais, as células HT-29.

Globalmente, os resultados demonstram que os EVT possuem um papel protetor contra a inflamação não comprometendo a viabilidade celular. Esta conclusão é mecanisticamente suportada pela interferência com vias celulares de inflamação. De facto, o EVT inibiu a degradação de I $\kappa$ B induzida por TNF- $\alpha$ , suprimiu parcialmente a sobreprodução de IL-8 e preveniu a expressão de iNOS induzida por citocinas o que levou a uma significativa redução da sobreprodução de  $\bullet$ NO. O EVT também diminui os níveis de nitração de tirosina e a expressão de ocludina foi reduzida e alterada. Para além disso EVT também afectam a via de sinalização do Nrf2 aumentando a sua translocação para o núcleo e aumentando a expressão dos seus genes alvo.

**Palavras-chave:** Doenças inflamatórias intestinais; Extratos polifenólicos de vinho tinto; Efeitos anti-inflamatórios; Óxido Nítrico; Nrf2.

# Chapter 1: Introduction

## 1.1 - Inflammatory Diseases of the Gastrointestinal Tract

Inflammatory diseases of the gastrointestinal (GI) tract comprehend a great variety of illnesses with different symptoms and manifestations in the different parts of the GI tract. Gastrointestinal inflammation influences the function of the mucosal layer that lies closest to the luminal contents and also alters the ability of the mucosa to resist injury and its capacity to undergo repair once injury has occurred (Wallace J. L. et al, 2001).

### 1.1.1 - Inflammatory Bowel Diseases

#### *-Definition and etiology*

Inflammatory bowel diseases (IBDs) are a group of chronic inflammatory diseases of the gastrointestinal tract which major forms are ulcerative colitis and Crohn's diseases that have been well characterized by clinical, pathological, endoscopic and radiological features (Xavier R. J. et al, 2007). These diseases are characterized by a deregulated synthesis and release of a variety of proinflammatory mediators, including cytokines, reactive oxygen species (ROS) and nitric oxide (\*NO), resulting in a disruption of epithelial barrier, excessive tissue injury and persistent inflammatory state (Scaldaferri F. et al, 2007).

The etiology of IBD remains unclear but is thought to involve a combination of environmental, genetic and immunobiological factors. Both ulcerative colitis and Crohn's diseases are polygenic diseases, genome-wide scans have identified multiple genes that contribute to diseases susceptibility. Genetic factors discovered to date affect barrier function and innate and adaptive immunity (Xavier R. J. et al, 2007).

The onset of IBD typically occurs in the second and third decades of life. The existent data suggest that the worldwide incidence rate of ulcerative colitis varies greatly between 0.5–24.5/100,000 persons, while that of Crohn's disease varies between 0.1–16/100,000 persons worldwide, with the prevalence rate of IBD

reaching up to 396/100,000 persons (Lakatos P.L., 2006). This group of diseases compromise severely the life quality of patients and could also be a risk factor to the development of other pathologies such as colorectal cancer.

### ***-Major forms***

#### Ulcerative colitis

Ulcerative colitis is a relapsing, non-transmural inflammatory disease restricted to the colon. It is characterized by severe inflammation and concomitant production of a complex mixture of inflammatory mediators with the development of extensive superficial mucosal ulceration. Histopathological features include the presence of a significant number of neutrophils within the lamina propria and the crypts where they form micro-abscesses (fig.1). Depletion of goblet cell mucin is also common (Baumgart D. C. et al, 2007).

#### Crohn's disease

Crohn's disease is a relapsing transmural inflammatory disease of the gastrointestinal mucosa that can affect the entire gastrointestinal tract. Usual presentations include the discontinuous involvement of various portions of the gastrointestinal tract and the development of complications including strictures, abscesses or fistulas. This disease is characterized by aggregation of macrophages that frequently form non-caseating granulomas (fig. 1) (Baumgart D. C. et al, 2007).

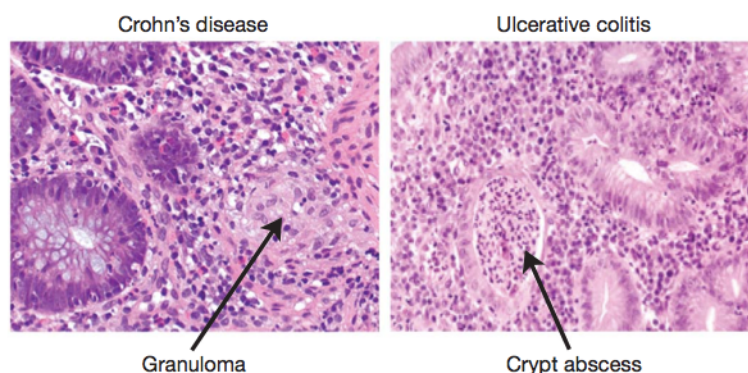


Figure 1: "Histologic hallmarks of IBD: clues to immunopathogenesis. Left panel, Crohn's disease—biopsy from a terminal ileum with active disease. The figure illustrates a discrete granuloma composed of compact macrophages, giant cells and epithelioid cells. Surrounding the nodule there is marked infiltration of lymphoid cells, plasma cells and other inflammatory cells, but there is no necrosis. Right panel, Ulcerative colitis—colonic mucosal biopsy taken from a patient with active disease. The crypt abscess is composed of transmigrated neutrophils and the surrounding epithelium exhibits features of acute mucosal injury" (Xavier R. J et al, 2007).

### **1.1.2 - The intestinal barrier in IBD**

The intestinal barrier is a dynamic and complex structure that ensures the intestinal homeostasis separating the intestinal contents from the host tissues, regulating the nutrient absorption and allowing interactions between the resident bacterial flora and the mucosal immune system. This barrier is composed by a thick mucus layer containing antimicrobial products, intestinal epithelial cells (IECs) and an underlying set of cells (mesenchymal cells, dendritic cells, lymphocytes and macrophages) (Roda G. et al, 2010).

#### ***Mucus layer***

The mucus layer is synthesized by goblet cells and it is composed of a large and complex variety of molecules. It acts as a chemical barrier preventing pathogen invasion. The viscosity of the mucus given by the presence of glycosylated mucins and trefoil factors (TFFs) is the most important feature in the protection of the mucosa. Other constitutive elements of the mucus are secretory immunoglobulins produced by B-lymphocytes, antimicrobial peptides, such as defensins and lectins, secreted by Paneth cells, antimicrobial protease inhibitors synthesized by epithelial cells and enterocyte hydrophobic phospholipids. In IBD is observed a decrease in the mucus viscosity with reduction of TFFs and the thinning of the mucus layer what leads to a lessened capability of the mucus layer to limit antigenic traffic and bacterial translocation in the lamina propria (Roda G. et al, 2010).

#### ***Epithelial junctions***

The intestinal permeability is regulated through the epithelial junctions consisted by desmosomes, adherent junctions (AJs) and tight junctions (TJs). They are necessary for maintenance of intercellular adhesion and regulation of paracellular transport being responsible to limit the access of microbes to host tissues and to mediate the antigenic traffic from the lumen to the lamina propria where they are processed, presented and eliminated (Roda G. et al, 2010).

Impairment of the tight junctions seems to have higher impact in IBD pathogenesis (Clayburgh D. et al, 2004). Tight junctions consist of a complex structure composed by different proteins such as transmembrane proteins like

occludin and proteins of the claudin family and cytoplasmic peripheral membrane proteins like ZO (zonula occludens)-1,2 and 3 (Clayburgh D. et al, 2004).

Recent studies have showed that elevated levels of proinflammatory cytokines, observed in IBD, induce expression alterations of some proteins like claudin and occludin and reorganization of others what leads to an impairment of tight junctions and consequently loss of barrier function (Zeissig S. et al 2007; Mankertz J. et al, 2000).

### ***Defensins***

Defensins are antimicrobial peptides produced by the intestinal mucosa that contribute to maintaining host immunity and protect from pathological flora. Defensins causes micropores in bacterial membranes what leads to the lost of pathogen integrity and consequent antimicrobial activity (Clayburgh D. et al, 2004).

In IBD there is a deficiency in the defensin expression but if it is one of the causes or a consequence of the disease is yet to be known (Clayburgh D. et al, 2004).

### ***Intestinal epithelial cells (IECs)***

Intestinal epithelial cells can be considered important immunoeffector cells with the capacity to release cytokines, chemokines, and other molecules involved in antigen presentation and immune defence.

It is known that the innate immune system is able to recognize a limited set of conserved bacterial and viral motifs, pathogen-associated molecular patterns (PAMPs), through pattern recognition receptors (PPRs). The Toll-like receptor (TLR) and the nucleotide-binding oligomerization domain (NOD) are families of PPRs. IECs express several members of the TLR family (TLR 1,2,3,4,5) which recognize specific PAMPs and activate signal transduction through the NF- $\kappa$ B pathway (Yamamoto-Furusho J. et al , 2007).

Recognition by TLRs protects against pathogens and is carefully regulated to shut down a proinflammatory response to comensal organisms. Alterations of TLRs expression and polymorphisms have been described in IBD causing amplification of inappropriated immune responses (Yamamoto-Furusho J. et al , 2007).



IEC also secrete proinflammatory cytokines like interleukin-6 and and in the last ten years research groups have discovered that they also secrete cytokines that regulate inflammation with chemoattractant proprieties for leukocytes and neutrophils (chemokines) and more recently have also shown that the cells also secrete chemokines for monocytes and lymphocytes (Stadnyk A. W.,2002).

IECs are also involved in the antigen presentation activating subsets of T-cells with regulatory function and they cross talk with mucosal lymphocytes.

In IBD during acute inflammatory episodes, the mucosal lining displays a characteristic inflammatory infiltrate of mast cells, lymphocytes, macrophages and activated neutrophils. Elevated levels of effector immune cells such as activated CD4+ and CD8+ cytotoxic T cells, cytolytic intraepithelial lymphocytes and perforin and granzyme-containing T-cells has been linked with damage of the epithelial gut mucosa in IBD (Neuman M. G., 2004) The figure below summarizes the alterations observed in IBD (fig.2).

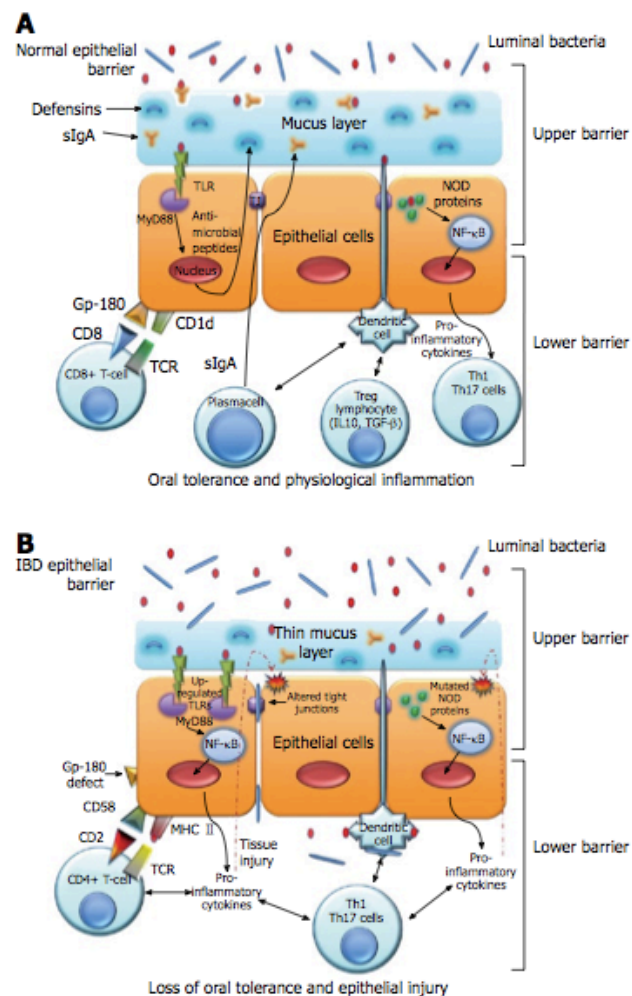


Figure 2: The epithelial barrier system. A: Normal epithelial barrier; B: Inflammatory bowel disease (IBD) epithelial barrier. TLR: Toll-like receptors; MyD88: myeloid differentiation factor 88; TJ: Tight junctions (Roda G. et al, 2010).

### **1.1.3 - IBD established and evolving therapies**

Nowadays, a specific causal treatment of IBD is still not available and the most currently drugs used in its treatment, particularly immunosuppressants and immunomodulators, are associated with significant side effects. The lack of effectiveness and the adverse effects of standard therapies have increased the need for the search of a new treatment that combines efficacy and safety. Patients often resort to the use of complementary and alternative medicines, particularly herbal therapies, with promising results (Rahimi R. et al, 2009).

During the last years, dietary polyphenols have been a focus of intensive research mainly directed to its antioxidant activity in the context of prevention of oxidative stress-induced diseases. More recently, alternative pathways for their health-promoting effects have been proposed, namely the modulation of signalling pathways in connection with physiological and pathophysiological conditions such as inflammatory processes.

An important source of polyphenols is wine, particularly red wine. In the last years numerous studies described health-promoting effects of polyphenols such as antibacterial, antifungal, antiviral, antineoplastic, hepatoprotective, immunomodulating and anti-inflammatory properties, however fundamental aspects of molecular mechanisms underlying their protective role remain unknown. A clear understanding of the molecular mechanisms of action of polyphenols is crucial in the valuation of these potent molecules as potential prophylactic and therapeutic agents in IBD.

## 1.2 – Polyphenols

### 1.2.1- Overview

Polyphenols are present in a wide variety of fruits, vegetables and beverages like tea and wine. They have been regarded as antioxidants, preventing the deleterious effects of oxidants and free radicals but more recently they are also recognized as modulators of cell signaling and inflammation (Rahman I. et al, 2006, Laranjinha J. et al, 2010; Laranjinha J. et al, 2001 ).

There are over 8000 structural variants of polyphenols but all these compounds have in common aromatic ring(s) bearing one or more hydroxyl moieties and its structure varies from simple molecules, like phenolic acids, to highly polymerized compounds, such as condensed tannins. They can be divided into at least 10 different classes based upon their chemical structure (Bravo L., 1998), being flavonoids the most widely distributed group of plant polyphenols. The most common structure of flavonoids is two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Rahman I. et al, 2006).

In figure 3 the hydroxyl group in the B ring ( $R_{4'}$  replaced by a H atom) gives the polyphenol the capacity to participate in redox reactions by donating the H atom, yielding a stable o-semiquinone radical due to the charge delocalization between the aromatic rings, particularly when there is a double bond in the position 2:3 of ring C.

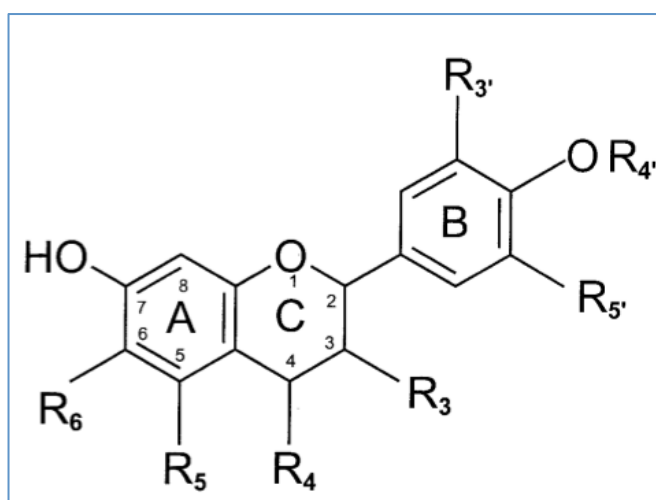


Figure 3 -General structure and numbering pattern for common food flavonoids. For most food flavonoids,  $R_{4'}=H$ ,  $R_5=OH$  and  $R_6=H$ . Exceptions include, biochanin A,  $R_{4'}=CH_3$ ; formononetin,  $R_{4'}=CH_3$ ,  $R_5=R_6=H$ ; glycitein,  $R_5=H$ ,  $R_6=OH$ ; and hesperitin,  $R_{4'}=CH_3$ . Additional individual flavonoids within each subclass are characterized by unique functional groups at  $R_3$ ,  $R_{3'}$ , and  $R_{5'}$  (Beecher G., 2003).

## 1.2.2 – Naturally occurring polyphenols in wine

Wine is an alcoholic beverage that contains a large amount of different polyphenols extracted from grapes during the process of vinification. In fact, red wine is one of the most abundant sources of polyphenols (Medic-Saric M. et al, 2009). Phenolic compounds in grapes and wines belong to the following major classes: stilbenes, flavan-3-ols, flavonols, anthocyanins, hydroxybenzoic acids, procyanidins and hydroxycinnamic acids (Rodrigo R. et al, 2010) (table 1). The phenolic composition of the wine is not only dependent of the grape variety from which is made but also on some external factors such as climate and winemaking technology.

Table 1- Wine polyphenolics chemical classification in decreasing content order (Rodrigo R. et al, 2010).

Category	Compound
<b>Flavonols</b>	Quercetin Myricetin Kaempferol Isorhamnetin
<b>Anthocyanins</b>	Cyanidin Malvidin Petunidin Delphindin Peonidin Pelargonidin
<b>Hydroxycinnamic acids</b>	Caffeic Ferulic p-coumaric Chlorogenic
<b>Hydroxybenzoic acids</b>	Gallic Ellagic Protocatechic Vanillic Syringic 3,4-dihydroxybenzoic p-hydroxybenzoic
<b>Flavanols</b>	(+)-catechin (-)-epicatechin
<b>Stilbenes</b>	Resveratrol Piceid
<b>Procyanidins</b>	Procyanidins A2, B1, B2, B3, B4

### **1.2.3- Absorption, bioavailability and metabolism of polyphenols**

The biological effects of polyphenols are of course dependent on their bioavailability that differs within each and every polyphenol. The chemical structure of the compound more than concentration determines the rate and extent of the absorption and the nature of the metabolites circulating in the plasma.

Polyphenols are at least partially absorbed through the gut barrier and the quantities of polyphenols found intact in urine vary from one polyphenol to another (Scalbert A. et al, 2002). The polyphenol structure has a major impact on intestinal absorption.

Most polyphenols are present in food in the form of esters, glycosides or polymers that cannot be absorbed in their native form. Therefore, before absorption they are hydrolysed by intestinal enzymes or by colonic microflora (Scalbert A. et al, 2002).

Once absorbed polyphenols undergo extensive modification; they are conjugated by methylation, sulfation and/or glucuronidation (D'Archivio M. et al, 2007). Then the metabolites circulate in the blood bound to plasma proteins particularly albumin. Additionally, the bioavailability of polyphenols is reported to be low due to their low absorption and rapid elimination by urine and bile.

#### **1.2.4- Polyphenols and cellular signaling**

In the last decades polyphenols have been the subject of an extensive research due to their observed biological effects in vitro such as free-radical scavenging, metal chelating properties and enzyme modulation abilities.

Earlier studies on polyphenols have viewed these compounds only from the perspective of antioxidants, however many other effects of polyphenols such as anti-inflammatory, anti-tumor, anti-atherogenic abilities could not be explained solely on the basis of their antioxidant properties. The extensive modifications that polyphenols undergo compromises their antioxidant properties but not their biological effects. More recent investigations showed that polyphenols may not merely exert their effects as free radical scavengers but may also modulate cellular signaling processes or may themselves serve as signaling agents (Laranjinha J. et al, 2010)

## **1.3- Polyphenols and inflammation**

### **1.3.1- Basic tenets of Inflammation**

Inflammation is a part of the nonspecific immune response that occurs in reaction to any type of bodily injury. It is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process.

The defining clinical features of inflammation are redness, warmth, swelling and pain that can be explained by increased blood flow, elevated cellular metabolism, vasodilatation, release of soluble mediators, extravasation of fluids and cellular influx.

Inflammation can be classified as either acute or chronic. In normal conditions inflammation is a self-limiting process but in some disorders the inflammatory process becomes continuous and consequently chronic inflammatory diseases develop.

The two kinds of inflammation have very specific characteristics: acute inflammation is characterized by infiltration of innate immune system cells, specifically neutrophils and macrophages while infiltration of T lymphocytes and plasma cells are features of chronic inflammation (Ferrero-Milani L. et al, 2007). Monocytes/macrophages play a central role in both.

### **1.3.2- Molecular mechanisms of polyphenols as anti-inflammatory agents**

There is convincing epidemiological and experimental evidence that dietary polyphenols have anti-inflammatory activity (Middleton E. et al, 2000). The detailed and particular mechanisms by which each polyphenol induces an anti-inflammatory action remain to be elucidated. However it is now known that these natural compounds can work as modifiers of signal transduction pathways to elicit their beneficial effects exerting anti-inflammatory activity by modulation of pro-inflammatory gene expression such as cyclooxygenase, lipoxygenase, nitric oxide synthases and several pivotal cytokines mainly by acting through nuclear factor-kappa B (NF- $\kappa$ B) and mitogen-activated protein kinase (MAPK) signalling (Santangelo C. et al, 2007).

An additional explanation is that polyphenols can modulate the activity of redox pathways and its components in a cell during normal and pathophysiological conditions, particularly acting on Nrf 2 (Nuclear factor erythroid 2- related factor 2) (Rahman I. et al, 2006).

It is important to note that the gastrointestinal tract is a compartment where the concentration of the dietary polyphenols might achieve its higher concentration in the body in an unmodified state and, therefore, where it might be expected polyphenol-dependent cellular modulatory processes via Nrf2, NF- $\kappa$ B and nitric oxide metabolism.



## **1.4- Nitric oxide**

### **1.4.1- Nitric oxide: characteristics, chemistry and biosynthesis**

#### ***-Characteristics***

In the past few decades the interest and research about nitric oxide ( $\bullet\text{NO}$ ) have been growing. It was first discovered in the eighties when several groups of researchers were trying to identify the endothelium derived relaxing factor (EDRF) (Furchgott R. et al, 1980; Ignarro et al, 1987; Palmer R. et al, 1987).

Nowadays,  $\bullet\text{NO}$  is recognized as a versatile cellular modulator involved in a wide variety of physiological and pathological events such as immunitary response, vasodilation and neuromodulation.

$\bullet\text{NO}$  is a peculiar radical species in many aspects, despite its extremely complex and diverse biological activity it is a simple molecule containing one nitrogen atom covalently bonded to an oxygen atom with one unpaired electron. It is one of the few gaseous signaling molecules and being a small hydrophobic molecule it can cross cell membranes without the help of channels or receptors as readily as molecular oxygen and carbon dioxide.  $\bullet\text{NO}$  is capable of penetrate rapidly across cell membranes and diffuses through considerable distances which means that it can affect a great number of biological processes.

#### ***-The chemistry of $\bullet\text{NO}$***

$\bullet\text{NO}$  has a very rich chemistry, despite not being a highly reactive compound it can react with several chemical species. To better understand its complex chemistry we can divide the effects that involve  $\bullet\text{NO}$  in direct and indirect (figure 4).

Direct effects results from direct reactions between  $\bullet\text{NO}$  and specific biological molecules (metal centers and radical species). These effects predominate at lower concentrations.

The reactions between  $\bullet\text{NO}$  and metal complexes can be classified in three types: (1) the direct reaction of  $\bullet\text{NO}$  with a metal center, (2)  $\bullet\text{NO}$  redox reaction with dioxygen metal complexes and (3) reduction of oxo-metalic complexes (Wink D. and Mitchell J., 1998). The reactions of NO with heme-iron containing proteins are the

most physiologically relevant and include the reaction with soluble guanylate cyclase (sGC) (Frieb A. et al, 2003), which plays an important role in neuromodulation and vasodilation.

$\bullet$ NO can also react with other radical species like carbon-centered radicals, lipid radicals and nitrogen dioxide (Wink D. and Mitchell J., 1998).

The indirect effects are defined as those resulting from the reactions of RNS, derived from  $\bullet$ NO, with various biological targets. These effects predominate at higher concentrations and usually result in nitroxidative stress. Usually, this involves the reaction of  $\bullet$ NO oxygen or with the superoxide radical ( $O_2^{\bullet-}$ ) ultimately forming products that are responsible for nitrative and nitroxidative stress with toxic effects in the cells.

In fact, most of the deleterious effects assigned to  $\bullet$ NO are supported by its reaction with  $O_2^{\bullet-}$  to produce peroxynitrite ( $ONOO^-$ ) in a diffusion-controlled reaction ( $k=1.9 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ ).  $ONOO^-$  acts as a powerful oxidant and nitrating agent modifying proteins, lipids and nucleic acids.

One important modification of proteins induced by  $ONOO^-$  is the formation of nitrotyrosine. The nitration of protein tyrosine residues to 3-nitrotyrosine is one of the molecular footprints left by the reactions of RNS with biomolecules (Radi R., 2004). This post-translational modification can alter protein function and it's being associated with acute and chronic diseases and can be a predictor of disease risk (Radi R., 2004). Despite the existence of repair mechanisms and the presence of in vivo reductants like GSH and the small fraction of nitrated proteins when compared to the total tissue protein, nitration can be focused on specific tyrosines resulting in modifications, encompassing loss or gain of protein function (Radi R., 2004). One example of the biological significance of protein tyrosine nitration is the loss of enzyme activity of the manganese superoxide dismutase (MnSOD) when nitrated by peroxynitrite in Tyr-34. Nitrated and inactivated MnSOD is found in both acute and chronic inflammatory processes (MacMillan-Crow L. et al, 1996).

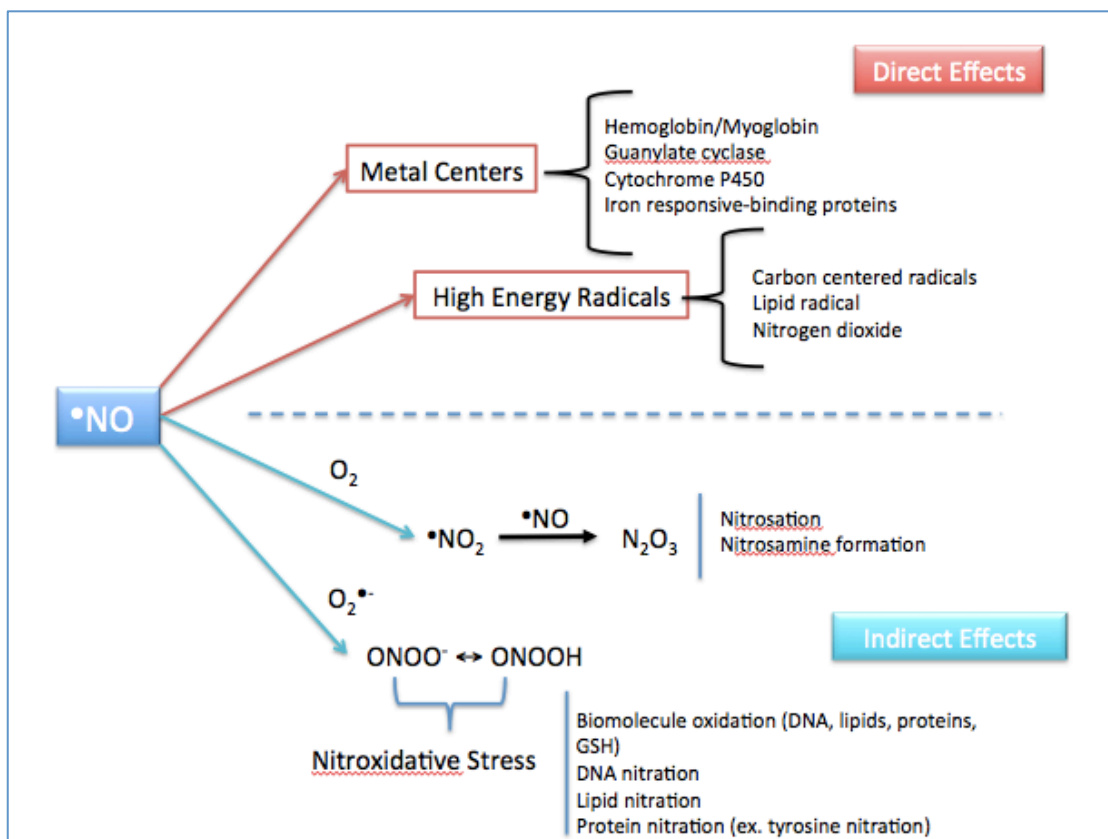


Figure 4- Chemical biology of nitric oxide: direct and indirect effects. Adapted from Wink D. and Mitchell J., 2001.

## ***-•NO Biosynthesis***

The biosynthesis of nitric oxide can occur in two different ways: enzymatic and non-enzymatic.

Nitric oxide synthase (NOS) had been considered the primary source of •NO in biological systems but there is also the contribution of enzyme-independent •NO generation.

### **Enzymatic •NO biosynthesis**

Three distinct isoforms of NOS have been identified: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). They are products of three distinct genes with different localization, regulation, catalytic properties and inhibitor sensitivity (Alderton W. et al, 2001). eNOS and nNOS are constitutively expressed and their activities are regulated by intracellular calcium concentrations via calmodulin. The function of iNOS is not affected by intracellular calcium concentrations because iNOS is bound to calmodulin even under resting intracellular calcium concentration. While eNOS and nNOS produce low fluxes of •NO for short periods of time, iNOS produces higher •NO levels for long periods of time.

### **Structure of the NOS**

In their active form NOS are dimeric and each monomer is associated with a calmodulin (CaM), they also contain relatively tightly-bound cofactors like (6R)-5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>), FAD, FMN and iron protoporphyrin IX (haem) (Alderton W. et al, 2001).

The NOS structure can be divided in two distinct domains: oxygenase domain and reductase domain.

The N-terminal oxygenase domain contains binding sites for haem, BH<sub>4</sub> and L-arginine and it's linked by a CaM-recognition site to a C-terminal reductase domain that has binding sites for FAD, FMN and NADPH (Alderton W. et al, 2001).

### **Reactions catalysed by NOS**

The synthesis of •NO requires L-arginine, NADPH and oxygen as substrates and the presence of other coenzymes/cofactors, as well as the presence of CaM.

Citrulline, NADP and  $\bullet$ NO are the products of the overall reaction (Knowles R. et al, 1994).

In this reaction electrons are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. In this domain they interact with the haem iron and  $\text{BH}_4$  at the active site to catalyse the reaction of oxygen with L-arginine, generating citrulline and  $\bullet$ NO as products. The electron flow through the reductase domain requires the presence of bound  $\text{Ca}^{2+}/\text{CaM}$  (figure 5).

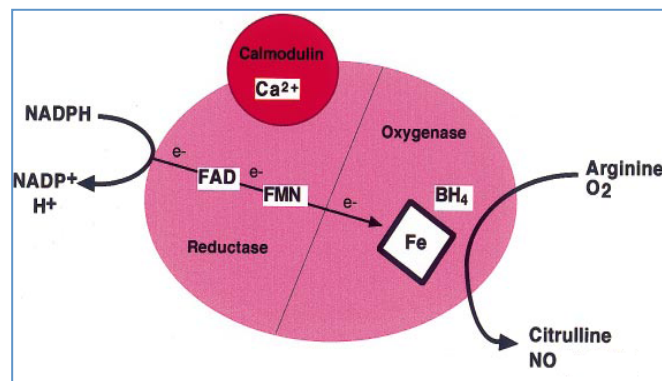


Figure 5-Overall reaction catalysed and cofactors of NOS (Alderton W. 2001).

### Regulation of NOS

NOSs are probably one of the more regulated enzymes in nature. Due to the wide variety of physiological roles of these enzymes regulation is complex. Activity, expression and localization are regulated by protein-protein interactions, alternative mRNA splicing and covalent modifications (Alderton W. et al, 2001).

The regulation of NOS activity is done by CaM, calcium, phosphorylation, protein inhibitor of NOS (PIN) and heat-shock protein 90 (Hsp90).

Table 2- Regulation of NOS (adapted from Alderton W. et al, 2001 ).

Type of regulation	nNOS	eNOS	iNOS
Alternate mRNA splicing	Yes	Not reported	Yes
Protein-protein interactions	CaM/ $\text{Ca}^{2+}$ , PDZ domains, PIN, caveolin-1, Hsp90	CaM/ $\text{Ca}^{2+}$ , caveolin-1 and -3, Hsp90, ENAP-1	CaM/ $\text{Ca}^{2+}$ , kalirin
Covalent modifications	Phosphorylation	Myristoylation, palmitoylation, phosphorylation	Not yet reported

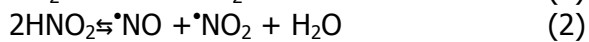
## Non-enzymatic •NO biosynthesis

A few years ago the inorganic anions nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) were thought to be inert and products of endogenous nitric oxide metabolism. However it is now known that they can be an alternative source of •NO to the classical L-arginine-•NO-synthase pathway particularly in hypoxic states (Lundberg J. et al, 2008).

Nitrate is present in several dietary sources like green leafy vegetables (lettuce, spinach) and nitrite appears majorly in cured meat and cereals, but the major source of nitrite is the reduction of nitrate in the oral cavity by commensal bacteria (Lundberg J. et al, 2008).

Under highly acidic conditions, which occur in the stomach, nitrite yields •NO and other reactive nitrogen species (Zweir J. et al, 1999).

This equilibrium can be represented by the following reactions:



•NO will leave the aqueous phase of the stomach being present in the expelled air. Measurements of •NO in the expelled air can be used as a non-invasive method for estimation of gastric acidity (Lundberg J. et al, 1994).

### **1.4.2- •NO and inflammation**

•NO is called a “double-edge-sword” or “Jekyll and Hide” because it can trigger pro- or anti-inflammatory effects. The apparent inconsistency could be explained by the multiple cellular actions of this molecule and the level and site of •NO production. Products based in •NO and not •NO directly are responsible for the opposite effects.

#### ***- Anti-inflammatory effects***

In small amounts •NO have been shown to exert anti-inflammatory activities. Several studies demonstrate that this molecule confers anti-adhesive properties to the endothelium inhibiting the adhesion of leucocytes (Kubes P. et al, 1991), decrease P-selectin expression by platelets, platelet adhesion, aggregation and microvascular thrombosis, reduce mast cell degranulation, lymphocyte proliferation and oxidant production by phagocytes and also can down-regulate macrophage cytokine production. All these effects reduce the inflammatory response and inhibit its progression (Clancy R. et al, 1998).

#### ***- Pro-inflammatory effects***

An excessive inflammatory cytokine production is responsible for the induction of iNOS, which produces large amounts of •NO (Clancy R. et al, 1998). An increased production of •NO has been implicated in diseases such as sepsis (Petros A. et al, 1991) and ulcerative colitis (Middleton S. et al, 1993). For that reason increased •NO overproduction is a reflection of an immune-activated state in which inflammatory cytokines and other mediators have up-regulated iNOS in diverse tissues.

Large amounts of •NO produced by cells in response to cytokines can destroy host tissues and impair cellular responses. The indirect effects of •NO, described previously, are specially implicated in these pro-inflammatory effects: the formation of reactive nitrogen species such as peroxynitrite triggers toxic events that culminate in cytotoxicity and nitration and nitrosation of several residues in diverse proteins, affecting their function (ex. iron-sulfur containing enzymes, disruption of mitochondrial respiration) (Clancy R. et al, 1998).

### **1.4.3 - •NO in the gastrointestinal tract**

Besides the cellular effects in the inflammation this radical also have tissue effects in the gastrointestinal tract. •NO has an effect on gastrointestinal secretion, permeability and blood flow (Lanas A., 2008).

Mucus and epithelial cell fluid contribute to gastrointestinal defense by acting as a physical barrier to damage caused by microbes, toxins and irritants and contributing to the protection of the epithelium from damage caused by acid and pepsin. The production of these fluids is enhanced by •NO (Sharma J. et al, 2007).

•NO , acting as a vasodilator, actuate in the microcirculation increasing mucosal blood flow. This fact results in the buffering of acid, dilution of toxins and stimulation of angiogenesis, all this effects are critical in mucosal protection (Bjorne H. et al, 2004).



## **1.5 - Nuclear factor-kappa B (NF- $\kappa$ B)**

### **1.5.1- Overview**

NF- $\kappa$ B is a pleiotropic transcription factor that plays a critical role in diverse cellular processes associated with proliferation, cell death, inflammation, as well as innate and adaptative immune responses. The NF- $\kappa$ B signalling pathway has been implicated in the pathogenesis of several inflammatory diseases including IBD (Rogler G. et al, 1998). It was first identified twenty years ago regulator of expression of the  $\kappa$ B light chain in B cells and target of vast research ever since.

In mammals the NF- $\kappa$ B family is constituted by five members, these include p65 (also called RelA), c-Rel, RelB, p50 and p52. These subunits only exist as homo or heterodimers in resting cells and all of them have a Rel homology domain (RHD) containing a nuclear localization sequence (NLS) involved in dimerization, sequence-specific DNA binding and interaction with the inhibitory I $\kappa$ B proteins (Hayden M. et al, 2008).

A large number of physiological stimuli are known to activate NF- $\kappa$ B (e.g. bacterial and viral products, pro-inflammatory cytokines, cellular receptors and ligands), which in turn activates an equally large number of target genes (e.g. chemokines, immune receptors, adhesion molecules).

### **1.5.2- Cascade and key molecules**

In resting conditions the majority of NF- $\kappa$ B dimers are maintained in a latent cytoplasmic form through binding to a member of the inhibitor of NF- $\kappa$ B (I $\kappa$ B) protein family (Verma I. et al, 1995), which includes I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$ , the p100 and p105 precursor proteins and the atypical members Bcl-3, I $\kappa$ BNS and I $\kappa$ B $\zeta$ .

The rate-limiting step in the activation of the NF- $\kappa$ B pathway is the release of the DNA binding subunits from the I $\kappa$ B proteins that act as chaperones sequestering the NF- $\kappa$ B into the cytoplasm. After receiving relevant stimulation that activates NF- $\kappa$ B, the I $\kappa$ B proteins are degraded and NF- $\kappa$ B is translocated to the nucleus where it acts as a transcription factor by binding to regulatory DNA sequences known as  $\kappa$ B sites (Wan F. et al, 2010).

Currently there are three distinct ways by which stimuli lead to generation of free NF- $\kappa$ B and NF- $\kappa$ B dependent transcription: the canonical, non-canonical and the atypical NF- $\kappa$ B activation pathways. The majority of inducers of NF- $\kappa$ B are known to operate via the canonical mechanism, the non canonical is crucial for lymphoid organogenesis and activated by very few inducers and the atypical mechanism is described as a response to DNA damage by agents that cause lesions in DNA such UV radiation and doxorubicin (Wan F. et al, 2010).

### ***-The canonical pathway***

Being activated by proinflammatory signals such as cytokines, pathogen-associated molecular patterns (PAMPs) and some danger-associated molecular patterns (DAMPs) the canonical pathways is particularly interesting in inflammatory conditions such as IBD. This signals work through different receptors belonging to the tumor necrosis factor (TNF) receptor (TNFR) and Toll-like receptor (TLR)-interleukin-1(IL-1) receptor (IL-1R) superfamilies (Shih V. et al, 2011) .

Upon receiving relevant stimulation that warrants activation of NF- $\kappa$ B, the I $\kappa$ B kinase (IKK) complex is activated by upstream kinases, like NIK, MEKK-1 and RIP through phosphorylation of specific serine residues in the activation domain of each IKK subunits. The IKK complex contains at least two kinases subunits (IKK1 and IKK2) and a non-catalytical associated modulator (called IKK $\gamma$  ,NEMO or IKKAP) (Karin M., 1999).

The canonical pathway is defined as being mediated by a NEMO-dependent IKK, in contrast with the noncanonical which doesn't requires NEMO.

Then the activated IKK complex phosphorylates I $\kappa$ B proteins on specific serine residues, triggering their ubiquitination and proteosomal degradation, which allows NF- $\kappa$ B translocation to the nucleus and activation of gene transcription (Wan F. et al, 2010) (figure 6).

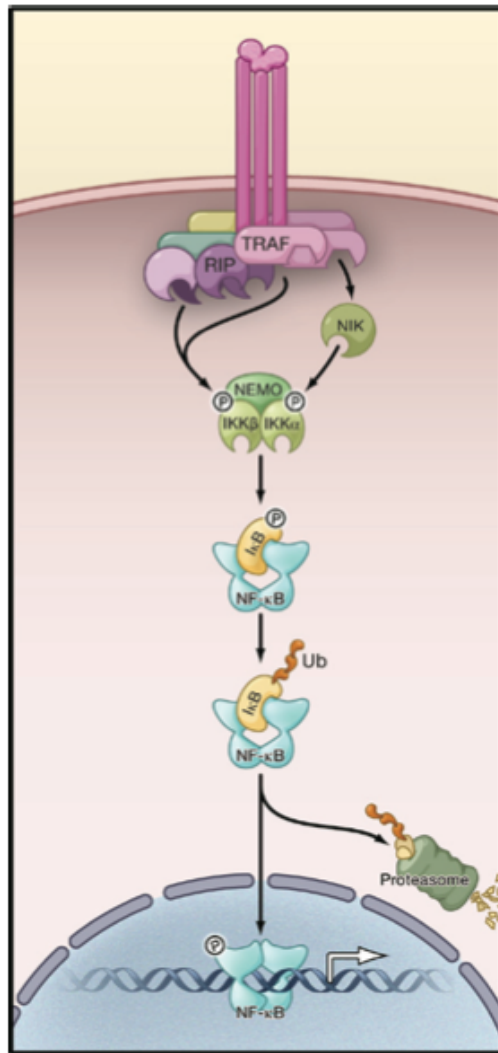


Figure 6 - NF- $\kappa$ B Signaling pathways (canonical pathway) (Hayden M. et al, 2004).

### 1.5.3- NF- $\kappa$ B target genes

NF- $\kappa$ B dimers bind to a set of related 10 bp DNA sites, collectively called  $\kappa$ B sites, to regulate the expression of many genes. The major classes of NF- $\kappa$ B target genes are cytokines and chemokines, immune receptors, adhesion molecules, stress response genes, regulators of apoptosis, transcription factors, growth factors, enzymes (e.g. collagenase and lysozyme) and cell cycle regulators (Pahl H., 1999).

## **1.6 - Nuclear factor erythroid 2- related factor 2 (Nrf2)**

### **1.6.1- Overview**

Nrf2 was originally identified in 1994 during a screen for proteins that bind to the control region of the  $\beta$ - globin gene (Moi P. et al, 1994).

This transcription factor is ubiquitously expressed in a wide range of cell types and tissues including liver, lung, intestine and kidney (Moi P. et al, 1994). It is part of the cap 'n' collar family of transcriptions factors that share a highly conserved basic region-leucine zipper (bZIP) structure (Moi P. et al, 1994) (figure 7).

The basic region upstream the leucine zipper is responsible for the DNA binding (Jaiswal A., 2004) and the acidic region is required for transcriptional activation. ARE-mediated transcriptional activation involves heterodimerization of Nrf2 with other bZIP proteins such as Jun and small Maf (Itoh K. et al, 1997; Venugopal R. et al, 1998).

The Nrf2 binding site is a subset of the antioxidant response elements (ARE), ARE's are regulatory sequences found on promoters of several detoxification genes.

This transcription factor mediates a broad-based set of adaptive responses to intrinsic and extrinsic cellular stress.

### **1.6.2-Negative regulation of Nrf2 by Keap1**

A cytosolic inhibitor of Nrf2 was identified in 1998 and named Keap1 (Kelch-like ECH-associated protein 1) (Itoh K. et al, 1999) (figure 7).

Keap1 has two discrete structural domains. One is the double glycine repeat (DGR) moiety (also known as the Kelch motif), and the other is the BTB protein interaction domain. The DGR region binds to the Nrf2- ECH homology 2 (Neh) domain of the N-terminal portion of Nrf2 whereas the other portion binds with the actin cytoskeleton causing Nrf2 sequestration in the cytoplasm and repression of his activity (Itoh K. et al, 1999). Keap1 also has three additional domains: the N-terminal region, the intervening region that is an especially cysteine-rich region and the C-terminal region (Jaiswal A., 2004).

Keap1 releases Nrf2 from sequestration during periods of oxidative stress or following exposure to electrophiles, The alliance between Keap1 and Nrf2 seems to function as a cellular redox sensor through which the activation of ARE-dependent genes in response to oxidative stress is regulated (Itoh K. et al, 1999). Keap1 releases Nrf2 from sequestration during periods of oxidative stress or following exposure to electrophiles and after dissociation, Nrf2 translocates to the nucleus allowing gene transcription.

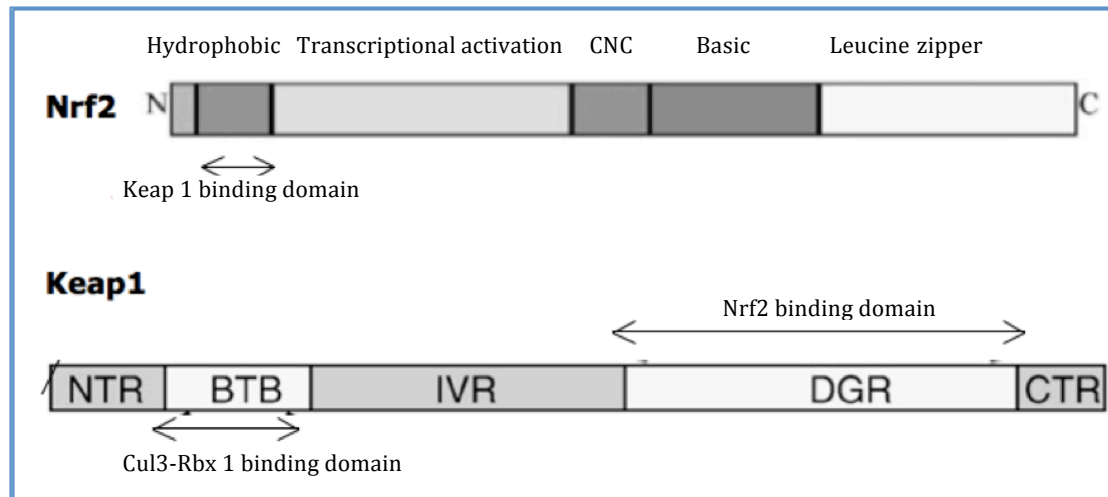


Fig. 7- Schematic presentation of various domains of Nrf2 and Keap1: NTR,N-terminal region; BTB,broad complex tramtrack bric-a-brac; IVR, intervening/linker region; DGR, Kelch domain/diglycine repeats; CTR, C-terminal region. (adapted from Kaspar J. et al, 2009)

### **-Dissociation**

Multiple steps appear to be important in triggering the dissociation of Nrf2 from Keap1. These events include modification of critical cysteine residues within Keap1 and phosphorylation of Nrf2.

Modification of Keap1 cysteines by ARE inducers is postulated to directly cause dissociation of the Keap1–Neh2 interaction, leading to Nrf2 nuclear accumulation. Dinkova-Kostova (Dinkova-Kostova A. et al, 2002) and Wakabayashi (Wakabayashi A. et al, 2004) studies have established the widely accepted model that electrophilic modification of Keap1 leads directly to dissociation of the Keap1–Nrf2 complex. However these studies have been performed using the murine Keap1 – Nrf2 system, in humans the direct disruption model is invalid, and the most reactive cysteines of human Keap1 are not the same as those reported for mouse Keap1 (Egglar A. et al,

2005). Egger study supports a model whereby electrophilic modification of Keap1 alone does not disrupt the Keap1–Nrf2 complex (Egger A. et al, 2005).

Although somewhat controversial, accumulating evidence suggests that phosphorylation of Nrf2 is also important for dissociation of Nrf2 from Keap 1. Multiple kinases (ex. MAP kinases) can phosphorylate Nrf2 and alter transcription of Nrf2 (Yu R. et al, 2000; Zipper L. et al 2000), however phosphorylation of Nrf2 by some protein kinases (ex. protein kinase C (Bloom D. et al, 2003)) does not alter transcription but only the dissociation process. Further research is yet required to elucidate the mechanistic details of the electrophilic signal transduction mechanism of Nrf2 nuclear accumulation.

### ***-Ubiquitination***

Nrf2 has a short half-life, approximately 13-20 minutes, due to constitutive ubiquitin-proteosomal degradation (McMahon M. et al, 2003; Nguyen T. et al, 2003).

Keap1 function as an adaptor protein in an ubiquitin-proteasome complex named Cul3-based E3 ligase complex. Keap1-Cul3 complexes act as Nrf2-specific E3 ubiquitin ligases that direct Nrf2 for polyubiquitination and destruction via proteasome (Cullinan S. et al, 2004).

For that reason Keap1 is responsible not only for sequestering Nrf2 but also for its normal proteasomal targeting and degradation.

### **1.6.3 - Other regulatory mediators of Nrf 2**

Apart from Keap1 there are other negative regulatory mediators of Nrf2 namely Bach 1 (BTB and CNC homology 1, basic leucine zipper transcription factor 1) and GSK3 $\beta$ /Fyn. They are very important in repressing Nrf2 downstream genes that are induced in response to oxidative/electrophilic stress.

Bach 1 is a transcription repressor that belongs to the cap'n'collar type of basic region leucine zipper factor family (CNC-bZip) (Oyake T. et al, 1996), ubiquitously expressed in tissues and distant related to Nrf2.

Bach 1 forms heterodimers with small Maf (Oyake T. et al, 1996) proteins that bind to the ARE (Dhakshinamoorthy S. et al, 2005) repressing gene expression.

Bach 1 competes with Nrf2 for binding to the ARE, which leads to the suppression of Nrf2 downstream genes (Dhakshinamoorthy S. et al, 2005).

Nuclear import of Nrf2 is followed by activation of a delayed mechanism involving glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), a multifunctional serine/threonine kinase, which controls the switching off of Nrf2 activation of gene expression. This enzyme phosphorylates Fyn at threonine residue(s), phosphorylated Fyn accumulates in the nucleus and phosphorylates Nrf2 at tyrosine 568 leading to nuclear export, ubiquitination, and degradation of Nrf2 (Jain A. et al, 2006; Jain A. et al, 2007).

### 1.6.4 - Molecular mechanisms underlying Nrf2 – mediated transcription

Into the nucleus Nrf2 can form heterodimers with a variety of transcriptional regulatory proteins, the complexes bind to motifs known as antioxidant or electrophile response elements (ARE/EpRE) located in the promoter or upstream promoter regions of detoxification genes.

The partners of Nrf2 are commonly members of the activator protein-1 (AP-1) family such as Jun and Fos (Venugopal R. et al, 1998) or the small Maf family of transcriptional factors (Itoh K. et al, 1997). Jun and Fos bind ARE sequences acting as positive and negative regulators, respectively, of ARE gene transcription. The precise role of small Maf proteins in the transactivation of ARE-containing genes remains yet controversial. Summarizing we can say that the Nrf2 driven transcription is influenced negatively or positively by the identity of the heterodimer partner.

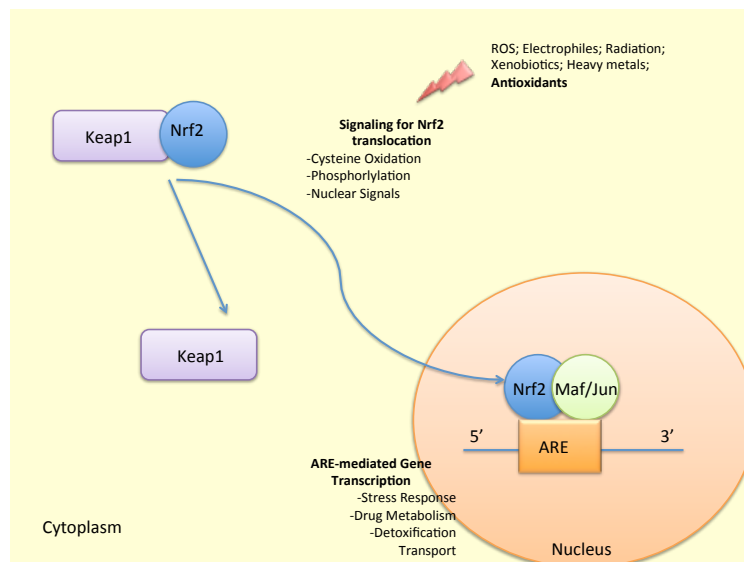


Figure.8- Regulation of Nrf2-mediated transcription

### 1.6.5 - Nrf2 regulated genes

The gene families affected by Nrf2, ARE-containing genes, have several cytoprotective actions: they can provide direct antioxidants, encode enzymes that directly inactivate oxidants, increase levels of glutathione synthesis and regeneration, stimulate NADPH synthesis, enhance toxin export through the multidrug-response transporters, enhance the recognition, repair, and removal of damaged proteins, elevate nucleotide excision repair, regulate expression of other transcription factors, growth factors and receptors, and molecular chaperones and inhibit cytokine-mediated inflammation (Wakabayashi N. et al, 2010).

Table 3 – Examples of Nrf2-responsive genes. Adapted from Aleksunes and Manautou (Aleksunes L. et al, 2007)

Cellular Process	Nrf2-related genes	Function
Glutathione Homeostasis	Glutamate-cysteine ligase (Gcl)	Catalyze formation of Y-glutamylcysteine
	Glutathione synthetase (GS)	Catalyze addition of glycine to Y-glutamylcysteine
	Glutathione-S-transferase (Gst)	Conjugate glutathione to chemicals
	Glutathione peroxidase – gastrointestinal (GI-GPx)	Reduce hydrogen peroxidase and alkyl hydroperoxides
Drug metabolism	NAD(P)H quinone oxidoreductase 1 (Nqo1)	Reduce quinones and endogenous antioxidants, scavenge superoxide
	UDP-glucuronosyltransferase (Ugt)	Catalyze addition of glucuronic acid to chemicals
	Microsomal epoxide hydrolase (mEH)	Hydrolyze epoxides
Stress Response	Heme oxygenase (Ho-1)	Catabolize heme to carbon monoxide, biliverdin and free iron
Iron metabolism	Ferritin	Sequester free iron
Excretion/Transporters	Multidrug resistance-associated proteins (Mrp)	Efflux chemicals across cell membrane
	Multidrug resistance proteins (Mdr)	Efflux chemicals across cell membrane



### 1.6.6 - Nrf2 interactions with additional pathways

Like other signalling pathways the Keap1-Nrf2 pathway does not function isolated, some of the protective effects may be mediated indirectly through cross talk with additional pathways affecting the cell fate (figure 9). Recent evidences suggest the existence of transcriptional cross-talk between Nrf2 and the arylhydrocarbon receptor, NF- $\kappa$ B , p53 and Notch pathways (Wakabayashi N. et al, 2010).

The cross talk between Nrf2 and NF- $\kappa$ B is an area of extensive research. The NF- $\kappa$ B pathway is involved in several processes such as inflammation, immune response, apoptosis and cell growth. Targets of NF- $\kappa$ B include genes classified as chemokines, cytokines, immunoreceptors, cell-adhesion molecules, stress-response genes, and regulators of apoptosis among many others. NF- $\kappa$ B was recently shown to prevent the transcription of Nrf2 dependent genes by reducing the availability of co-activator levels and promoting recruitment of a co-repressor (Liu G.H. et al, 2008).

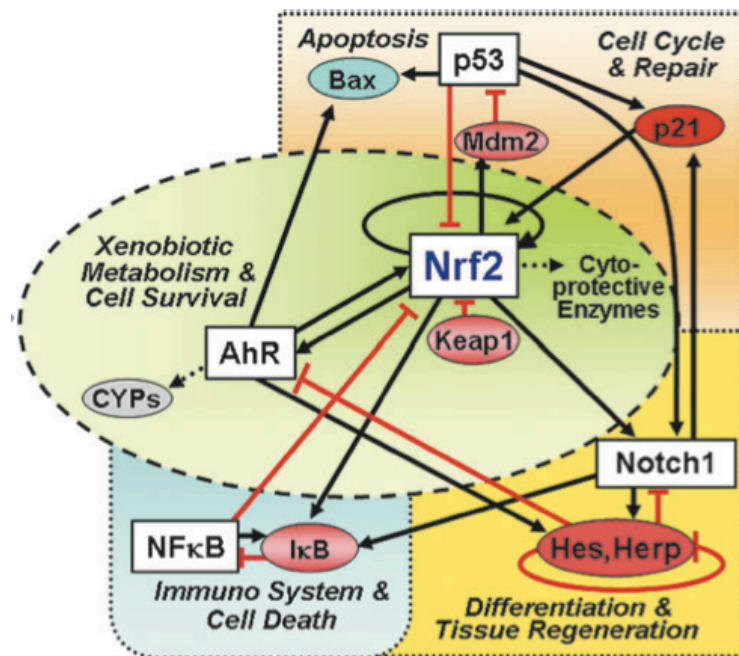


Fig.9- Possible means for regulation of cell survival and other cell-fate responses through interactions of Nrf2 with additional cell-signaling pathways, including AhR, NF- $\kappa$ B, p53, and Notch1. Adapted from Wakabayashi N. et al, 2010.

## Objectives

Considering that polyphenols are strong antioxidants but are also known to interfere in inflammatory signalling pathways and, on the other hand, Nrf2, NF- $\kappa$ B and  $\bullet$ NO metabolism are potential targets for polyphenols and regulators of inflammatory processes, our main goal is to investigate the potential anti-inflammatory effect of a red wine polyphenolic extract (RWE) against gastrointestinal inflammation by using cultured intestinal cell models. This is of biological significance for the prevention of chronic inflammation such as the inflammatory bowel disease. Confirmation of this hypothesis will provide new directions in the development of prevention and new treatments for reducing the extent of gastrointestinal inflammation.

Specifically, we propose to:

- Evaluate the potential anti-inflammatory effect of a RWE in HT-29 cells screening inflammatory parameters (activation of NF- $\kappa$ B, IL-8 production, iNOS induction and  $\bullet$ NO production).
- Evaluate if RWE could have other protective effects, including the protection against tyrosine nitration and modification of the occludin distribution in HT-29 cells stimulated with cytokines.
- Investigate if RWE could activate the Nrf2 pathway in HT-29 cells and if that could be the molecular mechanism involved in their anti-inflammatory action.

# Chapter 2: Materials and Methods

## 2.1- Materials

HT-29 cell line was purchased from Sigma-Aldrich (St Louis, MO, USA). Red wine extracts were obtained by Prof. Dr. Vítor Freitas (Chemistry Department, Faculty of Science, University of Porto, Porto, Portugal) (see annex 1 for detailed composition), DMEM- Modified Eagle Medium with Glutamax (Glutamax<sup>TM</sup>), foetal bovine serum and trypsin were purchased from Gibco (Grand Island, NY, USA). The enzyme-linked immunosorbent assay (Quantikine Immunoassay for Human IL-8) was obtained from R&D Systems (Minneapolis, MN, USA). Secondary monoclonal antibodies (anti-mouse, anti-rabbit, anti-goat) conjugated with alkaline phosphatase were purchased from abcam (Cambridge, CB, UK), primary monoclonal antibody anti-iNOS was purchased from Santa Cruz (Santa Cruz, CA, USA), primary monoclonal antibody anti-I $\kappa$ B was obtained from Cell signaling, primary monoclonal antibody anti-Nrf2 was obtained from R&D Systems (Minneapolis, MN, USA) and primary monoclonal antibody anti-actin was purchased from Sigma-Aldrich (St Louis, MO, USA). Alexa Fluor anti-mouse and anti-rabbit IgG were obtained from Invitrogen (Carlsbad, CA, USA). Polyvinylidene difluoride membranes and ECF substrate were purchased from Amersham/GE Healthcare (Buckinghamshire, BKM, UK). All other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA) with the highest purity available.

## **2.2-Methods**

### **2.2.1- Cell Culture**

HT-29 cells are an epithelial cell line derived from human colonic adenocarcinoma that is able to express differentiation features characteristic of mature intestinal cells. Cell cultures were grown in DMEM medium without antibiotics supplemented with foetal bovine serum (10% vol/vol) onto 75 cm<sup>2</sup> flasks and maintained at 37°C, in a humidified atmosphere of 5% CO<sub>2</sub>. Twenty-four hours before any experiment cells were deprived meaning that they were washed with phosphate-buffered saline (PBS) and cultured in fresh medium without foetal bovine serum.

### **2.2.2- Analysis of cell viability**

Cell viability was assessed using the MTT assay. The MTT assay is a colorimetric assay based on the reduction of the yellow dye MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) to formazan, an insoluble intracellular blue product, by cellular dehydrogenases (Denizot F., and R. Lang. 1986).

In this assay cells were cultured in 24 wells plates under specific conditions, after that the medium was removed and cells were washed twice with PBS. Then MTT was added to each well (final concentration of 0,5 mg/ml) and culture plates were incubated for 1h at 37°C. After the incubation, MTT was removed and 1 ml of DMSO was added and mixed until the formazan crystals dissolve. The extent of MTT reduction was measured spectrophotometrically at 540 nm in a plate reader.

### **2.2.3- Interleukin-8 (IL-8) analysis**

Levels of IL-8 protein secreted to the medium by the HT-29 cells were determined by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions.

In this assay a monoclonal antibody against IL-8 was pre-coated onto a microplate, then standards and samples were added to the wells and any IL-8 present bound to the immobilized antibody. After washing away any unbound substance an enzyme-linked polyclonal antibody specific for IL-8 was added to the wells and another wash to remove any unbound antibody-enzyme reagent was

performed. Afterwards a substrate solution was added to the wells and colour develops in proportion to the amount of IL-8 bound in the initial step. Color development is then stopped and the intensity of the colour was measured at 450 nm.

#### **2.2.4- Preparation of whole cell, cytoplasmic and nuclear lysates**

To the preparation of whole cell lysates after the incubation under the specified conditions, the cells into the plates were washed twice with PBS, resuspended in ice cold lysis buffer [50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% (w/v) glycerol, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 1mM NaVO<sub>4</sub>, 5mM NaF, 1 mM PMSF, 1/100 (v/v) proteases cocktail inhibitor] and maintained in ice for 15 minutes. Lysates were then centrifuged at 14 000 rpm for 10 minutes at 4°C and supernatants were subsequently collected and stored at -80°C.

To the preparation of cytoplasmic lysates cells were also washed twice with PBS, resuspended in ice cold cytoplasmic extracts buffer [10mM Tris-HCl pH 7.5, 10 mM NaCl, 3mM MgCl<sub>2</sub>, 0,5%(v/v) Igepal, 1mM NaVO<sub>4</sub>, 5mM NaF, 1 mM PMSF, 1/100 (v/v) proteases cocktail inhibitor], maintained in ice for 5 minutes and centrifuged at 5 000 rpm for 5 minutes at 4°C and supernatants were subsequently collected. To the preparation of nuclear lysates the pellet obtained in the last step was resuspended in ice cold nuclear extracts buffer [20 mM Hepes pH 7.5, 300 mM NaCl, 5mM MgCl<sub>2</sub>, 0,2 mM EDTA, 1 mM DTT, 20%(v/v) glycerol, 1mM NaVO<sub>4</sub>, 5mM NaF, 1 mM PMSF, 1/100 (v/v) proteases cocktail inhibitor], maintained in ice for 5 minutes and centrifuged at 14 000 rpm for 20 minutes at 4°C and supernatants were subsequently collected. Cytoplasmic and nuclear lysates were stored at -80°C.

#### **2.2.5- Protein determination**

The protein content of the extracts was determined by the Bio-Rad protein assay dye, using bovine serum albumin as the standard. This assay is based on the colour change of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein (Bradford dye-binding procedure) (Bradford M., 1976).

## 2.2.6- Western Blot

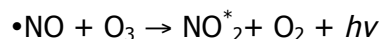
Equal amount of protein present in the lysates were analysed by Western blot.

Proteins were first denatured and then separated by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) at 150V. The separated proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane for 2 h at 200 mA. To avoid non-specific binding, membranes were blocked for 1 h at room temperature with 5% (w/v) non-fat dried milk in TBS-T buffer [25 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween 20].

Membranes were then incubated overnight at 4°C with primary monoclonal antibody (dilution 1:1 000) (anti-actin, anti-iNOS, anti-I $\kappa$ B, anti-Nrf2). After six washes of ten minutes with TBS-T, membranes were incubated with phosphatase alkaline-labelled secondary antibody (dilution 1:20 000) for 1.5 h at room temperature. Membranes were washed again six times with TBS-T. The bands were revealed with ECF and visualized in a Typhoon system. The images were analysed with the Quantity One software (BioRad). B-actin was used as a loading control.

## 2.2.7- Nitric Oxide production determination

In aqueous solution, nitric oxide rapidly degrades to nitrite, which accumulates in the medium. Nitrite in the cell culture supernatants is reduced by an iodide/tri-iodide containing reaction mixture to nitric oxide, which is released into the gas phase. Then NO was measured by chemiluminescence. By this method NO reacts with ozone (O<sub>3</sub>) to produce nitrogen dioxide in excited state (NO<sub>2</sub><sup>\*</sup>) which returns to the ground state emitting light (*hν*). Light emission is linearly related to the NO content of the sample.



Gas phase chemiluminescence is highly sensitive to quantify •NO and nitrosated compounds in biological samples with low levels of these species.

The reaction mixture (45 mmol/L potassium iodide (KI) and 10 mmol/L iodine (I<sub>2</sub>) in glacial acetic acid) was kept at 56°C in a septum-sealed reaction vessel, continuously bubbled with nitrogen. The outlet of the gas stream was passed through a scrubbing bottle containing sodium hydroxide (1 mol/L; 0°C) in order to

trap traces of acid and iodine before transfer into the detector (CLD 88 Eco Medics, Switzerland).

Sodium nitrite standards and sample aliquots (100  $\mu$ L) are injected into the reaction mixture by Hamilton syringes. \*NO signal output was sampled at 2 Hz. Peak integration is performed using the EDAQ Power Chrom software.

## **2.2.8- Immunohistochemistry**

HT-29 cells were cultured in twenty-four wells plates onto glass coverslips. After incubation under their specified conditions, the cells were washed with PBS, fixed with 4% (w/v) paraformaldehyde in PBS during ten minutes and stored if needed at 4°C with PBS into the well. Permeabilization and blockage were made incubating the glass coverslips into de plates with PBS containing 0,1% of saponin and 5% of fetal bovine serum during 30 minutes. Subsequently, cells were incubated with the primary antibody, washed twice with PBS for 5 minutes and then incubated with the appropriated fluorescent secondary antibody. After another two washes, the glass coverslips were mounted in microscope slides using a mounting medium with PBS:glycerol (50/50) and Hoescht 33258 (5 mmg/ml).

The stained cells that remained attached to the plates were visualized using a Nikon fluorescence microscope employing a suitable filter.

## **2.2.9- Glutathione determination**

Concentrations of oxidized (GSSG) and reduced (GSH) forms of glutathione were determined using a fluorometric method described by Hissin and Hilf (Hilf P. and Hilf R., 1976). This method measures both, oxidized (GSSG) and reduced (GSH) glutathione using *o*-phthalaldehyde (OPT) as a fluorescent reagent and takes advantage of the reaction of GSH with OPT at pH 8 and GSSG with OPT at pH 12. GSH can be complexed to N-ethylmaleimide (NEM) to prevent interferences of GSH with measurement of GSSG.

To the sample preparation, cells were washed twice with ice cold PBS, resuspended in ice cold PBS-EDTA ( $\text{KH}_2\text{PO}_4$  100 mM, EDTA 5mM pH 8). Then ice cold  $\text{HClO}_4$  0,6M was added and the mixture was maintained in ice for 5 minutes. After vigorous vortex, the suspension was centrifuged at 14 000 rpm for 5 minutes

at 4°C. Supernatants were collected and the pellet was resuspended in NaOH 1N for protein quantification. Samples were stored at -80°C.

For GSH measurement, GSH standard solution or sample was added to phosphate-EDTA buffer (NaH<sub>2</sub>PO<sub>4</sub> 100mM, EDTA 5mM pH = 8) and OPT. The mixture was remained at room temperature for 15 min and then fluorescence detection was done with excitation and emission at 350 and 420 nm, respectively.

For GSSG measurement the sample was added to NEM and remained 30 minutes and then NaOH 100 mM was added and also OPT. The mixture was remained at room temperature for 15 min and then fluorescence detection was performed.

### **2.2.10- Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 5.00 software. All the results are representative of at least three independent experiments. Data are expressed as mean  $\pm$  SEM. Comparisons between multiple groups were performed with a one-way ANOVA or by a Student's t-test. A value of p lower than 0.05 was considered statistically significant.

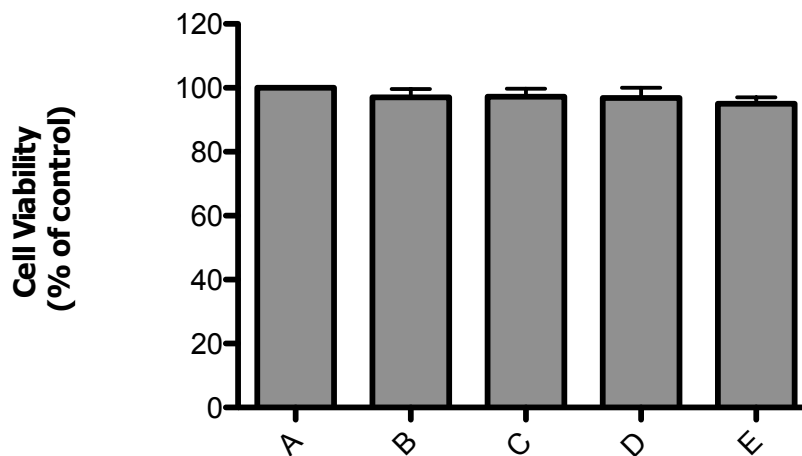


## Chapter 3: Experimental Results

### 3.1- Red Wine Extract does not affect the viability of HT-29 cells

Before examining the potential benefit of RWE in intestinal inflammation, we tested the cytotoxic profile of a RWE (the phenol content expressed in catechin equivalents of the selected RWE is 222 mg/g). For these propose, HT-29 cells were incubated with several concentrations of RWE (100, 200, 400 and 600  $\mu\text{g/ml}$ ) for twenty-four hours and then cell viability was evaluated by the MTT assay which principle was presented in the previous section.

As shown in figure 10 RWE did not affect the viability of HT-29 cells at the concentrations tested.



**Figure 10. - Effect of Red Wine Extract (RWE) on HT-29 cell viability (MTT assay).**

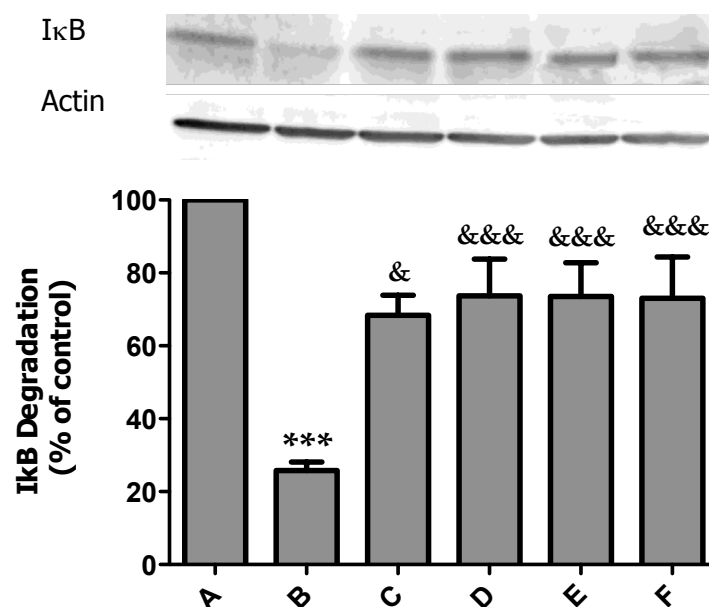
Cells were treated with four different concentrations of RWE (100, 200, 400 and 600  $\mu\text{g/ml}$ ) for 24 hours. Cell viability was measured by following the extent of MTT reduction. Data represent the mean  $\pm$  S.E.M. of at least four independent experiments run in duplicate and are expressed as percentage of control cells (100%). A-control; B-100  $\mu\text{g}$  RWE/ml; C-200  $\mu\text{g}$  RWE/ml; D-400  $\mu\text{g}$  RWE/ml; E-600  $\mu\text{g}$  RWE/ml.

### 3.2 - Red Wine Extract inhibits I $\kappa$ B degradation induced by TNF- $\alpha$ in HT-29 cells

NF- $\kappa$ B signaling pathway has been implicated in the pathogenesis of several inflammatory diseases, namely in IBD. I $\kappa$ B's are a class of inhibitor protein that sequester NF- $\kappa$ B dimers in the cytosol, preventing its nuclear translocation. I $\kappa$ B degradation lead to the release of NF- $\kappa$ B, which translocates to the nucleus, leading to the expression of pro-inflammatory proteins. Therefore, I $\kappa$ B degradation is an indirect marker for the activation of NF- $\kappa$ B.

To investigate the effect of RWE in the NF- $\kappa$ B pathway, HT-29 cells were incubated or not with RWE and then stimulated with TNF- $\alpha$  (70 ng/ml) and the levels of I $\kappa$ B degradation were determined by Western Blot in the different situations.

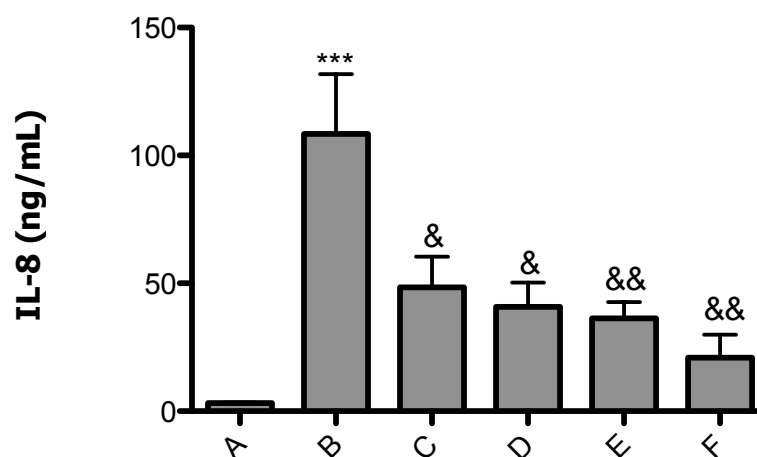
As shown in figure 11, TNF- $\alpha$  induced a rapid degradation (15 min) of the I $\kappa$ B protein. However, a pre-incubation with several concentrations of RWE (100, 200, 400 and 600  $\mu$ g/mL) resulted in almost complete inhibition of I $\kappa$ B degradation.



**Figure 11. - Effect of Red Wine Extract (RWE) on I $\kappa$ B degradation in TNF- $\alpha$ -stimulated HT-29 cells.** Cells were pretreated with four different concentrations of RWE (100, 200, 400 and 600 mg/ml) for 30 min and then stimulated with TNF- $\alpha$  (70 ng/ml) for 15 min. Cytosolic extracts were analyzed by Western blot using anti-human I $\kappa$ B antibody (Cell Signaling). In the bar graph, the relative expression of I $\kappa$ B normalized to the actin level was expressed as mean  $\pm$  SEM from at least five independent experiments. Statistical significance: \*\*\* $p$ <0.001 as compared to control cells; &&& $p$ <0.001 and & $p$ <0.05 as compared to cells stimulated with cytokines. A-Control; B-Cytokines; C-Cytokines +100  $\mu$ g RWE/ml; D-Cytokines +200  $\mu$ g RWE/ml; E-Cytokines +400  $\mu$ g RWE/ml; F-Cytokines +600  $\mu$ g RWE/ml.

### 3.3- Red Wine Extract inhibits IL-8 production by TNF- $\alpha$ -stimulated HT-29 cells

The NF- $\kappa$ B pathway activation begins gene transcription of inflammatory mediators, namely IL-8, a chemokine that plays a major role in mediating the inflammatory burst and massive infiltration of the mucosa by polymorphonuclear leukocytes. To investigate the effect of RWE in the production of pro-inflammatory proteins, the secretion of IL-8 by HT-29 cells was analysed by using enzyme-linked immunosorbent assay (Elisa). In the control situation IL-8 production is very reduced ( $3.22 \pm 0.2$  ng/ml). TNF- $\alpha$  induces IL-8 overproduction ( $108.42 \pm 23.45$  ng/ml). As shown in figure 12, RWE significantly reduced the TNF- $\alpha$  -induced production of IL-8 in a dose dependent manner.

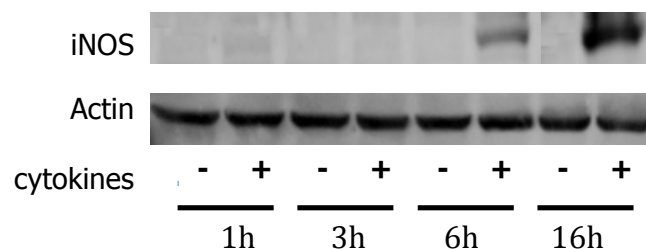


**Figure 12- Effect of Red Wine Extract (RWE) on IL-8 production by TNF- $\alpha$  -stimulated HT-29 cells.** Cells were pretreated with four different concentrations of RWE (100, 200, 400 and 600  $\mu$ g/ml) for 30 min and then stimulated with TNF- $\alpha$  (70 ng/ml) for 24 hours. IL-8 protein secretion in culture supernatant was quantified by using an Elisa kit (R&D Systems). Data represent the mean  $\pm$  SEM from at least three independent experiments. Statistical significance: \*\*\* $p < 0.001$  as compared to control cells; && $p < 0.005$  and & $p < 0.05$  as compared to cells stimulated with cytokines. A-Control; B- TNF- $\alpha$ ; C- TNF- $\alpha$  +100  $\mu$ g RWE/ml; D- TNF- $\alpha$  +200  $\mu$ g RWE/ml; E- TNF- $\alpha$  +400  $\mu$ g RWE/ml; F- TNF- $\alpha$  +600  $\mu$ g RWE/ml.

### 3.4 - Red Wine Extract supresses iNOS expression and inhibits nitric oxide production induced by cytokines in HT-29 cells

Nitric Oxide ( $\bullet\text{NO}$ ) is a molecular mediator of many physiological processes including vasodilation, inflammation, immunity and neurotransmission. Large amounts of  $\bullet\text{NO}$  can destroy host tissues and impair cellular responses. It is known that an excessive inflammatory cytokine production can lead to the induction of iNOS and consequently to an excessive  $\bullet\text{NO}$  production.

HT-29 cells were incubated with a cocktail of cytokines (20 ng/ml  $\text{TNF-}\alpha$ ; 10 ng/ml IL-1; 60 ng/ml  $\text{INF-}\gamma$ ) for one, three, six and sixteen hours and then the iNOS levels were determined by Western Blot. As we can see in figure 13 only at six hours occurred the iNOS induction.



**Figure 13- iNOS expression induced by cytokines in HT-29 cells.** HT-29 cells were stimulated with cytokines (20 ng/ml  $\text{TNF-}\alpha$ ; 10 ng/ml IL-1; 60 ng/ml  $\text{INF-}\gamma$ ) for 1, 3, 6 and 16 h as indicated in figure. Results are representative of three independent experiments.

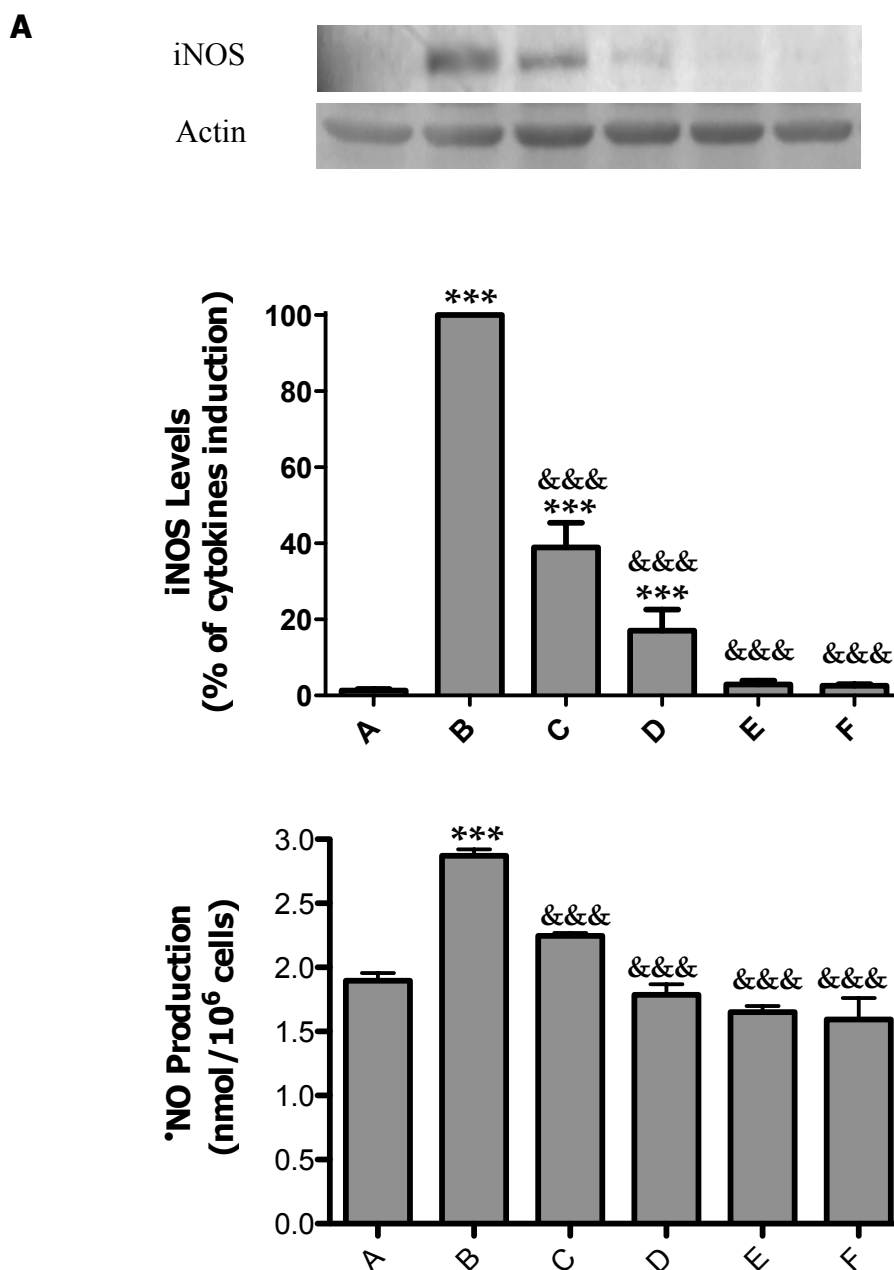
To investigate the effect of RWE in the iNOS expression, HT-29 cells were pre-treated with four different concentrations of RWE (100, 200, 400 and 600  $\mu\text{g/ml}$ ) for 30 min and then stimulated with cytokines (20 ng/ml  $\text{TNF-}\alpha$ ; 10 ng/ml IL-1; 60 ng/ml  $\text{INF-}\gamma$ ) for six hours and the iNOS levels were determined by Western Blot.

As shown in figure 14 A) expression of the iNOS protein was not detectable in unstimulated cells, but markedly increased six hours after cytokines treatment. Pre-treatment with RWE showed a significant concentration-dependent inhibition of iNOS protein expression in cytokines- stimulated cells.

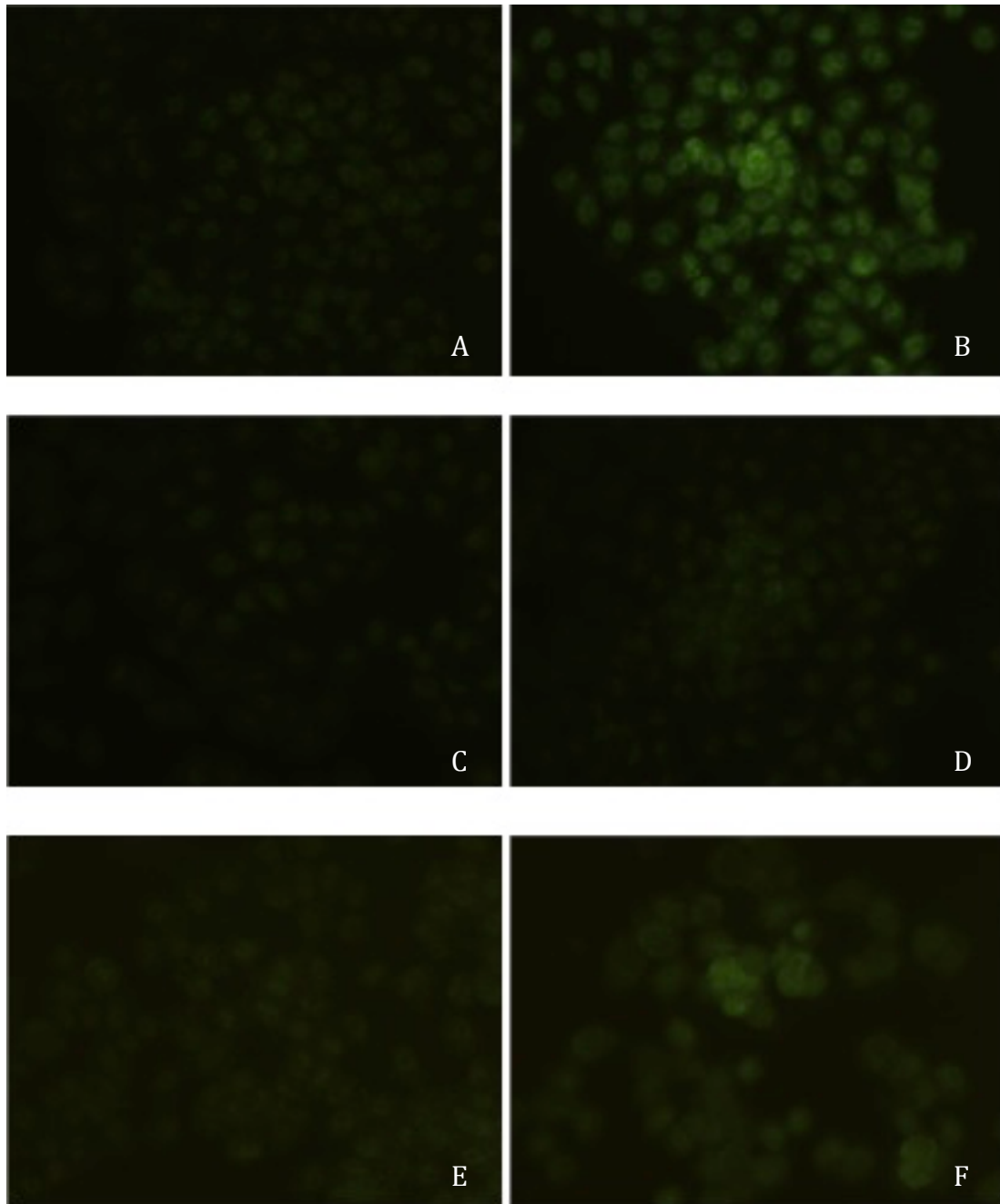
Considering cells only treated with cytokines as 100%, we can observe that pre-treatment with 100, 200, 400 and 600  $\mu\text{g/ml}$  of RWE decrease iNOS expression to 38.9, 17.05, 2.9 and 2.55 % respectively.

The •NO production was measured in the supernatants after sixteen hours of incubation with cytokines. Results showed an expectable increase of •NO production when cells were only treated with cytokines and a decrease to the control levels when cells were pre-treated with RWE (figure 14 B).

The induction of iNOS was also analyzed by immunohistochemistry (figure 15). Cells were also pre-treated with four different concentrations of RWE (100, 200, 400 and 600 µg/ml) for thirty minutes and then stimulated with cytokines (20 ng/ml TNF- $\alpha$ ; 10 ng/ml IL-1; 60 ng/ml INF- $\gamma$ ) for six hours. Results showed a higher induction of iNOS in cells treated only with cytokines and a visible decrease of the induction when cells were also pre-treated with RWE, even with lower concentrations of RWE.



**Figure 14- Effect of Red Wine Extract (RWE) on iNOS expression induced by cytokines in HT-29 cells.** Cells were pretreated with four different concentrations of RWE (100, 200, 400 and 600 mg/ml) for 30 min and then stimulated with cytokines (20 ng/ml TNF- $\alpha$ ; 10 ng/ml IL-1; 60 ng/ml INF- $\gamma$ ). **(A)** After 6 hours of incubation, total protein extracts were obtained and then analysed by Western blot using anti-iNOS antibody (Santa Cruz). In the bar graph, the relative expression of iNOS normalized to the actin level represents the mean  $\pm$  SEM from at least three independent experiments and are expressed as percentage of cells incubated with cytokines (100%). Statistical significance: \*\*\* $p < 0.001$  as compared to control cells; &&& $p < 0.001$  as compared to cells stimulated with cytokines. **(B)** After 16 hours of incubation, supernatants were collected and the  $\cdot$ NO production was measured by chemiluminescence. Statistical significance: \*\*\* $p < 0.001$  as compared to control cells; &&& $p < 0.001$  as compared to cells stimulated with cytokines. A-Control; B-Cytokines; C-Cytokines +100  $\mu$ g RWE/ml; D- Cytokines +200  $\mu$ g RWE/ml; E- Cytokines +400  $\mu$ g RWE/ml; F-Cytokines +600  $\mu$ g RWE/ml.



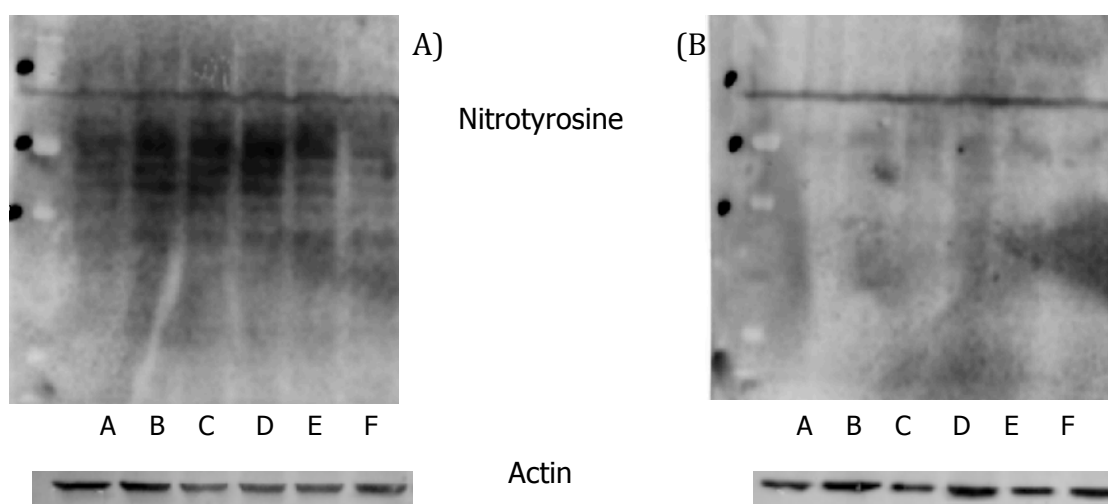
**Figure 15 - Immunostaining for iNOS:** Cells were pre-treated with four different concentrations of RWE (100, 200, 400 and 600 µg/ml) for 30 min and then stimulated with cytokines (20 ng/ml TNF- $\alpha$ ; 10 ng/ml IL-1; 60 ng/ml INF- $\gamma$ ) for 6 hours. A-Control; B-Cytokines; C-Cytokines +100 µg RWE/ml; D- Cytokines +200 µg RWE/ml; E- Cytokines +400 µg RWE/ml; F-Cytokines +600 µg RWE/ml.

### 3.5 - Red Wine Extract inhibits tyrosine nitration in cytokines-stimulated HT-29 cells

An increased production of  $\bullet\text{NO}$  would, via interaction with superoxide radical, yield the inflammatory stressor peroxynitrite, thus promoting a nitroxidative stress.

One of the major consequences of the peroxynitrite is the selective nitration of the tyrosine residues in specific proteins, affecting protein structure and function.

Therefore, as cytokines induced an overproduction of  $\bullet\text{NO}$  by HT-29 cells, we first analyzed, by western blot with an antibody anti-nitrotyrosine, if cytokines also induced nitration of the tyrosine residues in proteins. As we can see in figure 16 (lane B), cells incubated with cytokines showed higher levels of nitrated proteins as compared with control cells. To determine the effect of RWE in nitrotyrosination, HT-29 cells were pre-treated with RWE (100, 200, 400 and 600  $\mu\text{g}/\text{ml}$ ) for thirty minutes and then stimulated with cytokines (20 ng/ml  $\text{TNF-}\alpha$ ; 10 ng/ml IL-1; 60 ng/ml  $\text{INF-}\gamma$ ) for sixteen hours. RWE decreased the levels of tyrosine nitration in a dose dependent manner. Treatment with dithionite that converts nitrotyrosine in aminotyrosine proves that the bands present in figure 16 A) that disappear in figure 16 B) are really proteins with nitrated tyrosines.



**Figure 16: Effect of RWE on tyrosine nitration in cytokines-stimulated HT-29 cells:** HT-29 cells were pre-treated with different concentrations of RWE (100, 200, 400 and 600  $\mu\text{g}/\text{ml}$ ) and stimulated with cytokines (20 ng/ml  $\text{TNF-}\alpha$ ; 10 ng/ml IL-1; 60 ng/ml  $\text{INF-}\gamma$ ) for 16 hours then total extracts were obtained and western blot was performed. A) Without treatment with dithionite B) with dithionite treatment. A-control; B-Cytokines; C- Cytokines + 100  $\mu\text{g}$  RWE/ml; D-Cytokines +200  $\mu\text{g}$  RWE/ml; E-Cytokines +400  $\mu\text{g}$  RWE/ml; F-Cytokines +600  $\mu\text{g}$  RWE/ml.

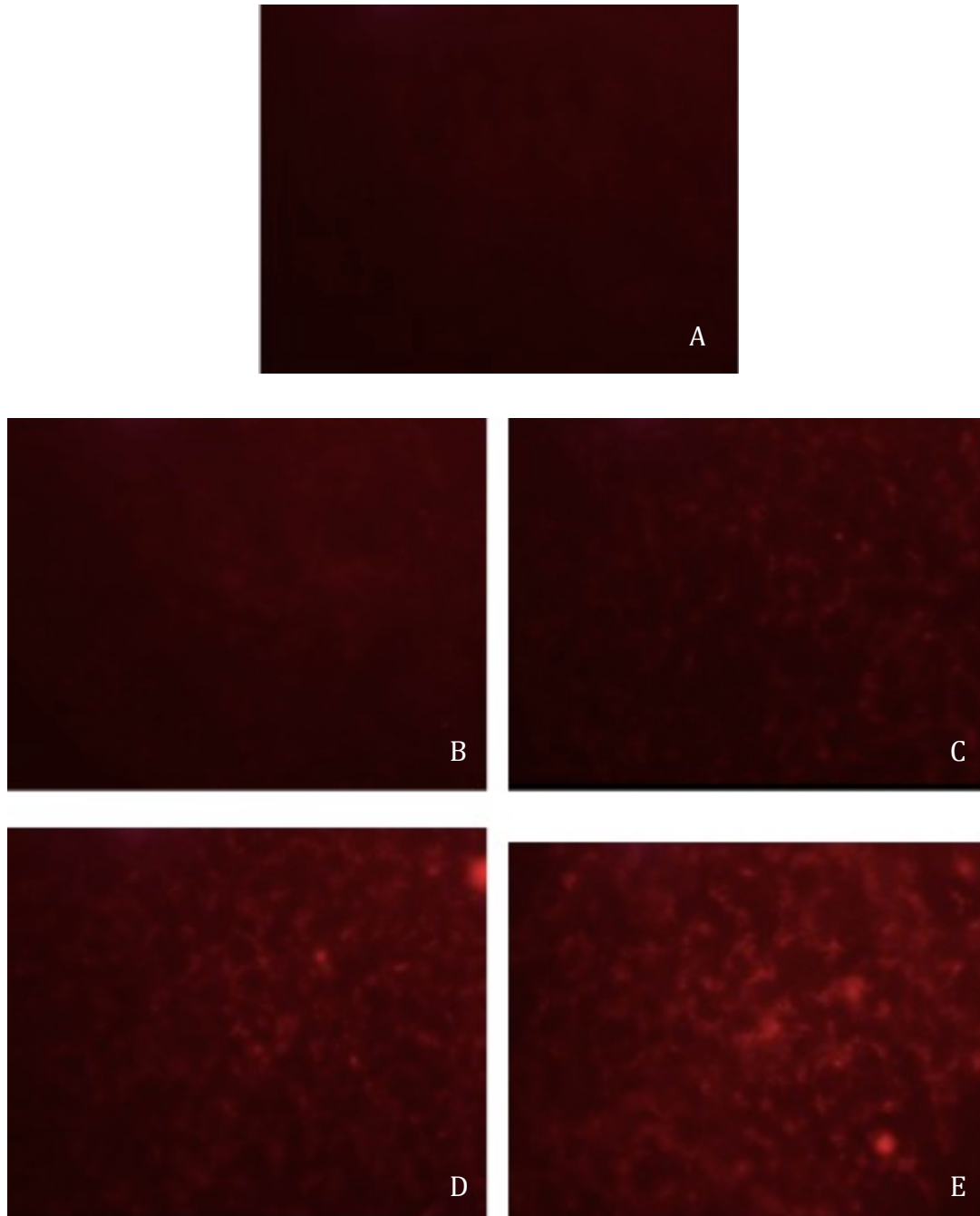


### **3.6 - Red Wine Extract modifies occludin expression and distribution in TNF- $\alpha$ -stimulated HT-29 cells**

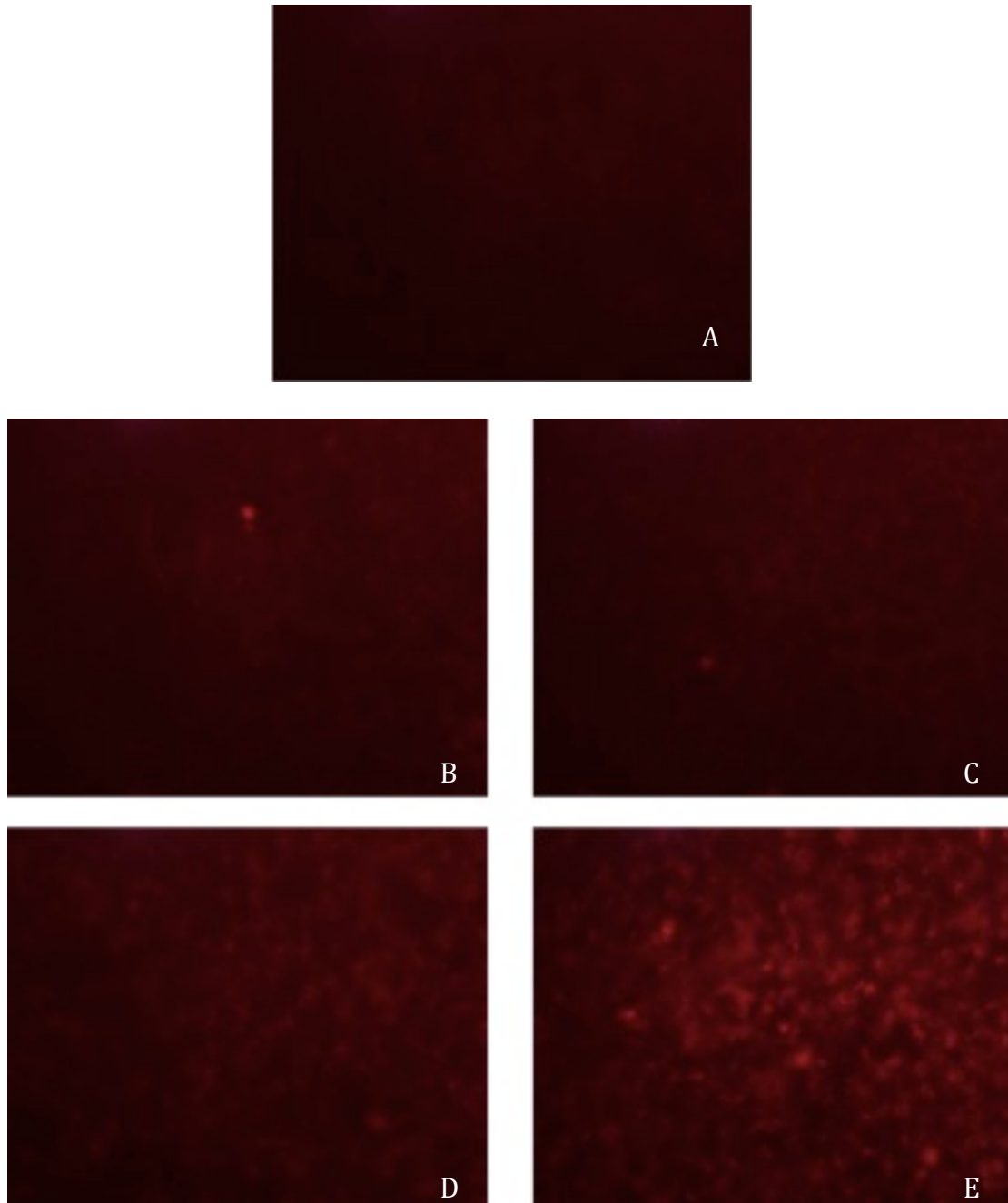
Intestinal epithelial cells (IECs) regulate the intestinal permeability through the epithelial junctions, which can be desmosomes adherent junctions or tight junctions (TJs). TJs are complex structures composed of different proteins such as occludins, proteins of the zonula occludens and proteins of the claudin family. In IBD we have an increase of pro-inflammatory cytokines that could lead to the impairment of tight junctions and consequently loss of barrier function.

To investigate the effect of RWE in the tight junctions the distribution and expression of occludin will be studied by immunocytochemistry in cells pre-treated with several RWE concentrations and stimulated with TNF- $\alpha$  (70 ng/ml) overnight (sixteen hours).

Immunocytochemistry results (figures 17,18) showed that RWE significantly alter de occludin expression and distribution in HT-29 cells, especially when HT-29 cells were stimulated with TNF- $\alpha$  and pre-treated with the higher concentrations of RWE. There is an increase of occludin levels around cells in a dose dependent manner.



**Figure 17 : Immunostaining for occludin:** Cells were pre-treated with four different concentrations of RWE (100, 200, 400 and 600 µg/ml) for 30 min and then stimulated with TNF- $\alpha$  (plus sign) and maintained overnight (16 h). A-control; B-100 µg RWE/ml; C-200 µg RWE/ml; D-400 µg RWE/ml; E-600 µg RWE/ml.



**Figure 18- Immunostaining for occludin:** Cells were pre-treated with four different concentrations of RWE (100, 200, 400 and 600  $\mu\text{g/ml}$ ) for 30 min and then stimulated with  $\text{TNF-}\alpha$  (plus sign) and maintained overnight. A- $\text{TNF-}\alpha$ ; B-  $\text{TNF-}\alpha$ +100  $\mu\text{g RWE/ml}$ ; C-  $\text{TNF-}\alpha$ +200  $\mu\text{g RWE/ml}$ ; D-  $\text{TNF-}\alpha$ +400  $\mu\text{g RWE/ml}$ ; E-  $\text{TNF-}\alpha$ +600  $\mu\text{g RWE/ml}$ .

## **3.7- Red Wine Extract affects the Nrf2 pathway in TNF- $\alpha$ -stimulated HT-29 cells**

### **3.7.1- Evaluation of Nrf2 translocation to the nucleus**

At normal conditions, Nrf2 resides in the cytoplasm bound to a cytosolic repressor Keap1. However, under nitroxidative stress conditions, Nrf2 breaks free from Keap1 and translocates into the nucleus enabling gene transcription.

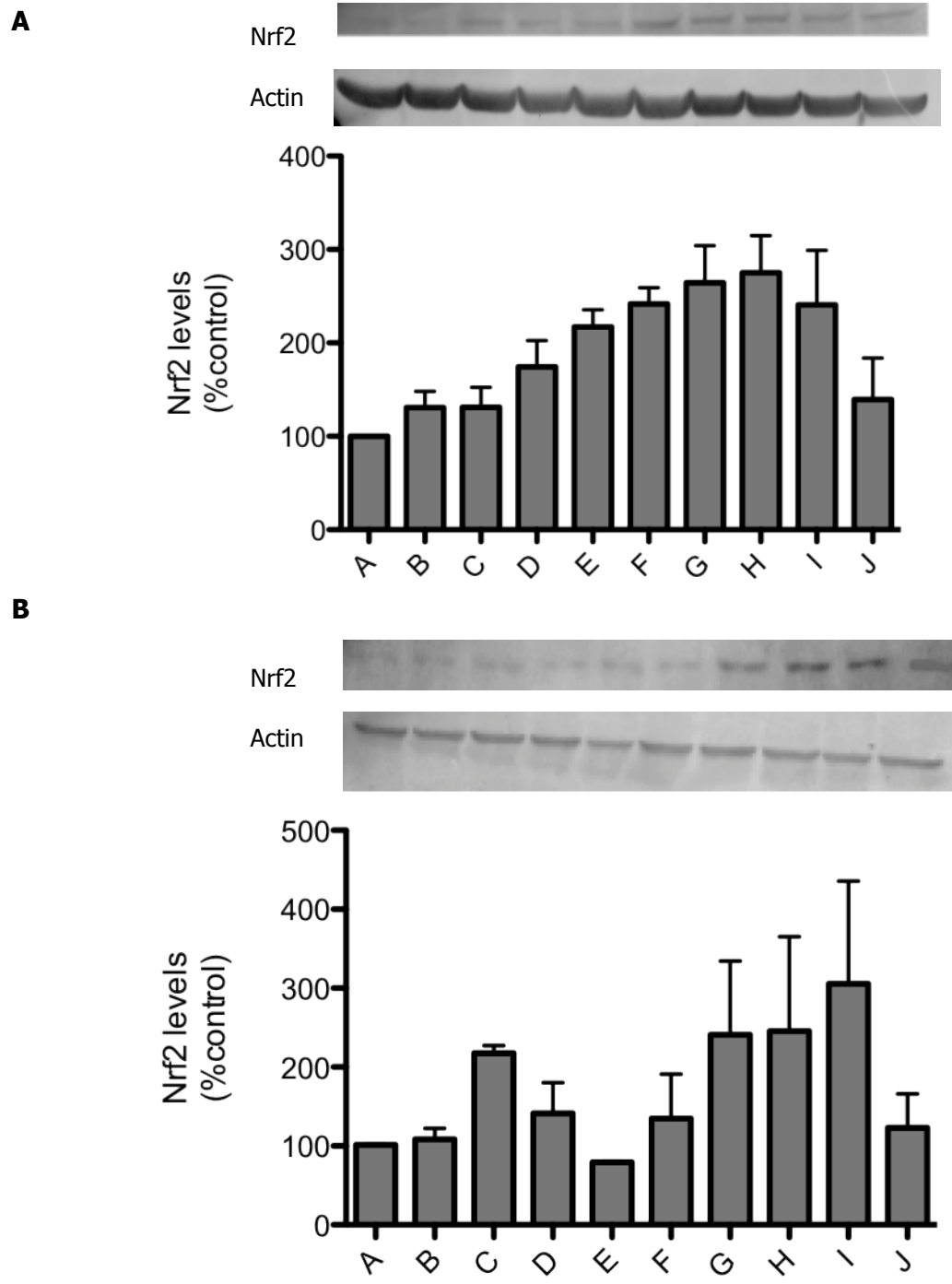
To investigate if the anti-inflammatory effect of RWE is related with the Nrf2 pathway activation, we analyse the levels of Nrf2 by Western Blot in the cytoplasm and in the nucleus at various times.

HT-29 cells were pre-treated with four different concentrations of RWE (100, 200, 400 and 600  $\mu\text{g/ml}$ ) for thirty minutes and then stimulated with TNF- $\alpha$  (70 ng/ml) for three hours.

Figure 19 show that treatment with RWE alone enhanced the Nrf2 levels in the cytoplasm, in a dose dependent manner. However, only treatment with 200 and 400  $\mu\text{g/ml}$  of RWE caused an increase in Nrf2 levels in the nuclear extracts ( $217.7\pm 9.5$  and  $141.5\pm 38$ , respectively). TNF- $\alpha$  increases cytoplasmatic Nrf2 levels ( $242\pm 17\%$ ) and also induced a slightly increase of the nuclear Nrf2 levels ( $135\pm 56$ ). Cells treated with 100, 200 and 400  $\mu\text{g/ml}$  of RWE and then stimulated with TNF- $\alpha$ -also presented higher cytoplasmatic Nrf2 levels. RWE (100, 200 and 400  $\mu\text{g/ml}$ ) potentiated the slightly increase of the nuclear Nrf2 levels induced by TNF- $\alpha$ .

Cells were also stimulated with TNF- $\alpha$  for thirty minutes, one and six hours but in these situations the Nrf2 levels in the cytoplasm and nucleus doesn't suffer significant variations (data not shown).

Results showed that RWE seems to increase the Nrf2 expression levels in the cytoplasm and Nrf2 translocation to the nucleus in both, non-stimulated and TNF- $\alpha$  stimulated HT-29 cells, being this effect stronger in the later case.



**Figure 19 -Effect of Red Wine Extract (RWE) on Nrf2 expression levels.** Cells were pretreated with four different concentrations of RWE (100, 200, 400 and 600 mg/ml) for 30 min and then stimulated with TNF- $\alpha$  for 3hours. **(A)** Cytoplasm; **(B)** Nucleus. After 3 hours of incubation, cytoplasmic and nuclear extracts were obtained and then analysed by Western blot using anti-Nrf2 antibody (R&D Systems). In the bar graphs, the relative expression of Nrf2 normalized to the actin level represents the mean  $\pm$  SEM from at least three independent experiments and are expressed as percentage of control cells (100%).  
 A-Control; B-100  $\mu$ g RWE/ml; C-200  $\mu$ g REW/ml; D-400  $\mu$ g RWE/ml; E-600  $\mu$ g RWE/ml; F-TNF- $\alpha$  s; G- TNF- $\alpha$ +100  $\mu$ g RWE/ml; H-TNF- $\alpha$ +200  $\mu$ g RWE/ml; I- TNF- $\alpha$ +400  $\mu$ g RWE/ml; J- TNF- $\alpha$ +600  $\mu$ g RWE/ml.

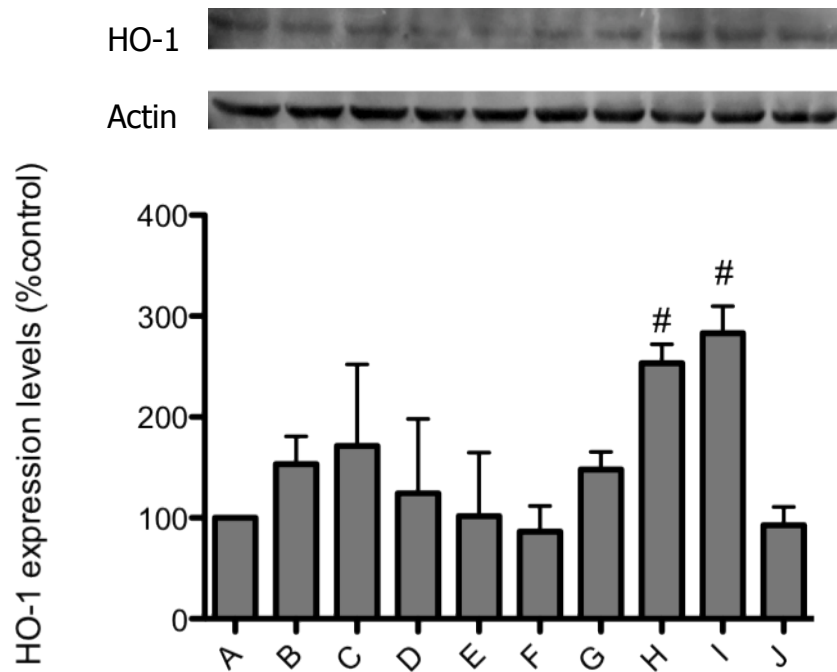
### **3.7.2- Evaluation of the Red Wine Extract effect in Nrf2 target genes**

Nrf2 regulates numerous genes through the antioxidant response element (ARE), such as Hemoxygenase 1 (HO-1) and glutathione related enzymes.

HO-1 is one of the Nrf2 downstream targets. HO-1 is an essential enzyme in heme catabolism. This enzyme is responsible for the heme degradation into iron, carbon monoxide and biliverdin, the latter being subsequently converted into bilirubin. Several positive biological effects exerted by this enzyme have gained attention, as anti-inflammatory, antiapoptotic, angiogenic, and cytoprotective functions are attributable to carbon monoxide and/or bilirubin.

So, as RWE seems to induce Nrf2 translocation to the nucleus, next we investigated the effect of RWE in the HO-1 levels by Western Blot. HT-29 cells were pre-treated with four different concentrations of RWE (100, 200, 400 and 600  $\mu\text{g/ml}$ ) for thirty minutes and then stimulated with TNF- $\alpha$  for one hour, three hours and six hours. The cytoplasmic extracts were obtained and western Blot was performed.

At one and three hours of stimulation with TNF- $\alpha$ , there were no significant alterations in the HO-1 expression levels (data not shown). At 6 hours of incubation, RWE alone, principally 200  $\mu\text{g/ml}$ , increased the levels of HO-1. The levels of HO-1 of cells pre-treated with 200 and 400  $\mu\text{g/ml}$  RWE and stimulated with TNF- $\alpha$  increase significantly when compared to the cells only stimulated with TNF- $\alpha$  (figure 20).



**Figure 20 -Effect of Red Wine Extract (RWE) on HO-1 expression levels.** Cells were pretreated with four different concentrations of RWE (100, 200, 400 and 600 mg/ml) for 30 min and then stimulated with TNF- $\alpha$  for 6 hours. After 6 hours of incubation, cytoplasmic extracts were obtained and then analysed by Western blot using anti-HO-1 antibody (Santa Cruz). In the bar graphs, the relative expression of HO-1 normalized to the actin level represents the mean  $\pm$  SEM from two independent experiments and are expressed as percentage of control cells (100%). Statistical significance: #  $p < 0.05$  as compared to cells stimulated with TNF- $\alpha$ .

A-Control; B-100  $\mu$ g RWE/ml; C-200  $\mu$ g REW/ml; D-400  $\mu$ g RWE/ml; E-600  $\mu$ g RWE/ml; F-TNF- $\alpha$  s; G- TNF- $\alpha$ +100  $\mu$ g RWE/ml; H-TNF- $\alpha$ +200  $\mu$ g RWE/ml; I- TNF- $\alpha$ +400  $\mu$ g RWE/ml; J- TNF- $\alpha$ +600  $\mu$ g RWE/ml.

Glutathione is one of the most important endogenous antioxidant produced by the cells, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as the maintenance of exogenous antioxidants such as vitamins C and E in their reduced (active) forms. It is also essential for the immune system to exert its full potential, e.g. (1) modulating antigen presentation to lymphocytes, thereby influencing cytokine production and the type of response (cellular or humoral) that develops, (2) enhancing proliferation of lymphocytes thereby increasing magnitude of response, (3) enhancing killing activity of cytotoxic T cells and NK cells, and (4) regulating apoptosis, thereby maintaining control of the immune response.

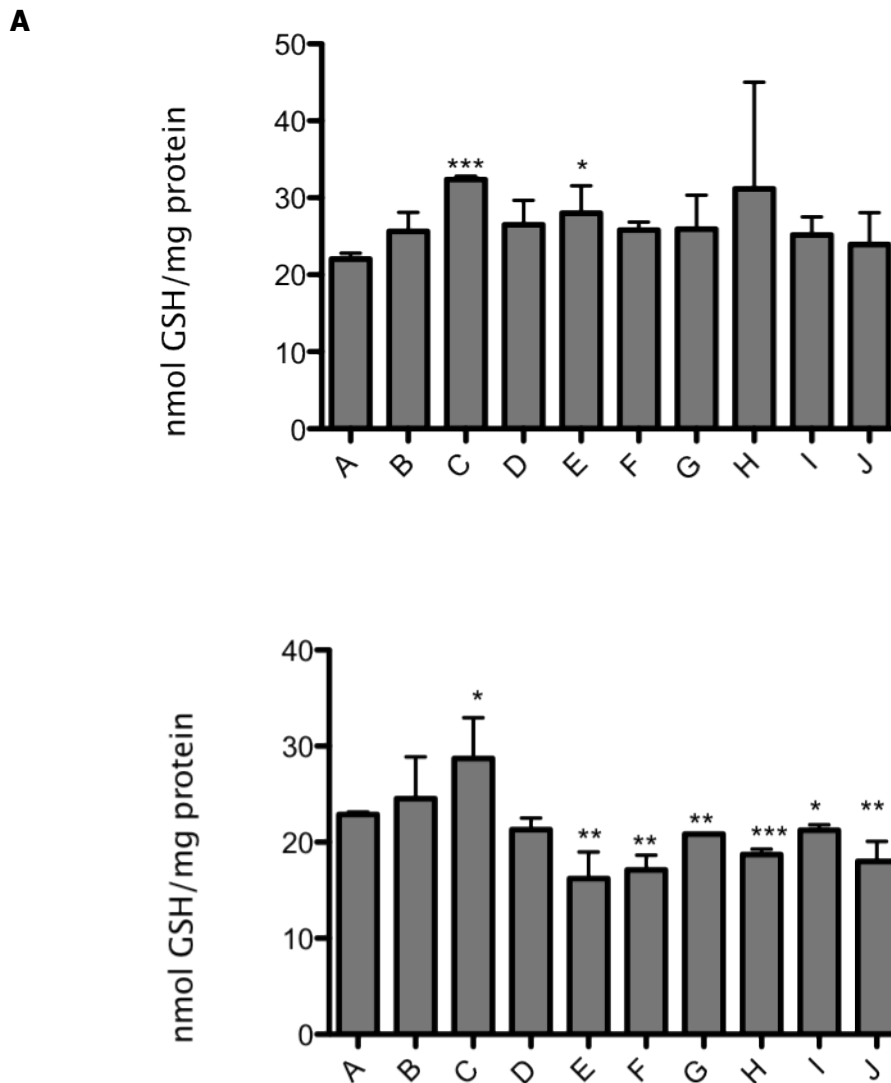
The oxidized and reduced glutathione levels in HT-29 cells pre-treated with four different concentrations of RWE (100, 200, 400 and 600  $\mu\text{g/ml}$ ) for thirty minutes and then stimulated cytokines (20 ng/ml TNF- $\alpha$ ; 10 ng/ml IL-1; 60 ng/ml INF- $\gamma$ ) for six hours or overnight were calculated by a fluorimetric method described by Hissin and Hilf. This method measures both oxidized (GSSG) and reduced (GSH) glutathione using *o*-phthalaldehyde (OPT) as a fluorescent reagent and takes advantage of the reaction of GSH with OPT at pH 8 and GSSG with OPT at pH 12. GSH can be complexed to N-ethylmaleimide (NEM) to prevent interferences of GSH with measurement of GSSG.

Results show that with six hours of stimulation reduced glutathione levels suffer a significant increase when cells are pre-treated with 200 and 600  $\mu\text{g/ml}$  of RWE but non stimulated with cytokines, addition of cytokines without pre-treatment with RWE induces a very little increase in the reduced glutathione levels. Pre-treatment with RWE in cytokines stimulated cells does not significantly alter the GSH levels when compared with cytokines stimulated cells levels although pre-treatment with 200  $\mu\text{g/ml}$  of RWE seems to increase GSH levels (figure 21 A).

With cytokines stimulation overnight results are different. In all conditions stimulated with cytokines the GSH levels decrease significantly. Pre-treatment with RWE extracts does not revert this effect but there are minor increases in GSH levels in cells pre-treated with RWE and cytokine stimulated when compared with cytokines stimulated cells without RWE pre-treatment (figure 21 B).

There is no significant impact in the oxidized glutathione levels with pre-treatment with RWE and six hours or overnight stimulation with cytokines (data not shown).





**Figure 21 - Effect of Red Wine Extract (RWE) on GSH levels in cytokines-stimulated HT-29 cells.** Cells were pretreated with four different concentrations of RWE (100, 200, 400 and 600  $\mu\text{g/ml}$ ) for 30 min and then stimulated with cytokines (20 ng/ml TNF- $\alpha$ ; 10 ng/ml IL-1; 60 ng/ml INF- $\gamma$ ) for **(A)** 6 hours, **(B)** Overnight. GSH levels were calculated using the fluorimetric method described by Hissin and Hilf. Data represent the mean  $\pm$  SEM from at least three independent experiments. Statistical significance: \*\*\* $p < 0.001$  as compared to control cells; \*\* $p < 0.005$  and \* $p < 0.05$  as compared to control cells. A-Control; B-100  $\mu\text{g}$  RWE/ml; C-200  $\mu\text{g}$  REW/ml; D-400  $\mu\text{g}$  RWE/ml; E-600  $\mu\text{g}$  RWE/ml; F-TNF- $\alpha$  s; G- TNF- $\alpha$ +100  $\mu\text{g}$  RWE/ml; H-TNF- $\alpha$ +200  $\mu\text{g}$  RWE/ml; I- TNF- $\alpha$ +400  $\mu\text{g}$  RWE/ml; J- TNF- $\alpha$ +600  $\mu\text{g}$  RWE/ml.

## Chapter 4: Discussion

Although the etiology of IBDs has not been fully elucidated, several studies suggested that genetic, environmental, microbial and immunologic factors are implicated in the pathogenesis of IBD. As other inflammatory disorders, IBD is characterised by a deregulated synthesis and release of a variety of proinflammatory mediators, including cytokines, reactive oxygen species (ROS) and nitric oxide ( $\text{NO}$ ), resulting in a disruption of epithelial barrier and excessive tissue injury.

A specific causal treatment of IBD is still not available and the most currently drugs used in its treatment have serious side effects that limit their use. Consequently, the development of new drug treatments that combine efficacy and safety is an important goal in IBD therapy.

In recent years, there has been a growing interest supported by a large number of experimental and epidemiological studies for the beneficial effects of dietary polyphenols in preventing chronic inflammatory conditions. Particularly, the efficacy of polyphenols in preventing and treating IBD has been suggested by several studies, using rodent models of IBD (Shapiro H. et al, 2007). In these studies acute or chronic colitis was induced by intrarectal administration of dinitrobenzene sulphate or trinitrobenzene sulphate, addition of dextran sulphate sodium in the drinking water or by knockout of the interleukin-2 genes. The administration of polyphenols was done orally or intraperitoneally before, during and/or after induction of colitis. Then rodents were killed and indices of disease were assessed between 48 h and 6 weeks after induction of colitis (Shapiro H. et al, 2007). The polyphenols tested (resveratrol, EGCG/green tea extract, curcumin,, quercetin and its naturally occurring glycones) reduced mortality rates, diminished colonic and extracolonic signs of disease, colon macropathology and micropathology and/or indices of inflammation and autoimmunity (eg, colonic myeloper-oxidase and NF-kB activity, increased TNF- $\alpha$ , IL-1b, IL-12, iNOS and reduced IL-10, Crohn's disease 4+ T cell and neutrophil infiltration) (Shapiro H. et al, 2007).

Additional studies performed in humans with ulcerative colitis or Crohn's disease using curcumin provided promising results, in terms of improved clinical symptoms, histopathology and laboratory indices and no serious adverse effects were reported (Holt P. et al, 2005; Hanai H. et al, 2006).

However a clear understanding of the molecular mechanisms of action of polyphenols is crucial in the evaluation of these molecules as potential prophylactic and therapeutic agents in IBD.

In this study, we show that a red wine extract, rich in polyphenols (the phenol content expressed in catechin equivalents of the selected RWE is 222 mg/g) may be useful in the prevention and/or treatment of intestinal inflammation

As epithelial cells play an important role in intestinal inflammation, in this study, we have used as cellular model, HT-29 cells, a colonic epithelial cell line. HT-29 cells are an epithelial cell line derived from human colonic adenocarcinoma that is able to express differentiation features characteristic of mature intestinal cells. When stimulated with cytokines they can serve as a model of intestinal inflammation.

In any study using compounds with potential beneficial effects it is important to evaluate their effect on cell viability to determine if the compound has a protective effect or whether it compromises cell viability, if so there will be not interest in proceed with the study. Results show that RWE doesn't compromise cell viability in any of the tested concentrations for twenty-four hours (figure 10). Additionally, results obtained by our group show that RWE significantly protect HT-29 cells against cytokines-induced cell death in a dose dependent manner, therefore the studies were carried on (data not shown).

Gastrointestinal inflammation is associated with a high production of proinflammatory cytokines, including TNF- $\alpha$ . Therefore, initially we tested TNF- $\alpha$  as anti-inflammatory stimulus in our cellular model. In our model of study, TNF- $\alpha$  induced I $\kappa$ B degradation (figure 11), leading to the NF- $\kappa$ B translocation to the nucleus, a clear sign that induces inflammation and for that reason is a suitable inflammatory stimulus for this study.

Since its discover, NF- $\kappa$ B has been suspected to play a key role in chronic and acute inflammatory diseases. NF- $\kappa$ B is clearly one of the most important regulators of pro-inflammatory gene expression. Synthesis of cytokines (e.g. TNF-  $\alpha$ , IL-1 $\beta$ , IL-6), chemokines (e.g. IL-8), adhesion molecules and acute phase proteins among others is mediated by NF- $\kappa$ B. NF- $\kappa$ B activation has been implicated in diverse inflammatory diseases, namely in inflammatory bowel disease. In fact, several studies have reported elevated NF- $\kappa$ B levels in intestinal mucosa of IBD patients (Ellis R. et al, 1998; Neurath M. et al, 1998; Rogler G. et al, 1998). The inhibition of NF- $\kappa$ B is generally considered as a useful strategy for treatment of inflammatory disorders, namely IBD, representing an important and very attractive therapeutic

target (Karin M. et al, 2004). Several polyphenols have been shown to exert their anti-inflammatory activity by modulating NF- $\kappa$ B activation and acting in multiple steps of the activation process (Rahman I. et al, 2006). For that reason the effect of RWE in I $\kappa$ B degradation was tested to find out whether the polyphenolic extract may interfere with the NF- $\kappa$ B pathway.

Results have shown that RWE inhibits I $\kappa$ B degradation (figure 11), preventing NF- $\kappa$ B translocation to the nucleus and consequent pro-inflammatory gene expression. I $\kappa$ B proteins are associated with the NF- $\kappa$ B in unstimulated cells, sequestering the NF- $\kappa$ B in the cytoplasm as an inactive non-DNA-binding form. Upon cell stimulation I $\kappa$ B proteins are rapidly phosphorylated by I $\kappa$ B kinase (IKK) complex on two serine residues that targets the inhibitor proteins for ubiquitination and subsequent degradation by the ubiquitin-proteasome pathway. NF- $\kappa$ B translocates to the nucleus inducing expression of various genes above mentioned.

Polyphenols can act at multiple steps of the NF- $\kappa$ B pathway and, in this particular case, RWE inhibit the I $\kappa$ B degradation but it was not studied if this occur as a direct effect on IKK or by interfering with the interaction of IKK with I $\kappa$ B. Both hypotheses were confirmed in other cellular models of inflammation with other polyphenols (Yang F. et al 2001; Mackenzie G. et al, 2004).

The modulation of the NF- $\kappa$ B cascade by polyphenols can also occur at late stages, namely by preventing the binding of NF- $\kappa$ B to DNA (Rasheed Z. et al, 2009), this aspect was not tested with RWE.

As a further development of this study it would be interesting to study exactly where RWE modulate the NF- $\kappa$ B cascade.. It is known that polyphenols can act simultaneously in both early and late steps of the NF- $\kappa$ B cascade (Rasheed Z. et al, 2009). RWE are a mixture of various polyphenols so is expectable that they can act in various steps of this pathway. Being aromatic compounds polyphenols might also affect the aryl hydrocarbon receptor (AhR) that interacts with the NF- $\kappa$ B regulating his activity (Potapovich A. et al, 2011) but this particular aspect of the NF- $\kappa$ B pathway was also not studied.

Knowing that NF- $\kappa$ B regulates pro-inflammatory gene expression, the levels of IL-8 were measured by Elisa. IL-8 is a primary inflammatory cytokine and one of the major mediators of inflammation. It is produced by many cells including epithelial cells in response to pro-inflammatory stimuli. This cytokine belongs to the CXC chemokine family and function as a chemoattractant for neutrophils and T-cells.

IL-8 has been implicated in various inflammatory diseases including ulcerative colitis (Zimmermann H. et al, 2011; Hauser W. et al, 2011). The inhibition of TNF- $\alpha$ -induced IL-8 overproduction by RWE in HT-29 (figure 12), suggested that RWE can interfere with the recruitment of neutrophils and T-cells and thus may impede progression and aggravation of intestinal inflammation. Expression of the IL-8 gene is dependent on the activation of transcription factor NF- $\kappa$ B, RWE shown to suppress NF- $\kappa$ B activity and consequently IL-8 overproduction.

Overproduction of  $\bullet$ NO occurs during the progression of various inflammatory diseases in the intestinal tract (Keklikoglu N. et al, 2008) due to the induction of the iNOS by excessive inflammatory cytokine production. Large amounts of  $\bullet$ NO produced by cells in response to cytokines can destroy host tissues and impair cellular responses. Compounds able to block iNOS-induced  $\bullet$ NO production by the suppression of iNOS induction are very attractive as anti-inflammatory agents. For that reason the effect of RWE in the iNOS induction was studied. Initially we challenged HT-29 cells with different concentrations of TNF- $\alpha$  for several periods of time and next we evaluated the iNOS induction by Western blot. TNF- $\alpha$  alone did not induce iNOS expression. Therefore, next, we examine the cytokines requirements for the iNOS induction in HT-29 cells and we conclude that it is necessary a mix of cytokines (20 ng/ml TNF- $\alpha$ ; 10 ng/ml IL-1; 60 ng/ml INF- $\gamma$ ) to induce iNOS expression.

Western Blot and immunohistochemistry results showed that RWE inhibits the expression of iNOS stimulated by cytokines in a concentration dependent manner (figure 14 A).

This effect is probably mediated by NF- $\kappa$ B inhibition, as reported to other polyphenols in previous studies where the expression of iNOS mRNA was reduced through prevention of the binding of NF- $\kappa$ B to the iNOS promoter, thereby inhibiting the induction of iNOS transcription (Lin Y. et al, 1997).

To further confirm that the inhibition of iNOS induction has a real effect in the  $\bullet$ NO production in cytokines-stimulated cells, the  $\bullet$ NO production was measured in the supernatants after twenty-four hours of incubation with cytokines. Results show an expectable increase of  $\bullet$ NO production when cells were only treated with cytokines and a decrease, to the control levels, when cells were pre-treated with RWE (figure 14 B). Initially, we used the Griess method for  $\bullet$ NO determination, but given its poor sensitivity, it could not be used. Therefore, chemiluminescence, a method with a high sensitivity and selectivity, was chosen as method for  $\bullet$ NO determination.

Indirect reactions of •NO occur when levels of •NO are very high and they are specially implicated in the pro-inflammatory effects: the formation of reactive nitrogen species such as peroxynitrite triggers toxic events that culminate in cytotoxicity and nitration and nitrosation of several residues in diverse proteins, affecting their function (ex. iron-sulfur containing enzymes, disruption of mitochondrial respiration) (Clancy R. et al, 1998).

Nitrotyrosine is a product of tyrosine nitration mediated by reactive nitrogen species and it is considered a marker of •NO-dependent, reactive nitrogen species-induced nitrate stress and an indicator of cell damage and inflammation.

The effect of RWE in nitrotyrosination was also investigated, and Western Blot results suggest that the higher concentrations of RWE effectively decrease the levels of tyrosine nitration (figure 16).

Treatment with dithionite, which converts nitrotyrosine in aminotyrosine, was used in the samples and Western Blot was performed with non-treated and treated samples to confirm that the bands present really correspond to proteins with nitrated tyrosines. The results obtained were only preliminary because the experiment could not be repeated more times due to the non-disponibility of more antibody, the aliquots of antibody could not be used more than one or two times because they lose their properties of marking. To further complete the results it would be interesting to perform Dot Blot because that technique would provide us a more accurate notion of the real quantity of nitrotyrosine in the sample and if in fact RWE protect against nitrotyrosination.

The microscopic observation of the cells into the plates treated with RWE lead to the suspicion that RWE could somehow affect the epithelial junctions. In fact, cells treated with RWE seems to be more condensate in aggregates than the control cells. Also, when cellular extracts were prepared, the suspension of cellular pellets was much more difficult.

Epithelial junctions regulate the intestinal permeability maintaining the intercellular adhesion and regulating paracellular transport, they could be desmosomes, adherent junctions (AJs) and tight junctions (TJs). Impairment of the tight junctions seems to have higher impact in IBD pathogenesis (Clayburgh D. et al, 2004). Elevated levels of pro-inflammatory cytokines, characteristic of IBD, lead to an impairment of tight junctions and consequently loss of barrier function.

To investigate if RWE could really have an effect in the tight junctions, particularly in an IBD situation, the occludin expression and distribution was observed by

immunocytochemistry in cells pre-treated with several RWE concentrations and stimulated with TNF- $\alpha$  overnight.

Results show that RWE significantly alter de occludin expression and distribution in HT-29 cells, especially when HT-29 cells were stimulated with TNF- $\alpha$  and pre-treated with the higher concentrations of RWE. There is an increase of occludin levels around cells in a dose dependent manner.

Occludin is an integral membrane protein specifically associated with tight junctions which forms a rate-limiting transport structure within the intercellular cleft and other studies demonstrate that this protein confers adhesiveness to the cells (Van Itallie C. et al, 1997; Kevil C. et al, 1998). In IBD the epithelial barrier is disrupted and a certain leakiness is observed in the tight junctions what makes any compound capable of revert this aspect very interesting as therapeutic agent.

The results suggest that RWE could have some effect in the tight junctions altering the occludin expression and distribution (figure 17 and 18) but more studies are necessary to confirm the results and to investigate the effect of RWE in other tight junctions proteins and in transepithelial permeability.

Nitroxidative stress inevitably accompanies any inflammation, changes the redox balance in cells and activates the redox-dependent signalling pathways. The transcription factor Nrf2 is the guardian of redox homeostasis and the gene families affected by Nrf2, ARE-containing genes, have several anti-inflammatory and cytoprotective actions. The role of Nrf2 in inflammatory diseases has gained attention in the last years particularly in cellular models like macrophages (Wang H. et al, 2003; Itoh K. et al 2004).

Knowing that dietary polyphenols have shown some beneficial effects in chronic inflammatory conditions and in particular having study the effect of RWE in some inflammatory parameters it was logical to study the effect of RWE in this particular pathway.

As previously explained, under normal conditions, Nrf2 resides in the cytoplasm bound to a cytosolic repressor Keap1. However, under nitroxidative stress conditions, Nrf2 breaks free from Keap1 and translocates into the nucleus enabling gene transcription. To investigate if the RWE suppress the inflammatory process by activating the Nrf2 pathway, the Nrf2 nuclear translocation was evaluated as well as the effect in the Nrf2 downstream targets HO-1 and glutathione.

This investigation had some technical problems. To determine if RWE has some effect in the translocation of Nrf2 to the nucleus the determination of the Nrf2 levels

in the cytoplasm and nucleus was performed by Western Blot. It is known that the molecular weight of Nrf2 is around 68 KDa and it was expectable that the band in the membranes would appear around that value. However with the first antibody used this didn't occur. The band occurred in a much lower molecular weight value what lead to question the reliability of the results. Other brand of antibody was then used, with this one, the band presumably correspondent to Nrf2 occurred at a molecular weight approximate of the real molecular weight of Nrf2.

Other challenge in the investigation was the choice of the assay duration times because the complete kinetics of the Nrf2 translocation to the nucleus is not fully understood. Choosing a time where the presumable increase of Nrf2 levels in the nucleus was visible was not easy.

In at concerns to the evaluation of Nrf2 translocation to the nucleus, results suggested that RWE increases the Nrf2 expression levels in the cytoplasm and Nrf2 translocation to the nucleus in both, non-stimulated and TNF- $\alpha$  stimulated HT-29 cells, being this effect stronger in the later case (figure 19).

However the expectable increase in the nuclear Nrf2 levels is not statistically significant (figure 19). It is important to note that every process that regulates gene transcription is also closely regulated. The entry of Nrf2 and the time of permanency into the nucleus should be no exception.

We must also take into account that western blot is not the most sensitive technique. It would be interesting to use other techniques to determine the levels of Nrf2 in the nucleus like ELISA. It was also interesting to determine if RWE really relieved Keap1 inhibition of Nrf2, this could be made by determining the Keap1 levels of expression by western blot. The ARE/Nrf2 complex could also be studied by electrophoretic mobility shift assay (EMSA) to determine if RWE enhances not only the Nrf2 translocation into the nucleus but also its binding to the ARE.

To further confirm the effect of RWE in the Nrf2 pathway, the Nrf2 downstream targets HO-1 and glutathione were studied.

In fact, after six hours of stimulation, RWE alone, principally 200  $\mu$ g/ml, increased the levels of HO-1. The levels of HO-1 in the cells pre-treated with 200 and 400  $\mu$ g/ml RWE and then stimulated with TNF- $\alpha$  also increase significantly when compared to the cells only stimulated with TNF- $\alpha$  (figure 20). These results are according to the results related with Nrf2 nuclear translocation leading to the conclusion that RWE enhances HO-1 expression most likely by the Nrf2 pathway.



However there were also some difficulties with the determination of the HO-1 expression levels, the bands in the western blot are not very visible even using various strategies in the western blot procedure to improve the quality of the results. For that reason and knowing that western blot is not the most accurate technique to gene expression determination it would be interesting to perform a quantitative real time PCR for HO-1 gene expression.

It is also important to consider that dietary polyphenols can regulate HO-1 expression via various transcription factors (Andreadi C. et al, 2006) which include AP-1 (via ERK and or JNK), NF- $\kappa$ B (Juan S. et al, 2005) as well as Nrf2 (Balogun E. et al, 2003). For that reason the increase in HO-1 expression levels cannot be fully attributed to the Nrf2 pathway.

In at concerns to the glutathione levels it is known that Nrf2 increases glutathione biosynthesis. The synthesis of glutathione is achieved by the consecutive action of the ATP-dependent enzymes,  $\gamma$ -glutamylcysteine synthetase and glutathione synthetase. It was reported that levels of both these key enzymes are affected by the activity of Nrf2 (Chan J. and Kwong M., 2000).

In a non-stimulated situation RWE seems to increase glutathione levels, however it does not have a major impact when cells are stimulated with cytokines for six hours or overnight (figure 21). Overnight stimulation induces cell death what leads to the observed decreases in the glutathione levels (figure 21 B). The fact that RWE increases glutathione levels in a non-stimulated situation is an evidence of the involvement of the Nrf2 pathway but it was expectable to see the same behaviour in a stimulated situation.

It would be important to also study the  $\gamma$ -glutamylcysteine synthetase and glutathione synthetase expression levels to have more informative results because the nitroxidative stress itself could lead to major glutathione depletion and even though the expression of synthetizing enzymes is increased this is not enough to increase total glutathione levels.

Taking in account all the results regarding the effect of RWE in the Nrf2 pathway there are compelling evidences that in fact this pathway is affected but further studies are needed to confirm these hypothesis.

# Conclusion

The presented work presents strong evidences that RWE can protect against intestinal inflammation and identifies possible molecular mechanisms involved in the defence.

Firstly, it was demonstrated that the viability of cells is not compromised when incubated with RWE. Under these conditions, RWE was shown to modulate cellular pathways preventing both the trigger and the propagation of critical inflammatory cascades. In particular, when incubated with the cellular model under inflammatory stimulus RWE prevented I $\kappa$ B degradation, inhibited the NF- $\kappa$ B pathway and prevented IL-8 overproduction as well as iNOS induction.

In addition to the interference with inflammatory cascades we have also searched for biomarkers of inflammation-associated nitrosative stress as other possible actions of RWE. In this regard it was shown that RWE prevented against tyrosine nitration. Additionally RWE interfered with occluding expression in the tight junctions.

This work also present some evidences that RWE can elicit their beneficial actions acting in the Nrf2 pathway but further studies are necessary to complement the obtained results.

All together the mechanistic and regulatory data shown here support that dietary compounds rich in polyphenols, particularly red wine, may provide a potential benefit for the prevention and treatment of inflammatory diseases such as IBD.

# References

- Alderton, W. K., C. E. Cooper, et al. (2001). "Nitric oxide synthases: structure, function and inhibition." *Biochem J* **357**(Pt 3): 593-615.
- Aleksunes, L. M. and J. E. Manautou (2007). "Emerging role of Nrf2 in protecting against hepatic and gastrointestinal disease." *Toxicol Pathol* **35**(4): 459-73.
- Andreadi, C. K., L. M. Howells, et al. (2006). "Involvement of Nrf2, p38, B-Raf, and nuclear factor-kappaB, but not phosphatidylinositol 3-kinase, in induction of hemeoxygenase-1 by dietary polyphenols." *Mol Pharmacol* **69**(3): 1033-40.
- Balogun, E., M. Hoque, et al. (2003). "Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element." *Biochem J* **371**(Pt 3): 887-95.
- Baumgart, D. C. and W. J. Sandborn (2007). "Inflammatory bowel disease: clinical aspects and established and evolving therapies." *The Lancet* **369**(9573): 1641-1657.
- Beecher, G. R. (2003). "Overview of dietary flavonoids: nomenclature, occurrence and intake." *J Nutr* **133**(10): 3248S-3254S.
- Bjorne, H. H., J. Petersson, et al. (2004). "Nitrite in saliva increases gastric mucosal blood flow and mucus thickness." *J Clin Invest* **113**(1): 106-14.
- Bloom, D. A. and A. K. Jaiswal (2003). "Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression." *J Biol Chem* **278**(45): 44675-82.
- Bradford, M.M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." *Anal Biochem* **72**:248-254.
- Bravo, L. (1998). "Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance." *Nutr Rev* **56**(11): 317-33.
- Chan, J. Y. and M. Kwong (2000). "Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein." *Biochim Biophys Acta* **1517**(1): 19-26.
- Clancy, R. M., A. R. Amin, et al. (1998). "The role of nitric oxide in inflammation and immunity." *Arthritis Rheum* **41**(7): 1141-51.
- Clayburgh, D. R., L. Shen, et al. (2004). "A porous defense: the leaky epithelial barrier in intestinal disease." *Lab Invest* **84**(3): 282-91.
- Cullinan, S. B., J. D. Gordan, et al. (2004). "The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase." *Mol Cell Biol* **24**(19): 8477-86.
- D'Archivio, M., C. Filesi, et al. (2007). "Polyphenols, dietary sources and bioavailability." *Ann Ist Super Sanita* **43**(4): 348-61.

Denizot, F., and R. Lang. (1986). Rapid colorimetric assay for cell growth and survival. J. Immunol. Methods **89**:271-277.

Dhakshinamoorthy, S., A. K. Jain, et al. (2005). "Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants." J Biol Chem **280**(17): 16891-900.

Dinkova-Kostova, A. T., W. D. Holtzclaw, et al. (2002). "Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants." Proc Natl Acad Sci U S A **99**(18): 11908-13.

Eggleter, A. L., G. Liu, et al. (2005). "Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2." Proc Natl Acad Sci U S A **102**(29): 10070-5.

Ellis, R. D., J. R. Goodlad, et al. (1998). "Activation of nuclear factor kappa B in Crohn's disease." Inflamm Res **47**(11): 440-5.

Ferrero-Miliani, L., O. H. Nielsen, et al. (2007). "Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation." Clin Exp Immunol **147**(2): 227-35.

Friebe, A. and D. Koesling (2003). "Regulation of nitric oxide-sensitive guanylyl cyclase." Circ Res **93**(2): 96-105.

Furchgott, R. F. and J. V. Zawadzki (1980). "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine." Nature **288**(5789): 373-6.

Hanai, H., T. Iida, et al. (2006). "Curcumin maintenance therapy for ulcerative colitis: randomized, multicenter, double-blind, placebo-controlled trial." Clin Gastroenterol Hepatol **4**(12): 1502-6.

Hauser, W., C. Schmidt, et al. (2011). "Depression and mucosal proinflammatory cytokines are associated in patients with ulcerative colitis and pouchitis - A pilot study." J Crohns Colitis **5**(4): 350-3.

Hayden, M. S. and S. Ghosh (2008). "Shared principles in NF-kappaB signaling." Cell **132**(3): 344-62.

Hissin, P. J. and R. Hilf (1976). "A fluorometric method for determination of oxidized and reduced glutathione in tissues." Anal Biochem **74**(1): 214-26.

Hogg, N. and B. Kalyanaraman (1999). "Nitric oxide and lipid peroxidation." Biochim Biophys Acta **1411**(2-3): 378-84.

Holt, P. R., S. Katz, et al. (2005). "Curcumin therapy in inflammatory bowel disease: a pilot study." Dig Dis Sci **50**(11): 2191-3.

Ignarro, L. J. (1989). "Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein." Circ Res **65**(1): 1-21.

Ignarro, L. J., G. M. Buga, et al. (1987). "Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide." Proc Natl Acad Sci U S A **84**(24): 9265-9.

Itoh, K., M. Mochizuki, et al. (2004). "Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-Delta(12,14)-prostaglandin j(2)." Mol Cell Biol **24**(1): 36-45.

- Itoh, K., N. Wakabayashi, et al. (1999). "Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain." *Genes Dev* **13**(1): 76-86.
- Itoh, K., T. Chiba, et al. (1997). "An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements." *Biochem Biophys Res Commun* **236**(2): 313-22.
- Jain, A. K. and A. K. Jaiswal (2006). "Phosphorylation of tyrosine 568 controls nuclear export of Nrf2." *J Biol Chem* **281**(17): 12132-42.
- Jain, A. K. and A. K. Jaiswal (2007). "GSK-3 $\beta$  acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2." *J Biol Chem* **282**(22): 16502-10.
- Jaiswal, A. K. (2004). "Nrf2 signaling in coordinated activation of antioxidant gene expression." *Free Radic Biol Med* **36**(10): 1199-207.
- Juan, S. H., T. H. Cheng, et al. (2005). "Mechanism of concentration-dependent induction of heme oxygenase-1 by resveratrol in human aortic smooth muscle cells." *Biochem Pharmacol* **69**(1): 41-8.
- Karin, M. (1999). "How NF- $\kappa$ B is activated: the role of the I $\kappa$ B kinase (IKK) complex." *Oncogene* **18**(49): 6867-74.
- Karin, M., Y. Yamamoto, et al. (2004). "The IKK NF- $\kappa$ B system: a treasure trove for drug development." *Nat Rev Drug Discov* **3**(1): 17-26.
- Kaspar, J. W., S. K. Niture, et al. (2009). "Nrf2:INrf2 (Keap1) signaling in oxidative stress." *Free Radic Biol Med* **47**(9): 1304-9.
- Keklikoglu, N., M. Koray, et al. (2008). "iNOS expression in oral and gastrointestinal tract mucosa." *Dig Dis Sci* **53**(6): 1437-42.
- Kevil, C. G., N. Okayama, et al. (1998). "Expression of zonula occludens and adherens junctional proteins in human venous and arterial endothelial cells: role of occludin in endothelial solute barriers." *Microcirculation* **5**(2-3): 197-210.
- Knowles, R. G. and S. Moncada (1994). "Nitric oxide synthases in mammals." *Biochem J* **298** ( Pt 2): 249-58.
- Kubes, P., M. Suzuki, et al. (1991). "Nitric oxide: an endogenous modulator of leukocyte adhesion." *Proc Natl Acad Sci U S A* **88**(11): 4651-5.
- Lakatos, P. L. (2006). "Recent trends in the epidemiology of inflammatory bowel diseases: up or down?" *World J Gastroenterol* **12**(38): 6102-8.
- Lanas, A. (2008). "Role of nitric oxide in the gastrointestinal tract." *Arthritis Res Ther* **10** Suppl 2: S4.
- Laranjinha, J. (2001). "Caffeic acid and related compounds: biochemical and cellular effects", in *Handbook of Antioxidants. Biochemical, Nutritional and Clinical Aspects*, 2nd edition, (Cadenas E. and Packer L. Eds.) Marcel Dekker Inc., New York. pp. 279-302
- Laranjinha, J. (2010). "Translation of Chemical Properties of Polyphenols into Biological Activity with Impact on Human Health", in *Recent Advances in Polyphenol Research*. Volume 2 (eds C. Santos-Buelga, M. T. Escribano-Bailon and V. Lattanzio), Wiley-Blackwell, Oxford, UK.

- Lefer, A. M. and D. J. Lefer (1999). "Nitric oxide. II. Nitric oxide protects in intestinal inflammation." Am J Physiol **276**(3 Pt 1): G572-5.
- Lin, Y. L. and J. K. Lin (1997). "(-)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB." Mol Pharmacol **52**(3): 465-72.
- Liu, G. H., J. Qu, et al. (2008). "NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK." Biochim Biophys Acta **1783**(5): 713-27.
- Lundberg, J. O., E. Weitzberg, et al. (1994). "Intragastric nitric oxide production in humans: measurements in expelled air." Gut **35**(11): 1543-6.
- Lundberg, J. O., E. Weitzberg, et al. (2008). "The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics." Nat Rev Drug Discov **7**(2): 156-67.
- Mackenzie, G. G., F. Carrasquedo, et al. (2004). "Epicatechin, catechin, and dimeric procyanidins inhibit PMA-induced NF-kappaB activation at multiple steps in Jurkat T cells." FASEB J **18**(1): 167-9.
- MacMillan-Crow, L. A., J. P. Crow, et al. (1996). "Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts." Proc Natl Acad Sci U S A **93**(21): 11853-8.
- Mankertz, J., S. Tavalali, et al. (2000). "Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma." J Cell Sci **113** ( Pt 11): 2085-90.
- McMahon, M., K. Itoh, et al. (2003). "Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression." J Biol Chem **278**(24): 21592-600.
- Medic-Saric, M., V. Rastija, et al. (2009). "From functional food to medicinal product: systematic approach in analysis of polyphenolics from propolis and wine." Nutr J **8**: 33.
- Middleton, E., Jr., C. Kandaswami, et al. (2000). "The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer." Pharmacol Rev **52**(4): 673-751.
- Middleton, S. J., M. Shorthouse, et al. (1993). "Increased nitric oxide synthesis in ulcerative colitis." Lancet **341**(8843): 465-6.
- Moi, P., K. Chan, et al. (1994). "Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region." Proc Natl Acad Sci U S A **91**(21): 9926-30.
- Neuman, M. G. (2004). "Signaling for inflammation and repair in inflammatory bowel disease." Rom J Gastroenterol **13**(4): 309-16.
- Neurath, M. F., C. Becker, et al. (1998). "Role of NF-kappaB in immune and inflammatory responses in the gut." Gut **43**(6): 856-60.
- Nguyen, T., P. J. Sherratt, et al. (2003). "Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome." J Biol Chem **278**(7): 4536-41.

- Oyake, T., K. Itoh, et al. (1996). "Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site." *Mol Cell Biol* **16**(11): 6083-95.
- Pahl, H. L. (1999). "Activators and target genes of Rel/NF-kappaB transcription factors." *Oncogene* **18**(49): 6853-66.
- Palmer, R. M., A. G. Ferrige, et al. (1987). "Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor." *Nature* **327**(6122): 524-6.
- Petros, A., D. Bennett, et al. (1991). "Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock." *Lancet* **338**(8782-8783): 1557-8.
- Potapovich, A. I., D. Lulli, et al. "Plant polyphenols differentially modulate inflammatory responses of human keratinocytes by interfering with activation of transcription factors NFkappaB and AhR and EGFR-ERK pathway." *Toxicol Appl Pharmacol*.
- Radi, R. (2004). "Nitric oxide, oxidants, and protein tyrosine nitration." *Proc Natl Acad Sci U S A* **101**(12): 4003-8.
- Rahimi, R., S. Mozaffari, et al. (2009). "On the use of herbal medicines in management of inflammatory bowel diseases: a systematic review of animal and human studies." *Dig Dis Sci* **54**(3): 471-80.
- Rahman, I., S. K. Biswas, et al. (2006). "Regulation of inflammation and redox signaling by dietary polyphenols." *Biochem Pharmacol* **72**(11): 1439-52.
- Rasheed, Z., N. Akhtar, et al. (2009). "Polyphenol-rich pomegranate fruit extract (POMx) suppresses PMACI-induced expression of pro-inflammatory cytokines by inhibiting the activation of MAP Kinases and NF-kappaB in human KU812 cells." *J Inflamm (Lond)* **6**: 1.
- Roda, G., A. Sartini, et al. (2010) "Intestinal epithelial cells in inflammatory bowel diseases." *World J Gastroenterol* **16**(34): 4264-71.
- Rodrigo, R., A. Miranda, et al. (2010). "Modulation of endogenous antioxidant system by wine polyphenols in human disease." *Clin Chim Acta* **412**(5-6): 410-24.
- Rogler, G., K. Brand, et al. (1998). "Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa." *Gastroenterology* **115**(2): 357-69.
- Santangelo, C., R. Vari, et al. (2007). "Polyphenols, intracellular signalling and inflammation." *Ann Ist Super Sanita* **43**(4): 394-405.
- Scalbert, A., C. Morand, et al. (2002). "Absorption and metabolism of polyphenols in the gut and impact on health." *Biomed Pharmacother* **56**(6): 276-82.
- Scaldaferri, F. and C. Fiocchi (2007). "Inflammatory bowel disease: Progress and current concepts of etiopathogenesis." *Journal of Digestive Diseases* **8**(4): 171-178.
- Shapiro, H., P. Singer, et al. (2007). "Polyphenols in the treatment of inflammatory bowel disease and acute pancreatitis." *Gut* **56**(3): 426-35.
- Sharma, J. N., A. Al-Omran, et al. (2007). "Role of nitric oxide in inflammatory diseases." *Inflammopharmacology* **15**(6): 252-9.
- Shih, V. F., R. Tsui, et al. (2011). "A single NFkappaB system for both canonical and non-canonical signaling." *Cell Res* **21**(1): 86-102.

- Stadnyk, A. W. (2002). "Intestinal epithelial cells as a source of inflammatory cytokines and chemokines." *Can J Gastroenterol* **16**(4): 241-6.
- Van Itallie, C. M. and J. M. Anderson (1997). "Occludin confers adhesiveness when expressed in fibroblasts." *J Cell Sci* **110 ( Pt 9)**: 1113-21.
- Venugopal, R. and A. K. Jaiswal (1998). "Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes." *Oncogene* **17**(24): 3145-56.
- Verma, I. M., J. K. Stevenson, et al. (1995). "Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation." *Genes Dev* **9**(22): 2723-35.
- Wakabayashi, N., A. T. Dinkova-Kostova, et al. (2004). "Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers." *Proc Natl Acad Sci U S A* **101**(7): 2040-5.
- Wakabayashi, N., S. L. Slocum, et al. (2010). "When NRF2 talks, who's listening?" *Antioxid Redox Signal* **13**(11): 1649-63.
- Wallace, J. L. and L. Ma (2001). "Inflammatory mediators in gastrointestinal defense and injury." *Exp Biol Med (Maywood)* **226**(11): 1003-15.
- Wan, F. and M. J. Lenardo "The nuclear signaling of NF-kappaB: current knowledge, new insights, and future perspectives." *Cell Res* **20**(1): 24-33.
- Wang, H., T. O. Khor, et al. (2003). "Role of Nrf2 in suppressing LPS-induced inflammation in mouse peritoneal macrophages by polyunsaturated fatty acids docosahexaenoic acid and eicosapentaenoic acid." *Mol Pharm* **7**(6): 2185-93.
- Wink, D. A. and J. B. Mitchell (1998). "Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide." *Free Radic Biol Med* **25**(4-5): 434-56.
- Xavier, R. J. and D. K. Podolsky (2007). "Unravelling the pathogenesis of inflammatory bowel disease." *Nature* **448**(7152): 427-434.
- Yamamoto-Furusho, J. K. and D. K. Podolsky (2007). "Innate immunity in inflammatory bowel disease." *World J Gastroenterol* **13**(42): 5577-80.
- Yang, F., H. S. Oz, et al. (2001). "The green tea polyphenol (-)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal epithelial cell line IEC-6." *Mol Pharmacol* **60**(3): 528-33.
- Yang, F., H. S. Oz, et al. (2001). "The green tea polyphenol (-)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal epithelial cell line IEC-6." *Mol Pharmacol* **60**(3): 528-33.
- Yu, R., C. Chen, et al. (2000). "Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism." *J Biol Chem* **275**(51): 39907-13.
- Zeissig, S., N. Burgel, et al. (2007). "Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease." *Gut* **56**(1): 61-72.
- Zimmermann, H. W., S. Seidler, et al. (2011). "Interleukin-8 is activated in patients with



chronic liver diseases and associated with hepatic macrophage accumulation in human liver fibrosis." PLoS One **6**(6): e21381.

Zipper, L. M. and R. T. Mulcahy (2000). "Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes." Biochem Biophys Res Commun **278**(2): 484-92.

Zweier, J. L., A. Samouilov, et al. (1999). "Non-enzymatic nitric oxide synthesis in biological systems." Biochim Biophys Acta **1411**(2-3): 250-62.

# Annexes

## Annex 1: Detailed Composition of the Red Wine Extract used in the experiments

<b>Total proantocianic tannins</b>	4,1 g/L
<b>Total fenols</b>	222 mg/L
<b>Procyanidins</b>	
B1	351,36 mg/L
B2	158,70 mg/L
B3	6,98 mg/L
B4	17,02 mg/L
C1	47,13 mg/L
B2-gallate	51,66 mg/L
(+)-catechin	8,21 mg/L
(-)-epicatechin	3,66 mg/L
<b>Anthocyanins</b>	
delphinidin 3-glucoside	73,49 mg/L
cyanidin 3-glucoside	0,00 mg/L
petunidin 3-glucoside	89,75 mg/L
peonidin 3-glucoside	20,89 mg/L
malvidin 3-glucoside	378,91 mg/L
delphinidin 3-acetylglucoside	21,82 mg/L
cyanidin 3-acetylglucoside	10,47 mg/L
petunidin 3-acetylglucoside	15,49 mg/L
peonidin 3-acetylglucoside	17,51 mg/L
delphinidin 3-cumaroilglucoside	116,68 mg/L
malvidin 3-acetylglucoside	4,85 mg/L
malvidin 3-cafeoilglucoside	4,66 mg/L
peonidin 3-cumaroilglucoside	9,53 mg/L
malvidin 3-cumaroilglucoside	83,95 mg/L

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