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**Flavonoids with potential anti-inflammatory activity: *in vitro* structure/activity studies of their effects in pro-inflammatory signalling systems**

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Trabalho realizado sob a orientação do Professor Doutor José Luís Fontes da Costa Lima e da Professora Doutora Eduarda das Graças Rodrigues Fernandes

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Assinatura do autor,

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# Abstract

The term inflammation comprises the physiologic response of the organism to unchecked tissue damage from infection or injury. In spite of being a body's natural defence, the inflammatory response may contribute to the development of pathologic processes when the anti-inflammatory defences of the organism are not enough to contain and antagonize the deleterious activities of the overproduced pro-inflammatory mediators. Chronic inflammatory diseases, like rheumatoid arthritis, Crohn's disease, Alzheimer's disease, diabetes, among others, are responsible for the permanent affliction, disability, and, many times, premature death of millions of patients. They constitute a heavy burden for modern society. Unfortunately, the traditional steroidal or non-steroidal anti-inflammatory therapies are either not enough or associated with too many side effects. New therapeutic approaches are urgently required – more efficient and safer new drugs.

In recent years, flavonoids have been associated with various health benefits, including its anti-inflammatory effects. These properties associated with their ubiquitous distribution in nature, and their presence in the great majority of foods, as part of our daily diet, made flavonoids great value-added molecules. Taking in account these potential anti-inflammatory properties, flavonoids started to be considered a valuable alternative to modulate and prevent inflammatory processes; and moreover, to be the base to the synthesis of more potent and efficient anti-inflammatory drugs. The work developed in this PhD thesis intends to extend and rationalize the current knowledge on the alleged anti-inflammatory properties of flavonoids by elucidating the mechanism of action related with their structure (structure-activity relationship). For this purpose, a group of 24 flavonoids were selected, belonging to three flavonoid classes, flavones, flavanones and flavonols.

The first approach was based on the modulation of human neutrophils' oxidative burst. All the flavonoids under study effectively inhibited the production of reactive oxygen species, in this *in vitro* model. The structural feature that determined the greater activity was the presence of a catechol group in B-ring.

In a second approach, the ability of flavonoids to inhibit the leukotriene B<sub>4</sub> production was evaluated. Flavonoids with a catechol group in B-ring were shown to be the most effective. In this same work, the type of inhibition of lipoxygenase and the interaction between the flavonoids and this enzyme were evaluated. It was clear that both the substituents in B-ring and A-ring influenced these parameters.

The third work concerned the study of the inhibition of the production of prostaglandin (PG)E<sub>2</sub>, via cyclooxygenase (COX)-1 and -2. There were obvious differences between the flavonoids tested. Selective inhibitors of COX-1 and COX-2 were found. The best

inhibitors of PGE<sub>2</sub> production – via COX-2 were also excellent inhibitors of the production of the studied cytokines.

Finally the pro-apoptotic activity of the selected flavonoids was evaluated and all of them revealed to induce neutrophils' apoptosis, via caspase pathways, although no mitochondrial involvement could be observed, suggesting a key role of the extrinsic apoptotic pathway.

The experimental studies performed in the scope of this thesis allowed the conclusion that among the tested flavonoids, the ones with a catechol group in B-ring revealed overall best anti-inflammatory activities. Indeed, flavonoids 3',4'- dihydroxyflavone, 5,3',4'- trihydroxyflavone, 7,3',4'-trihydroxyflavone, and luteolin are undoubtedly good modulators of all the pro-inflammatory mediators evaluated.

**Keywords:** Flavonoids, Human Neutrophils, Lipxygenase, Cyclooxygenases, Apoptosis.

## Resumo

O termo “inflamação” engloba uma resposta fisiológica do organismo a agressões tecidulares por infecção ou ferimento. Apesar de ser uma resposta natural de defesa, a resposta inflamatória pode contribuir para o desenvolvimento de processos patológicos quando as defesas anti-inflamatórias do organismo não são suficientes para conter e antagonizar os efeitos deletérios dos mediadores inflamatórios. As doenças crônicas inflamatórias, como a artrite reumatóide, a doença de Crohn, a doença de Alzheimer, a diabetes, entre outras são responsáveis pelo permanente sofrimento, incapacidade e, muitas vezes, pela morte prematura de milhões de doentes, constituindo assim um pesado fardo para a sociedade. Infelizmente, as terapias anti-inflamatórias tradicionais, com fármacos esteroides e não-esteroides, não são suficientes ou estão associadas a demasiados efeitos secundários. São assim necessárias novas abordagens terapêuticas urgentes – novos fármacos mais eficientes e seguros.

Recentemente, os flavonoides têm vindo a ser associados a vários benefícios em saúde, incluindo os seus efeitos anti-inflamatórios. Estas propriedades, associadas à sua ampla distribuição na natureza, e presença na maioria dos produtos alimentares, como parte integral da dieta do dia-a-dia, têm feito dos flavonoides moléculas de excelente valor acrescentado. Tendo em conta estas potenciais propriedades anti-inflamatórias, os flavonoides começaram a ser considerados uma alternativa válida na modulação e prevenção do processo inflamatório; e ainda como base para a síntese de fármacos anti-inflamatórios mais potentes e eficientes. O trabalho desenvolvido nesta tese de doutoramento pretende ampliar e tornar mais racional o conhecimento corrente acerca das propriedades anti-inflamatórias dos flavonoides, através da relação entre o seu mecanismo de ação e a sua estrutura (relação estrutura-atividade). Com esta finalidade, foi seleccionado um grupo de 24 flavonoides, pertencentes a três classes diferentes, flavonas, flavanonas e flavonois.

A primeira abordagem baseou-se na modulação do *burst* oxidativo de neutrófilos pelos flavonoides. Todos os flavonoides em estudo inibiram de forma efectiva a produção de espécies reativas de oxigénio neste modelo *in vitro*. A característica estrutural que determinou uma melhor actividade foi o grupo catecol no anel B.

No segundo trabalho foi estudada a capacidade dos flavonoides para inibirem a produção do leucotrieno B<sub>4</sub>. Mais uma vez, os melhores inibidores desta produção foram os flavonoides com o grupo catecol no anel B. Neste mesmo trabalho foi avaliada o tipo de inibição da lipoxigenase e a interacção entre esta e os flavonoides. Ficou claro que quer os substituintes no anel A, quer os do anel B influenciavam estes parâmetros.

O terceiro trabalho residiu no estudo da inibição da prostaglandina (PG)E<sub>2</sub>, via ciclooxigenase (COX)-1 e -2. Observaram-se diferenças óbvias entre os flavonoides testados. Foram encontrados inibidores selectivos das COX-1 e COX-2. Os melhores inibidores da PGE<sub>2</sub>-via COX-2 foram também excelentes inibidores da produção das citocinas estudadas.

Finalmente, foi avaliada a actividade pro-apoptótica dos flavonoides seleccionados e todos eles revelaram induzir a apoptose dos neutrófilos, pelas vias das caspases, embora não tenha sido observado o envolvimento mitocondrial, sugerindo um papel-chave da via extrínseca da apoptose.

Os estudos experimentais realizados no âmbito desta tese permitem concluir que de entre os flavonoides estudados, aqueles que possuem um grupo catecol no anel B, foram os que revelaram uma melhor actividade anti-inflamatória global. De facto, os flavonoides 3',4'- dihidroxiflavone, 5,3',4'-trihidroxiflavone, 7,3',4'-trihidroxiflavone e a luteolin foram, indubitavelmente, bons moduladores de todos os mediadores inflamatórios avaliados.

**Keywords:** Flavonoides, Neutrófilos Humanos, Lipoxigenase, Ciclooxigenases, Apoptose.

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# Abbreviations

|                  |                                                                  |
|------------------|------------------------------------------------------------------|
| A23187           | calcium ionophore                                                |
| AA               | arachidonic acid                                                 |
| ABAH             | 4-aminobenzoyl hydrazide                                         |
| ADAM(s)          | A disintegrin and metalloproteinase(s)                           |
| ADAMTS(s)        | A disintegrin and metalloproteinase with thrombospondin motif(s) |
| Amplex red       | N-acetyl-3,7-dihydroxyphenoxazine                                |
| AP-1             | activator protein 1                                              |
| APF              | 2-[6-(4'-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid         |
| ASC              | apoptosis-associated speck like protein                          |
| ATL              | aspirin/triggered lipoxins                                       |
| ATP              | adenosine triphosphate                                           |
| BAFF             | B cell activating factor                                         |
| CAM(s)           | cell adhesion molecule(s)                                        |
| CAT              | catalase                                                         |
| CBP              | CREB-binding protein                                             |
| CCL              | chemokine (C-C motif) ligand                                     |
| CCCP             | carbonyl cyanide 3-chlorophenylhydrazone                         |
| CCR              | CC chemokine receptors                                           |
| CD40L            | CD40 ligand                                                      |
| cGMP             | cyclic guanosine monophosphate                                   |
| COX              | cyclooxygenase                                                   |
| cPLA2            | cytoplasmic phospholipase A2                                     |
| CRE(s)           | cAMP-responsive element(s)                                       |
| CREB             | cAMP response element-binding protein                            |
| CRP              | C-reactive protein                                               |
| CXCR             | CXC chemokine receptors                                          |
| Cys LTs          | cysteinyl leukotrienes                                           |
| D <sub>2</sub> O | deuterium oxide                                                  |
| DAG              | diacylglycerol                                                   |
| DAMP(s)          | damage-associated molecular pattern molecule(s)                  |
| DMSO             | dimethylsulfoxide                                                |
| DNA              | deoxyribonucleic acid                                            |
| DP               | prostaglandin D receptors                                        |

|                |                                                  |
|----------------|--------------------------------------------------|
| DPPH           | 2,2-diphenyl-1-picrylhydrazyl                    |
| DSS            | dextrane sulfate sodium                          |
| DTT            | <sub>DL</sub> -dithiothreitol                    |
| EGF            | epidermal growth factor                          |
| EIA            | enzyme immunoassay                               |
| eNOS (or NOS3) | endothelial cell nitric oxide synthase           |
| EP             | prostaglandin E receptor                         |
| EPO            | eosinophil peroxidase                            |
| ERK            | extracellular signal-regulated kinase            |
| FADD           | Fas-associated protein with Death Domain         |
| FcεRI          | immunoglobulin E receptor                        |
| FGF            | fibroblast growth factor                         |
| FLAP           | 5-lipoxygenase-activating protein                |
| FP             | prostaglandin F receptor                         |
| FPRL           | G protein–coupled formyl peptide receptor like   |
| GC             | guanylate cyclase                                |
| GCSF           | granulocyte colony stimulating factor            |
| GlyCAM         | glycan-bearing cell adhesion molecule            |
| GM-CSF         | granulocyte macrophage colony-stimulating factor |
| GPCR(s)        | G protein-coupled receptor(s)                    |
| GPx            | glutathione peroxidase                           |
| GR             | glutathione reductase                            |
| GRO            | growth-regulating oncogene                       |
| GSH            | glutathione                                      |
| GSH-Px         | plasma glutathione peroxidase                    |
| GTPase         | guanosine triphosphatase                         |
| HBSS           | Hank's balanced salt solution                    |
| HETE           | hydroxyeicosatetraenoic acid                     |
| HGF            | hepatocyte growth factor                         |
| HIF            | hypoxia-induced factor                           |
| HLA            | human leukocyte antigen                          |
| HMGB           | high mobility group box                          |
| HPETE          | hydroperoxyeicosatetraenoic acid                 |
| HRP            | peroxidase from horseradish                      |
| ICAM           | intercellular adhesion molecule                  |
| IFN            | interferon                                       |
| Ig(s)          | immunoglobulin(s)                                |

|                         |                                                 |
|-------------------------|-------------------------------------------------|
| IGF                     | insulin-like growth factor                      |
| IgSF                    | immunoglobulin gene superfamily                 |
| IKK                     | I $\kappa$ B kinase                             |
| IL                      | interleukin                                     |
| IL-8R                   | interleukin 8 receptor                          |
| iNOS (macNOS, or NOS2)  | nitric oxide synthase                           |
| IP                      | prostacyclin receptor                           |
| IP3                     | inositol 1,4,5-trisphosphate                    |
| iPLA2                   | Ca <sup>2+</sup> - independent phospholipase A2 |
| IRAK1                   | interleukin-1 receptor-associated kinase 1      |
| JAK                     | Janus kinases                                   |
| JNK                     | c-Jun N-terminal kinase                         |
| KGF                     | keratinocyte growth factor                      |
| LAMP                    | lysosome-associated membrane proteins           |
| LOX                     | lipoxygenase                                    |
| LPS                     | lipopolysaccharide                              |
| LT(s)                   | leukotriene(s)                                  |
| Lucigenin               | N,N'-dimethyl-9,9'-biacridinium dinitrate       |
| LX(s)                   | lipoxin(s)                                      |
| MAC                     | membrane attack complex                         |
| MadCAM                  | mucosal addressin cell adhesion molecule        |
| MAPK                    | mitogen-activated protein kinase                |
| MBL                     | mannose-binding lectin                          |
| MCP                     | monocyte chemoattractant protein                |
| MEM                     | methoxyethoxymethyl                             |
| MIP                     | macrophage inflammatory protein                 |
| MMP(s)                  | metalloproteinase(s)                            |
| mPGES                   | microsomal prostaglandin E synthase             |
| MPO                     | myeloperoxidase                                 |
| NADPH                   | nicotinamide adenine dinucleotide phosphate     |
| NAP                     | neutrophil activating peptide                   |
| NDGA                    | nordihydroguaiaretic acid                       |
| NEMO (or IKK $\gamma$ ) | nuclear factor kappa B essential modulator      |
| NETs                    | neutrophils extracellular traps                 |
| NF- $\kappa$ B          | nuclear factor kappa B                          |
| NFAT                    | nuclear factor of activated T-cells             |
| NK cells                | natural killer cells                            |

|                  |                                                                                      |
|------------------|--------------------------------------------------------------------------------------|
| NLRs             | nucleotide oligomerization domain (nucleotide oligomerization domain)-like receptors |
| NMR              | nuclear magnetic resonance                                                           |
| nNOS (or NOS1)   | neuronal nitric oxide synthase                                                       |
| NOD              | nucleotide oligomerization domain                                                    |
| NOS              | nitric oxide synthase                                                                |
| NOX(s)           | non-phagocytic oxidase(s)                                                            |
| MPTP             | 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridin                                          |
| Nrf2             | nuclear factor E2-related factor 2                                                   |
| NSAID(s)         | non-steroidal anti-inflammatory drug(s)                                              |
| PAF              | platelet-activating factor                                                           |
| PAMP(s)          | pathogen-associated molecular pattern(s)                                             |
| PBMC(s)          | peripheral blood mononuclear cell(s)                                                 |
| PBS              | Dulbecco's phosphate buffer saline, without calcium chloride and magnesium chloride  |
| PDGF             | platelet derived growth factor                                                       |
| PECAM(s)         | platelet-endothelial cell adhesion molecule(s)                                       |
| PG(s)            | prostaglandin(s)                                                                     |
| PGDH             | 15-hydroxyprostaglandin dehydrogenase                                                |
| PGI <sub>2</sub> | prostacyclin                                                                         |
| PGIS             | prostacyclin synthase                                                                |
| PHA              | phytohaemagglutinin                                                                  |
| phox             | phagocyte oxidase                                                                    |
| PI3K             | phosphatidylinositol 3-kinase                                                        |
| PIP3             | phosphatidylinositol (3,4,5)-trisphosphate                                           |
| PK               | protein kinase                                                                       |
| PLA2             | phospholipase A2                                                                     |
| PMA              | phorbol-12-myristate-13-acetate                                                      |
| PMN(s)           | polymorphonuclear leukocyte(s)                                                       |
| PORS             | pro-oxidant reactive species                                                         |
| PPAR(s)          | peroxisome proliferator-activated receptor(s)                                        |
| PRRs             | pattern recognition receptors                                                        |
| PSGL             | selectin glycoprotein ligand                                                         |
| PTK              | tyrosine protein kinase                                                              |
| PXR              | pregnane X receptor                                                                  |
| Rac2             | Ras-related C3 botulinum toxin substrate 2                                           |

|                         |                                                               |
|-------------------------|---------------------------------------------------------------|
| RANTES                  | regulated on activation, normal T cell expressed and secreted |
| RNS                     | reactive nitrogen species                                     |
| ROS                     | reactive oxygen species                                       |
| S1P                     | sphingosine1-phosphate                                        |
| SAA                     | serum amyloid A                                               |
| SAP                     | serum amyloid P component                                     |
| SAR                     | structure-activity relationship                               |
| SMAD(s)                 | mother(s) against decapentaplegic                             |
| SOD                     | superoxide dismutase                                          |
| sPLA2                   | secretory phospholipase A2                                    |
| STAT(s)                 | signal transducer(s) and activator(s) of transcription        |
| STD- <sup>1</sup> H NMR | saturation transfer difference <sup>1</sup> H NMR             |
| Syk                     | spleen tyrosine kinase                                        |
| TAK                     | TGF- $\beta$ activated kinase                                 |
| TBARS                   | thiobarbituric acid reactive substances                       |
| TEAC                    | trolox equivalent antioxidant capacity                        |
| TF                      | tissue factor                                                 |
| TGF- $\beta$            | transforming growth factor beta                               |
| TIMP(s)                 | tissue inhibitor of metalloproteinase(s)                      |
| TLRs                    | toll-like receptors                                           |
| TNBS                    | trinitrobenzene sulfonic acid                                 |
| TNF                     | tumor necrosis factor                                         |
| TNFR                    | tumor necrosis factor alpha receptors                         |
| TPA                     | 12-O-tetradecanoylphorbol-13-acetate                          |
| TRADD                   | tumor necrosis factor receptor type 1-associated death domain |
| TRAF                    | TNF receptor associated factor                                |
| TSLP                    | thymic stromal lymphopoietin                                  |
| TX                      | thromboxane                                                   |
| TXBSI                   | thromboxane synthase inhibitor                                |
| VCAM                    | vascular cell adhesion molecule                               |
| VEGF                    | vascular endothelial growth factor                            |

## Symbols

|           |                           |
|-----------|---------------------------|
| $K_i$     | inhibition constant       |
| $K_m$     | Michaelis-Menten constant |
| $V_{max}$ | maximum enzyme velocity   |

## ***CHAPTER I. General Introduction***

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### **I.1. Introduction**

Inflammation has become one of the hottest areas of biomedical research. An immense array and variety of inflammatory mechanisms are known to underlie serious chronic diseases and conditions. It is known that inflammation triggers and/or accelerates rheumatoid arthritis, osteoarthritis, inflammatory bowel diseases and is often involved in cardiovascular, neurodegenerative, and cancer diseases. With so many diseases at stake, affecting so many people, developing effective and safe anti-inflammatory drugs should be a top priority. It is true that a myriad of anti-inflammatory molecules are in the therapeutic market, but it is also true the traditional steroidal or non-steroidal anti-inflammatory therapies are either not enough or associated with too many side effects. New therapeutic approaches are urgently required – more efficient and safer new drugs.

Flavonoids, a large class of polyphenols, comprise a wide group of molecules, with a great structural variety, organized in subclasses. In recent years, the interest in those various classes of flavonoids has been growing due to their various beneficial healthy and pharmacological properties. These health promoting properties are probably due to their ability to interfere with pro-oxidant and pro-inflammatory systems, preventing their deleterious consequences.

In this thesis a large group of selected flavonoids, belonging to three different flavonoid subclasses, flavones, flavanones, and flavonols, were evaluated concerning their ability to modulate pro-oxidant systems, key enzymes, and signalling pathways that are involved in inflammatory processes, to extend and rationalize the current knowledge on the alleged anti-inflammatory properties of flavonoids by elucidating the mechanism of action related with their structure (structure-activity relationship).

## I.2. General and specific objectives of the thesis

The main objective of this thesis was to evaluate the modulating effect of a group of selected flavonoids, in *in vitro* models of inflammation, namely on the oxidative burst of human neutrophils, neutrophil's apoptosis, as well as in pro-inflammatory enzymes, and pro-inflammatory signalling pathways in whole human blood. We expect to contribute for the definition of the best structure-activity relationship and consequently for the development of better and safer anti-inflammatory drugs.

As specific objectives, we aimed to evaluate the effects of a series of flavonoids in:

- The oxidative burst of human neutrophils.
- The activity of LOX in human neutrophils.
- The apoptotic effect in human neutrophils and respective pathways.
- The activities of COX-1 and COX-2 enzymes in human whole blood.
- The production of pro-inflammatory cytokines and chemokines, in human whole blood.

## I.3. Outline of the thesis

This thesis is organized in four main chapters:

### **Chapter I. General introduction**

This chapter is divided in three sections: the first section (I.1) includes a brief theoretical background of the thesis theme and the general motivation and objectives; the second section (I.2) comprises the main and the specifics objectives of this thesis; the last section (I.3) covers a general description of the other chapters, namely their organization and structure.

### **Chapter II. Theoretical Background**

This chapter includes one section (II.1.), which comprises a review article, providing a comprehensive description of the inflammatory process and its mediators. This article also provides the state of the art concerning the main anti-inflammatory effects of flavonoids.

### **Chapter III. Original Research**

This chapter is divided in four sections corresponding to published (III.1, III.2, III.3) and one submitted (III.4) original manuscripts, which resulted from the experimental studies designed to answer the questions that derived from the general and specific objectives of this thesis.

### **Chapter IV. Discussion and Conclusions**

This chapter is divided in three sections: the first one (IV.1) consists in an integrated discussion of the performed original research; the second one (IV.2) comprises the general conclusions of the thesis and the third section (IV.3) contains the references.

## ***CHAPTER II. Theoretical Background***

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### **II.1. Pro-inflammatory pathways: the modulation by flavonoids**

Manuscript submitted for publication

## **Pro-inflammatory pathways: the modulation by flavonoids**

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**Abstract**

Inflammation is a natural, carefully orchestrated response of the organism to tissue damage, involving various signalling systems and the recruitment of inflammatory cells. These cells are stimulated to release a myriad of mediators that amplify the inflammatory response and recruit additional cells. These mediators present numerous redundancies of functions, allowing a broad and effective inflammatory response, but simultaneously make the understanding of inflammation pathways much difficult. The extent of the inflammatory response is usually self-limited, although it depends on the balance between the pro- and anti-inflammatory signals. When that equilibrium is dislocated, a more widespread inflammatory response may take place.

Flavonoids have been shown to be possible alternatives to the traditionally molecules used as anti-inflammatory agents. In fact, the biological activities of flavonoids include the modulation of the diverse phases of inflammatory processes, from the gene transcription and expression to the inhibition of the enzymatic activities and the scavenging of the reactive species. In the present manuscript, the inflammatory network and the flavonoids' broad spectrum of action in many of the analysed inflammatory pathways are reviewed.

**Keywords:** Flavonoids, Inflammatory pathways, Eicosanoids, Reactive species, Cytokines, Transcription factors.

## **1. Introduction**

### **1.1. Inflammation**

#### **1.1.1. The Concept**

The word inflammation comprises the physiologic response of the organism to unchecked tissue damage from infection or injury; the inflammatory response is a body's natural defence.<sup>1,2</sup> Historically, there are Egyptian papyrus dated around 3000 BC already describing clinical features of inflammation. However, it was only in the first century AD that a Roman writer, Celsus, first listed the four cardinal signs of inflammation: rubor (redness), tumor (swelling), calor (heat) and dolor (pain).<sup>3</sup> In the 19<sup>th</sup> century a fifth sign was added, loss of function (*functio laesa*). This addition is attributed to Rudolf Virchow, however some authors attribute it to Galen's work during the 2nd century. Even though the latter has referred the disturbed function, he did not define the term loss of function as a specific symptom of inflammation, as Virchow did.<sup>2,4</sup> Along the 19<sup>th</sup> century, other fundamental developments were made: the definition of inflammation not as a disease but as a nonspecific response in the host, the discovery of phagocytosis mechanism and the process of phagocytic cells' recruitment to the injured area to engulf invading bacteria, and the discovery of molecules that mediate the vascular changes of inflammation. These events corresponded to the underlying basis for the important findings of chemical mediators of inflammation and the use of anti-inflammatory drugs in clinical medicine.<sup>3</sup>

Inflammation is a carefully orchestrated response of the organism, involving endothelial cells, epithelial cells and the recruitment of inflammatory cells such as neutrophils, monocytes, macrophages, and, in some cases, lymphocytes.<sup>1</sup> The local cells along with the recruited ones are stimulated to release a myriad of mediators that amplify the inflammatory response and recruit additional cells.<sup>5</sup> Several functional redundancies have been noted for these mediators that allow a broad and effective response, but simultaneously hinder the understanding of the inflammation's pathways.<sup>2</sup> Regardless the self-limited physiology of the inflammatory response, it depends on the balance between the pro- and anti-inflammatory signals, and when that equilibrium is dislocated, a more widespread inflammatory response takes place.<sup>1</sup> At this point, it is crucial to support the inflammation resolution with molecules that act as effective modulators of the various processes sheltered in the inflammation network.

#### **1.1.2. Acute Inflammation vs Chronic Inflammation**

The inflammatory process may be classified as acute or chronic, according to the nature and the effectiveness of the initial response. The acute inflammation is defined as a transient and initial response to injury. It is rapid in onset and is of short duration, lasting

for hours or a few days. It is characterized by the release of numerous chemical mediators, and leads to the exudation of fluid and plasma proteins (edema) and the migration of leukocytes, predominantly neutrophils.<sup>3,6</sup> Acute inflammation may have different stimuli / causes: a) infections (bacterial, viral, fungal, parasitic) and microbial toxins; b) immune reactions, or hypersensitivity reactions, where the immune system damage the individual's own tissues; c) tissue necrosis from various causes, ischemia, trauma, physical and chemical injury (e.g. thermal injury, irradiation, exposure to some environmental chemicals); d) hypoxia, which often underlies cell injury and it is mediated by a protein called hypoxia-induced factor-1 $\alpha$  (HIF-1 $\alpha$ ), which is produced by cells deprived of oxygen and activates the transcription of many genes involved in inflammation; e) other stimuli: foreign bodies (e.g. splinters, dirt, sutures), because they cause traumatic tissue injury or carry microbes. The importance of this inflammatory process is related to the difficulty in the elimination of the stimuli, resulting in the persistence of the inflammation.<sup>3,7,8</sup> When the acute inflammation is not enough to remove the invaders, it can progress to a chronic phase. The self-limiting response is turned into a loop, where the damage by itself attracts more pro-inflammatory mediators, in a resistant process to the normal feedback mechanisms.<sup>9</sup>

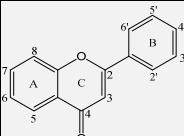
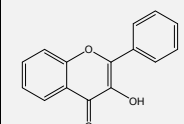
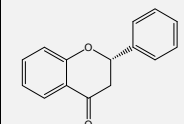
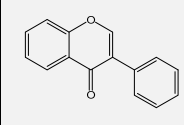
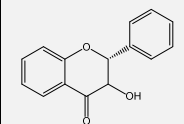
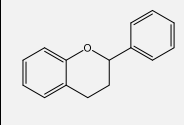
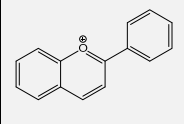
Chronic inflammation is defined as a prolonged inflammation (weeks to months), where the tissue injury and the attempts to repair coexist.<sup>8</sup> As described before, the chronic inflammation may follow the acute. However, it may also begin insidiously, as a low-grade response, without any manifestations of an acute reaction. This last form of chronic inflammation is associated with diseases such as rheumatoid arthritis, atherosclerosis, tuberculosis, and pulmonary fibrosis. It has also been implicated in the progression of cancer and in diseases such as Alzheimer disease.<sup>9</sup> The chronic inflammation may have different stimuli / causes: a) infections by microorganisms that are difficult to eradicate is the most common cause; b) immune-mediated inflammatory diseases, as autoimmune diseases, which are caused by an excessive and inappropriate activation of the immune system; and c) prolonged exposure to potentially toxic agents, sterile, either exogenous (e.g. toxic plasma lipid components, that at least in part cause atherosclerosis) or endogenous (e.g. inhalation of particulate silica for prolonged periods).<sup>3,6,8</sup> Chronic inflammatory diseases affect many people around the globe. The development of effective and safe anti-inflammatory drugs should be a top priority. However, the traditional steroidal or non-steroidal anti-inflammatory therapies are either not enough or associated with too many side effects. In recent years, flavonoids have attracted increasing interests due to their various beneficial pharmacological properties, probably due to their antioxidant and anti-inflammatory effects.

## 1.2. Flavonoids

### 1.2.1. Definition, Classification and Nomenclature

Flavonoids are part of the vast family of polyphenols, and were discovered by Albert Szent-Györgyi, while he was working on the isolation of vitamin C.<sup>10</sup> They are bioactive molecules with important functions in plant cells. Flavonoids are the most widely distributed polyphenols in plants and fruits and an essential part of human diet. Flavonoids are structurally derived from benzo-γ-pyrone displaying a diphenylpropane (C6-C3-C6) basic structure, which consists of two benzene rings (A and B rings) linked by a pyran heterocyclic ring (C ring).<sup>11</sup> There are various classes of flavonoids where they are grouped according to the oxidation and pattern of substitution of the C ring. What distinguishes the different flavonoids in the same class is the pattern of substitution of the A and B rings.<sup>12</sup> Table 1 summarizes some dietary sources of flavonoids, according to their classes.

**Table 1** - Classification, structure and dietary sources of some flavonoids.

| Flavonoids Class | Structure                                                                           | Flavonoid (examples)                                 | Dietary sources                                            | References |
|------------------|-------------------------------------------------------------------------------------|------------------------------------------------------|------------------------------------------------------------|------------|
| Flavones         |  | Chrysin, apigenin, rutin, luteolin                   | Fruit peel, red wine, red pepper, tomato skin, buckwheat   | 12         |
| Flavonols        |  | Quercetin, kaempferol, myricetin, fisetin, galangin  | Onion, red wine, olive oil, berries, grapes, broccoli, tea | 12         |
| Flavanones       |  | Naringin, naringenin, eriodictyol, hesperidin        | Lemons, oranges, grapes                                    | 12         |
| Isoflavones      |  | Genistin, daidzin                                    | Soy                                                        | 12         |
| Flavanonols      |  | Taxifolin, dihydrokaempferol                         | Onion                                                      | 13         |
| Flavans          |  | Flavan-3-ol (catechin), flavan-4-ol, flavan-3,4-diol | Tea                                                        | 12         |
| Anthocyanidins   |  | Apigenidin, cyanidin, malvidin, delphinidin          | Cherry, strawberry, easberry                               | 12         |

Flavonoids can also be found in commercially available products, namely as standardized phytotherapeutic extracts (ex. green tea extracts), part of dietary supplements (ex. quercetin and resveratrol capsules) or as isolated products.<sup>14</sup> In the last few years the number of studies with flavonoids has been increasing considerably, not only due to their multiple biological properties, but also due to their promising use as alternative drugs in a myriad of diseases related to chronic inflammation (viral infections<sup>15</sup>, cancer, cardiovascular diseases<sup>16</sup>, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, asthma, atherosclerosis, metabolic syndrome, and brain and skin inflammation<sup>17</sup>) and as food supplements. One of the known biological effects of flavonoids is their anti-inflammatory activity. In this work the inflammatory network and the flavonoids' broad spectrum of action in many of the analysed inflammatory pathways are reviewed.

## **2. Cells mediating the inflammatory response**

The inflammatory process begins with the release of various mediators from the resident cells, including, mast cells, interstitial fibroblasts and vascular endothelial cells. This signalling changes the local adhesion molecules' profile and creates a chemotactic gradient that recruits cells from the blood stream.<sup>5</sup> The cells mediating the inflammatory response are represented in figure 1.

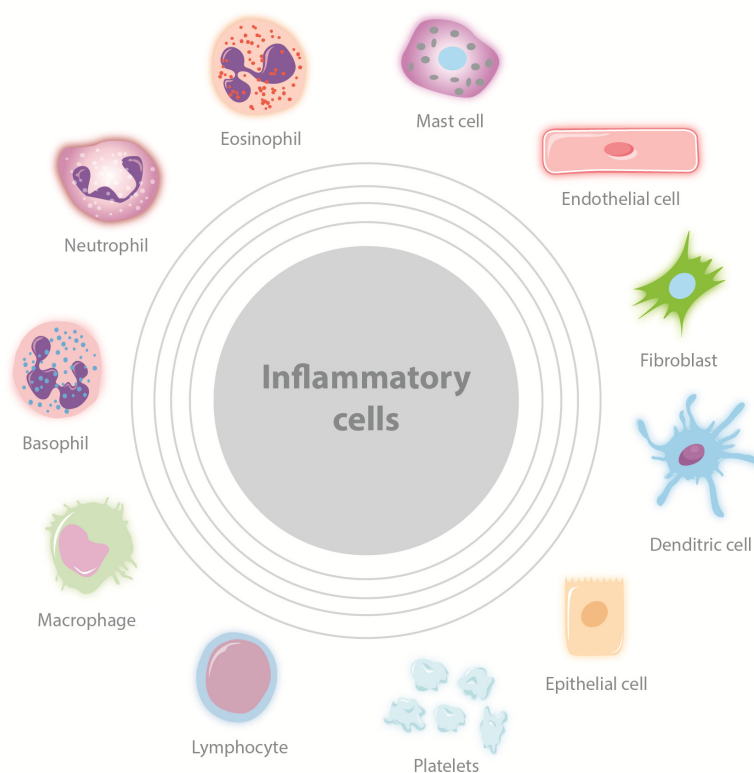


**Figure 1** - Cells involved in the inflammatory response.

## **2.1. Granulocytes**

Neutrophils, eosinophils and basophils compose the granulocytes, so called because of the presence of granules, actually highly developed vacuolar structures, in their cytoplasm. They are also known as polymorphonuclear leukocytes (PMNs) group, named for the nucleus' multilobulated shape. They play an important role in the inflammatory response. They are the first cells migrating from the circulation and arriving at the inflammatory site. The most relevant are neutrophils and eosinophils, even though basophils also participate in allergic reactions.<sup>4,6</sup>

### **2.1.1. Neutrophils**

Neutrophils are the major cell type in most acute inflammatory diseases, as they are the body's first line defence, but they are also found in some chronic inflammatory diseases. Their primary function is to contain and kill invading pathogens.<sup>6</sup> In general, the following sequential cellular events take place in acute inflammation, all involving neutrophils, being designated as neutrophil activation (Figure 2):

1. the peripheral blood neutrophils are subdivided in two pools, a circulating one and a marginating one, adhered to the endothelial cells. These pools are altered along the process due to the activation or inactivation of the neutrophils by a variety of mediators that are released;
2. chemotaxis: is the process of calling the neutrophils to the inflammation site, which is mediated by the chemotactic mediators, which bind to neutrophil receptors, causing the release of calcium, increasing neutrophil motility;
3. margination: the neutrophils are pushed to the periphery of the vessels by the action of fibrinogen and erythrocytes aggregated into *rouleaux* in the venules;
4. rolling: the neutrophils' rolling occurs in the venules by the action of selectins adhesion molecules to which neutrophils establish a weak binding, causing them to "roll" (bind-detach, bind-detach) along the surface;
5. adhesion: the integrins adhesion molecules (ligands) are responsible for the adhesion of the neutrophils to the venular endothelial cells;
6. *diapedesis*: the neutrophils move, transmigrate, across the venular endothelium basement membrane to the interstitial tissue. This movement is possible due to the pores generated within the endothelial cells themselves; however, neutrophils may also pass between endothelial cells by disruption of cell-cell junctions. This is possible by the interactions between adhesion molecules found on neutrophils and endothelial cells known as platelet-endothelial cell adhesion molecules (PECAMs);
7. phagocytosis: consists in a multistep process that comprises the opsonization, ingestion, and killing of the pathogens. Phagocytosis may be triggered by two

mechanisms: a) by direct neutrophil recognition of pathogen-associated molecular patterns (PAMPs) found in bacteria or viruses; these small molecule motifs are recognized by toll-like receptors (TLRs) and other pattern recognition receptors (PRRs); b) via opsonization, consisting in the modification of the surface of the pathogen by immunoglobulins (Igs) or complement components, or both, that are later recognized by the receptors expressed by neutrophils, low-affinity FcγRIIa and high-affinity FcγRIIIb. After the recognition, neutrophils project pseudopodia in all directions around the particle. The pseudopodia meet one another on the opposite side and fuse, creating an enclosed compartment that contains the phagocytized particle. Then this compartment invaginates to the inside of the cytoplasm and breaks away from the outer cell membrane to form a free-floating *phagocytic vesicle*. A single neutrophil can typically phagocytize 3 to 20 bacteria before the neutrophil itself becomes inactivated and dies.<sup>3,8,18,19</sup>

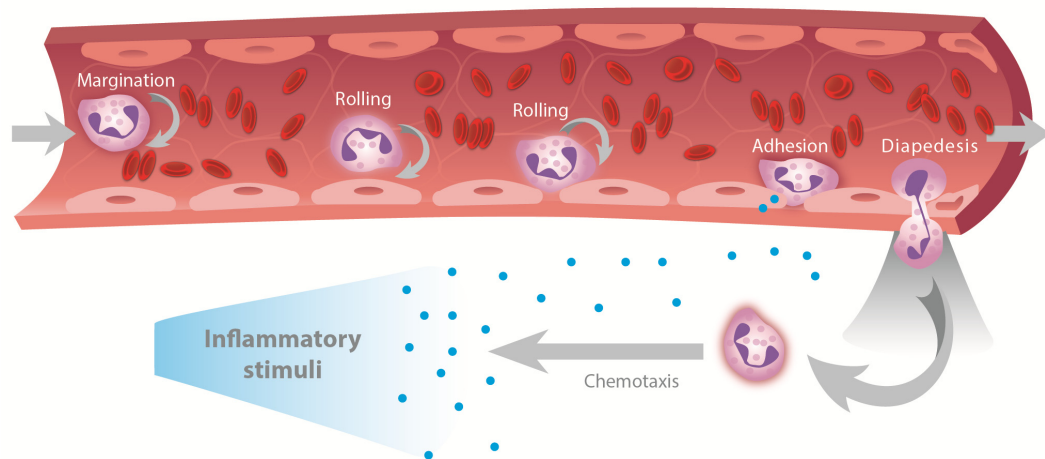


**Figure 2** - The process of neutrophil activation. <sup>Adapted from 8</sup>

Neutrophils are highly specialized cells that present cytoplasmic granules containing an impressive arsenal of microbicidal agents that are released upon cell activation.<sup>6</sup> As soon as a neutrophil phagocytizes a bacteria or extracellular material, the azurophilic granules fuse with the phagosome, creating a phagolysosome, directing the content to the ingested target. The lysosomes, azurophilic granules, contain a variety of proteases, including elastase, matrix metalloproteinase (MMP)-3,-8, and -9, cathepsins, and lysozyme. However, on one hand, the azurophilic granules differ from the true lysosomes because they lack lysosome-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2) and the mannose-6-phosphate receptor system; on the other hand, specific granules retain a variety of membrane-associated proteins (cytochromes, signalling molecules, and

receptors), they are reservoirs of proteins destined for topologically external surfaces of phagocytic vacuoles and the plasma membrane.<sup>4,19</sup>

Neutrophils contain a membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that is assembled and activated during the inflammatory response leading to the formation of reactive oxygen species (ROS) and nitrogen (RNS) by the rapid consumption of oxygen, a process designated as oxidative burst, as will be described below. Furthermore, neutrophils also contain a key enzyme designated myeloperoxidase (MPO), which has microbicidal activity but has also an important role in tissue injury that occurs in the event of inflammatory diseases. As mentioned before, neutrophils are chemoattracted to the site of inflammation; this is done by chemoattractants, which include leukotriene B<sub>4</sub> (LTB<sub>4</sub>), platelet-activating factor (PAF), interleukin (IL)-8, and the complement split product C5a. The concentrations of these chemoattractants and their gradient of distribution ascertain the migration of neutrophils. At low concentrations of the chemoattractants, the neutrophils migrate toward regions with higher concentrations. At high concentrations, they lead to the cease of the neutrophils' migration and induce their activation; this happens for example at the bacterial source of the gradient. The neutrophils present another receptor, namely for growth factors, colony-stimulating factors, and cytokines, which may also modulate neutrophils' function.<sup>4</sup> More recently, another way of action of neutrophils have been described. It consists in the release of neutrophils extracellular traps (NETs), which are extracellular fibrillar networks, produced by neutrophils, composed by nuclear chromatin associated with granule proteins. These NETs are produced in response to the infectious pathogens and inflammatory mediators. The bacteria become trapped in these NETs and are killed, avoiding the spread of bacteria. This function leads to cell death as the nuclei of the neutrophils are lost in the process.<sup>4,6,20</sup>

### **2.1.2. Eosinophils**

Eosinophils are found in great number in extravascular tissue, in contrast to neutrophils that are much more common in the bloodstream. The eosinophils need to be recruited by chemokines, and eotaxin is an especially important chemokine in this recruitment.<sup>3</sup> Eosinophils do not phagocytose the pathogens; they liberate their content alongside them, including four distinct granule cationic proteins: major basic protein, eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin, and inflammatory mediators: lipid mediators, such as leukotriene C<sub>4</sub>(LTC<sub>4</sub>) and PAF, which may cause dysfunction to parasites but also destruction / lysis of other cells, contributing to tissue damage. These granulocytes are viewed as effector cells of allergic responses

and parasite elimination. They are also present in the inflammatory response generated by allergic or immediate hypersensitivity reactions, as basophils and mast cells.<sup>1,4</sup>

### **2.1.3. Basophils**

Basophils represent less than 1 % of leukocytes and are the least abundant in the granulocyte population.<sup>21</sup> During the inflammatory response, granulocytes migrate from the blood into tissues in response to chemoattractants, such as IL-8 produced by neutrophils, or eotaxin by eosinophils and by other basophils.<sup>22</sup> Once in the periphery, these granulocytes can be activated by a variety of signals, including those mediated by cytokines [IL-3, IL-33, IL-18, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF)], antibodies (IgE, IgD and IgG), allergens, proteases, TLR ligands and complement factors (C5a). When activated, granulocytes rapidly produce and secrete a myriad of mediators, namely: histamine, LTC<sub>4</sub>, PAF, all major mediators of acute bronchoconstriction, chemokines and cytokines [IL-4, IL-5, IL-13, and thymic stromal lymphopoietin (TSLP)]. As an example, the cytokine IL-3 enhances the production of IL-4 and IL-13 by basophils, in response to the IgE-mediated activation. Basophils are also capable of activate B cell populations, by increasing the production of IL-4 and B cell activating factor (BAFF) and by the induction of IgM, IgG and IGA, mediated by IgD. Also IL-4 and IL-13 can be produced via TLR2 ligand by basophils' recognition of PAMPs.<sup>21</sup> Overall, basophils play their main role in allergic reactions.<sup>4</sup>

### **2.2. Monocytes / Macrophages**

Monocytes are a heterogeneous population, which begin to migrate in the extravascular tissues quite early in acute inflammation. This process of monocyte adhesion to, and diapedesis through, the vascular endothelial cells is similar to that of neutrophils, but monocytes respond to a different set of chemotactic factors, including the chemokines, chemokine (C-C motif ligand 5, also known as, regulated on activation, normal T cell expressed and secreted (CCL5 or RANTES), CCL2, CCL7, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1 alpha and 1 beta (MIP-1 $\alpha$  and -1 $\beta$ ), and the growth factors, transforming growth factor beta (TGF- $\beta$ ) and platelet-derived growth factor (PDGF). When a monocyte reaches the extravascular tissue, it undergoes a transformation into a larger phagocytic cell, the macrophage.<sup>1,3,23</sup>

Macrophages constitute a bridge between the innate and adaptive immune system being their basic functions, the constant immune surveillance and the orchestration of the immune response. Macrophages recognize the pathogens through the surface expressed receptors for PAMPs, including TLRs, formyl peptide receptors, and scavenger receptors,

many of which are also found on neutrophils. Macrophages also have in their surface, receptors for multiple opsonins, including the mannose receptor (that directly recognizes glycoproteins on microbial surfaces), CR1, CR3, CR4, FcγRI, FcγRII, and FcγRIII. To exert their functions, macrophages are activated, and this may happen by the action of interferon gamma (IFN-γ), complement components, immune complexes, lipopolysaccharide (LPS), and cytokines, such as IL-1β, tumor necrosis factor alpha (TNF-α), and IL-6. The mechanisms of destruction of the microbes are similar to the neutrophils: proteases (e.g. lysosomal enzymes, elastase and collagenase), ROS and RNS, oxidative stress products, and chloramines. Activated macrophages also produce a number of inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-12, and others), chemokines (including IL-8, MCP-1, MCP-2, RANTES, and others), complement cascade proteins (C1, C2, C3, and C4), eicosanoids [e.g. prostaglandin (PG)E<sub>2</sub>, thromboxane (TX)A<sub>2</sub>, prostacyclin (PGI<sub>2</sub>), LTB<sub>4</sub>, and PAF] that may contribute to the propagation of inflammation. In addition, activated macrophages produce pro-coagulant factors such as tissue factor (TF) and plasmin inhibitor (or alpha 2-antiplasmin), linking the inflammatory and clotting responses.<sup>3,4</sup> These cells also have the ability to engulf pathogens and apoptotic cells, but they are capable of phagocytose much larger particles and also, after digesting particles, they can extrude the residual products and often survive and function for many more months.<sup>18</sup>

### **2.3. Lymphocytes**

The term lymphocyte comprises three cellular types: natural killer (NK) cells, B cells and T cells. NK cells are effector lymphocytes of the innate immune system; they control several types of tumors and microbial infections by limiting their spread and subsequent tissue damage. NK cells have a cell-mediated function, based in reciprocal interactions with dendritic cells, macrophages, T cells and endothelial cells.<sup>24</sup> B cells and T cells are the main type of lymphocytes and are part of the adaptive immune response; the first ones participate in the humoral immunity and the last ones in cell-mediated immunity.<sup>25</sup>

As so, lymphocytes intervene both in antibody-mediated and cell-mediated immune responses. During inflammation, the cytokines produced by macrophages (TNF-α and IL-1β) promote the recruitment of lymphocytes to the inflammatory site. For this migration, the antigen-stimulated lymphocytes of different types (T and B cells) use various adhesion molecules, namely selectins, integrins and their ligands. The interaction between lymphocytes and macrophages, in a bidirectional way, establishes a stage of persistent inflammatory response and, as such, the inflammatory reaction tends to be chronic and severe. Thus, macrophages display antigens to T cells and produce membrane molecules

(co-stimulators) and cytokines (e.g., IL-12) that stimulate T-cells. When activated, these cells produce various cytokines that recruit monocytes and IFN- $\gamma$  which is a powerful activator of macrophages.<sup>3</sup>

#### **2.4. Platelets**

Platelets are small and anucleate cellular fragments in circulation. Their primary function is to maintain homeostasis; they rapidly respond to vascular injury, become activated and induce platelet aggregation and thrombus formation. Platelets have the ability to modulate early inflammation, pointing out their vital role in this inflammatory phase, and straightening the link between inflammation and coagulation.<sup>4</sup> Despite the platelet granules content in pro-coagulant factors, Willebrand factor, fibrinogen, and factor V, those granules also contain inflammatory mediators. When platelets are activated, in the presence of inflammatory mediators such as PAF, they liberate various inflammatory mediators: (i) the CD40 ligand (CD40L), a primarily platelet-based immunomodulator of the TNF family that has only recently been shown to be expressed by activated platelets; it is responsible for the stimulation of vascular endothelial cells to produce cytokines (e.g. IL-1 and IL-18) and for the increased thrombotic activity; (ii) lipid mediators, namely TXA<sub>2</sub>, hydroxyeicosatetrenoate (12-HETE) and PAF; (iii) the neutrophil activating peptide 2 (NAP-2); (iv) sphingosine1-phosphate (S1P); (v) growth factors, namely PDGF and TGF- $\beta$ ; (vi) nitric oxide ( $\cdot$ NO); (vii) cytokines, including IL-1 $\beta$  and IL-7; (viii) chemokines, like the CXCL5, MCP-3, RANTES, MIP-1 $\alpha$ , platelet factor 4 (PF4); (ix) histamine; (x) serotonin; (xi) growth-regulating oncogene- $\alpha$  (GRO- $\alpha$ ); (xii) high mobility group box 1 (HMGB-1) and P-selectin. The P-selectin expressed in the surface of the platelets establishes a link to the selectin glycoprotein ligand-1 (PSGL-1) on leukocytes, creating a platelet-lymphocyte complex, which in turn leads to the activation of the lymphocytes. The P-selectin expression also results in the monocyte expression of TF and the release of inflammatory cytokines by monocytes. Platelets contribute to leukocyte-leukocyte and leukocyte-endothelial cell interactions.<sup>4,26</sup> Interestingly, it has been demonstrated that platelets are able to distinguish between their roles in immunity and hemostasis, being also able to respond differentially among various pathogen ligands.<sup>27</sup>

#### **2.5. Mast cells**

Mast cells are most commonly localized in perivascular locations within connective tissues. On their surface they expose the high affinity immunoglobulin E receptor (Fc $\epsilon$ RI) that binds the Fc portion of IgE antibody. In the presence of these stimuli, cells

degranulate.<sup>3</sup> The granular content of mast cells is similar to those of basophils, and, like basophils, mast cells may play a role in allergic reactions.<sup>4</sup> Mast cells are also present in chronic inflammatory reactions, and due to its ability to secrete a plethora of cytokines, they have both the ability to promote and limit inflammatory reactions in different situations. Mast cells are able to produce various inflammatory mediators, namely: pre-formed mediators that are granule associated (e.g. histamine), mediators synthesized *de novo* (e.g. LTC<sub>4</sub>, PAF, and PGD<sub>2</sub>), cytokines and chemokines [e.g. IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, TNF- $\alpha$ , vascular endothelial growth factor (VEGF), TGF- $\beta$ , MIP-1 $\alpha$ , and MCP-1].<sup>1,3</sup>

### **2.6. Epithelial cells**

Epithelial cells have an active role in the inflammation process. They are capable of escalate an immune response by the internalization of organisms and production and secretion of cytotoxic and microbicidal peptides. These cells may be induced by bacterial components, e.g. LPS, cytokines, TNF- $\alpha$  and IL-1 $\beta$ . This activation leads to the production of other inflammatory mediators, via nuclear factor kappa B (NF- $\kappa$ B) and I $\kappa$ B signalling pathways: cytokines (e.g. TNF- $\alpha$  and IL-1 $\beta$ ), chemokines (e.g. IL-8, MIP-2, CXC chemokines, MCP-1, IL-7 and IL-15), NO, adhesion molecules [e.g.  $\beta$ -integrins and intercellular adhesion molecule 1 (ICAM-1)] and a variety of receptors: TLRs (2 and 4), TNF- $\alpha$  receptors (TNFR1 and TNFR2), growth factor receptors and plasminogen activator receptors.<sup>1</sup>

### **2.7. Endothelial cells**

Endothelial cells are crucial to the regulation and propagation of the inflammatory response. The activated endothelial cells produce a variety of proteins that contribute to inflammation in different pathways: ROS and RNS, inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , and IL-6), chemokines (IL-8, RANTES and MIP-1), adhesion molecules [ICAM-1, ICAM-2, PECAM, vascular cell adhesion molecule 1 (VCAM-1), E-selectin, and P-selectin], proteases, leukotrienes (LTs) and prostaglandins (PGs) and growth factors (VEGF and TGF- $\beta$ ). Endothelial cells also express cytokine and chemokine receptors (TNFR1 and IL-1R), TLRs and pro-coagulants and protease-activated receptors. These cells also carry out alterations of the cytoskeleton and of the intercellular proteins for the leukocytes' migration and to alter the vascular permeability.<sup>1,4</sup>

### **2.8. Fibroblasts**

Fibroblasts are ubiquitously present in the human organism and are the main responsible for extracellular matrix production, and consequently by its effects, not only in tissue structural cells but also in hematopoietic cells. It is well demonstrated that fibroblasts are able to produce a myriad of inflammatory mediators, to which they can themselves respond, suggesting a central role in the regulation of the reparative response.<sup>28</sup> They express matrix metalloproteinases (MMPs) and cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ . They also synthesize collagen, required to tissue repair, but if it is laid down in an abnormal manner or excessive amount, it contributes to organ dysfunction. In this way, fibroblasts are intimately linked to the regulation and perpetuation of inflammation.<sup>1</sup>

### **2.9. Dendritic cells**

Dendritic cells may be characterized as the conductors of the immune response, as they internalize and process antigens and then display them in the surface, in conjunction with human leukocyte antigen (HLA) molecules, allowing the “presentation” to the lymphocytes, resulting in the latter activation.<sup>1</sup>

## **3. Effects of flavonoids on cells mediating the inflammatory response**

Flavonoids have the ability to interfere with the functions of cells mediating the inflammatory response and therefore interfere with the inflammatory process. Several studies with quercetin revealed its capacity to interfere with the inflammatory process, namely inhibition of the secretion of lysosomal enzyme from PMNs, induced by concanavalin A; inhibition of neutrophil degranulation as well as the catalytic activity of elastase. Along with quercetin, luteolin, apigenin and kaempferol have been described as potent inhibitors of  $\beta$ -glucuronidase and lysozyme release from activated neutrophils.<sup>16</sup> It is believed that, in general, flavonoids act as down-regulators of myeloid cell (mostly macrophage), meaning that they lower the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, pro-inflammatory cytokines IL-1 $\beta$ , TNF, IL-6, and other markers.<sup>17</sup> Isoflavones proved to inhibit tyrosine protein kinase (PTK), which is involved in phosphorylation of particular T cell proteins, interfering in T cell proliferation. Genistein promoted the inhibition of p56lck in T cells stimulated with phytohaemagglutinin/ phorbol-12-myristate-13-acetate (PHA/PMA), with subsequent reduction of IL-2 expression. It was also able to inhibit human peripheral mononuclear cell proliferation and IL-2 and LTB<sub>4</sub>

production. Luteolin, apigenin, myricetin and quercetin proved to modulate lymphocytes and neutrophils.<sup>29</sup> The detailed description of the flavonoids' effects in the different cells involved in inflammation, namely granulocytes, macrophages, lymphocytes, mast cells, epithelial cells, endothelial cells, dendritic cells and fibroblasts was revised in <sup>17</sup>. As this paragraph illustrates, all the phenomena involved in inflammation are linked. Therefore, it is a hard task to separate and categorize all the different inflammatory mediators and the flavonoids' isolated effects in each one of them. Along this paper we will try to discuss these effects for each main inflammatory mediator, although, as we will see, a vast number of works study more than one mediator together, because they are all linked.

## **4. Inflammatory mediators**

### **4.1. The complement cascade and effects of flavonoids**

The complement system was first discovered in 1899 by Bordet.<sup>4</sup> It is an ancient defense mechanism of the body that links innate immunity and the humoral arm of adaptive immunity.<sup>5</sup> This system is composed for more than thirty circulating and membrane-bound regulatory and constitutive glycoproteins that interact to recognize and kill pathogens. The tissue damage, when unregulated, leads to a deregulated activation of the complement cascade. An excessive activation of complement can be deleterious and has been implicated in the pathogenesis of multiple immune and inflammatory conditions. A wide variety of circulating glycoproteins and tissue membrane-bound proteins act to regulate the complement activity and down regulate its attack.<sup>30</sup>

The complement cascade present three distinct pathways (figure 3): a) the classical, b) the alternative and c) the mannose-binding lectin (MBL). A) The classical pathway is triggered by the interaction of antigen:antibody complexes with C1. In humans, only IgM and IgG (except IgG4) are effective in the activation of the classical pathway. However, there are other molecules capable of activate the classical pathway, via direct interaction with C1q, namely C-reactive protein (CRP), serum amyloid P component (SAP),  $\beta$ -amyloid, some gram-negative bacteria, some viruses, mycoplasmas, protozoa and intracellular components (e.g. DNA), mitochondrial membranes, cytoskeletal filaments and apoptotic cells. The overproduction of C3a and C5a during complement activation and the excessive formation of membrane attack complexes lead to the detrimental effect of complement. As such, a number of inhibitory molecules present in plasma or membrane-bound guarantee the prevention of an uncontrolled activation of the complement system. The C1 inhibitor is present in plasma and prevents the activation of C1s and C1r, antagonizing the classical pathway. In addition, C1 inhibitor also inhibits the MBL

pathway.<sup>4,30</sup> B) The alternative pathway is triggered by bacterial products (e.g. LPS), which results in the sequential activation of C3a, C5a and the membrane attack complex. Although antibodies can activate this pathway, they are not necessary for the activation, neither is lectin. This alternative pathway initiates with the deposition of active C3 on a surface that has no regulators of the complement present. This leads to the formation of C3b fragments and the process continues as an unconstrained amplification loop. This pathway cannot distinguish 'self from other'. Its specificity resides in the ability of host cells (but not bacteria) to defend themselves against indiscriminate complement activation.<sup>4,30</sup> C) The MBL pathway is triggered by the binding of bacterial sugars, such as mannose, to MBL protein, activating C4a and C5a and then joins the common pathway for the activation of the membrane attack complex. However, MBL recognizes certain pathogenic carbohydrates expressed on the surface of microorganisms, but does not recognize carbohydrates such as galactose and sialic acid, which are the terminal sugars expressed on mammalian glycoproteins, allowing the discrimination between 'self from other'. MBL is formed in the liver and are designated as complement-activating soluble pattern recognition molecules, i.e., it recognizes PAMPs in the microbes' surfaces.<sup>4,30</sup> The three pathways of the complement differ in their mechanisms of target recognition and initial activation but converge at the third component of complement, C3, with the proteolytic release of fragments and in the assembly of the membrane attack complex (MAC), formed by components C5b to C9, amplifying the inflammatory response and the mediated tissue injury.<sup>5</sup>

Generally, the functions of the complement cascade can be divided in also three categories: a) inflammation, b) phagocytosis and c) cell lysis. a) Inflammation: the components C3a, C5a and C4a (in a lesser extent) generate an inflammatory response. They are the cleavage products of the corresponding complement components that stimulate histamine release from mast cells and they can rapidly increase vascular permeability and cause vasodilation, as well as, leukocyte chemotaxis and activation. They are called anaphylatoxins because they have effects similar to those of mast cell mediators that are involved in the reaction called anaphylaxis. C3a and C5a also induce TNF and IL-1 $\beta$  release by peripheral blood mononuclear cells (PBMCs). In addition, C5a activates the lipoxygenase (LOX) pathway of arachidonic acid (AA) metabolism, causing additional release of inflammatory mediators, it embed chemotaxis, enzyme release and generation of ROS in neutrophils and monocytes, which participate in the microorganisms killing.<sup>5</sup> b) Phagocytosis: C3b and its cleavage product, inactive C3b, act as opsonins; they coat and opsonize cell membranes and soluble antigens for recognition and digestion by neutrophils and macrophages. c) Cell lysis: MAC (C5b-C9) deposits in cells and makes them permeable to water and ions and results in cells death (lysis).<sup>3,4,8</sup> Moreover, the MAC

formation has a stimulating effect on several nucleated cell types, namely induction of pro-coagulant activity in platelets and endothelial cells, pro-inflammatory activity in endothelial cells and smooth muscle cells, and their proliferation, release of eicosanoids from phagocytic cells, and triggering of signal transduction pathways.<sup>30</sup> Noteworthy, complement deficiencies cause susceptibility to bacterial infection, but also susceptibility to autoimmune diseases.<sup>4</sup>

Studies of the effects of flavonoids in the complement cascade are scarce, however it is described they reduce the activation of the complement system, consequently reducing the adhesion of inflammatory cells to the endothelium.<sup>29</sup> Very recently, a short communication stated the preliminary inhibitory mechanism of four flavonoids (quercitrin, afzelin, isoquercitrin and quercetin) in the complement activation cascade for the first time. All the three tested glycosides acted on all the tested components of the complement system, indicating that the glycoside moieties may be necessary to block C3 and C4 components. The single studied flavonol probably interacted with C1q, C2, C5 and C9.<sup>31</sup>

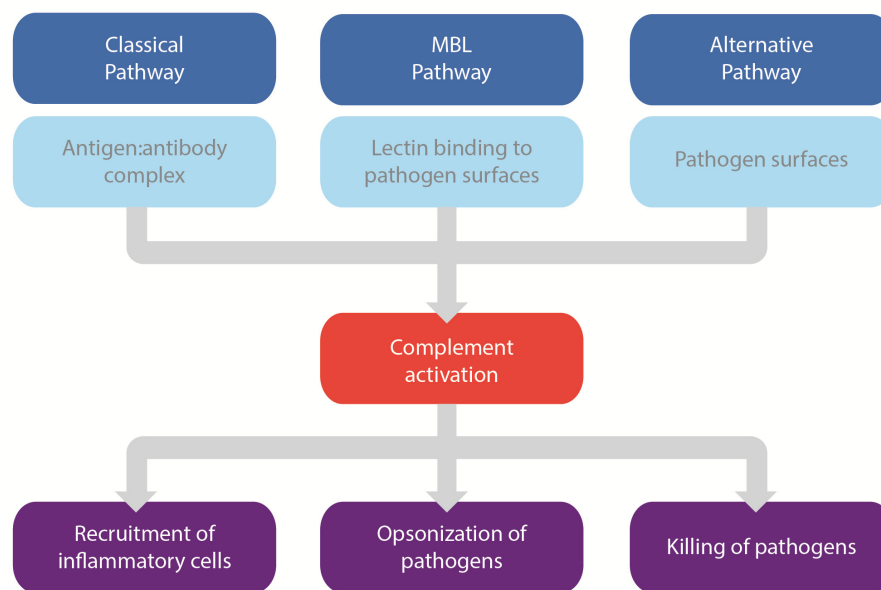


**Figure 3** - Schematic representation of the different pathways of complement activation and their effects. <sup>Adapted from 32</sup>

#### **4.2. Lipid mediators of inflammation**

One of the most important roles of membrane lipids in inflammation signalling is their ability to serve as substrates for the generation of hydrophobic pro-inflammatory and anti-inflammatory mediators. Phospholipase A2 (PLA2) is the key enzyme in the universe of lipids mediators. There are four types of PLA2: secretory PLA2 (sPLA2), which require  $\text{Ca}^{2+}$  in mM concentrations for optimal activity; cytoplasmic PLA2 (cPLA2), requiring  $\text{Ca}^{2+}$

in  $\mu\text{M}$  concentrations, which are AA-selective;  $\text{Ca}^{2+}$  - independent PLA2 (iPLA2), specificity for plasmalogen substrates; and PAF acetylhydrolase (PAF-PLA2), which has a series of isozymes specific for short chains. Under basal conditions, AA is released from the phospholipid membrane by iPLA2 and then is reacylated, i.e., reincorporated in the membrane, being unavailable for eicosanoids biosynthesis. When cells are stimulated by receptors activation, the intracellular levels of  $\text{Ca}^{2+}$  increase and the  $\text{Ca}^{2+}$  -dependent cPLA2 liberates arachidonate at a rate that exceeds the rate of reacylation. This leads to arachidonate metabolism by COX isoenzymes and by LOX.<sup>33</sup>

#### 4.2.1. Eicosanoids

Eicosanoids are lipid-derived molecules. They are not stored, but synthesized *de novo* from membrane lipids during cell activation by mechanical trauma, cytokines and growth factors, among others. Throughout this activation, the cPLA2 translocates to the nuclear envelope, endoplasmic reticulum and Golgi apparatus, where it releases the AA from the lipid bilayer. AA is a polyunsaturated fatty acid, normally covalently associated with cell membrane phospholipids.<sup>5</sup> The schematic steps of eicosanoids' production are explained in figure 4.

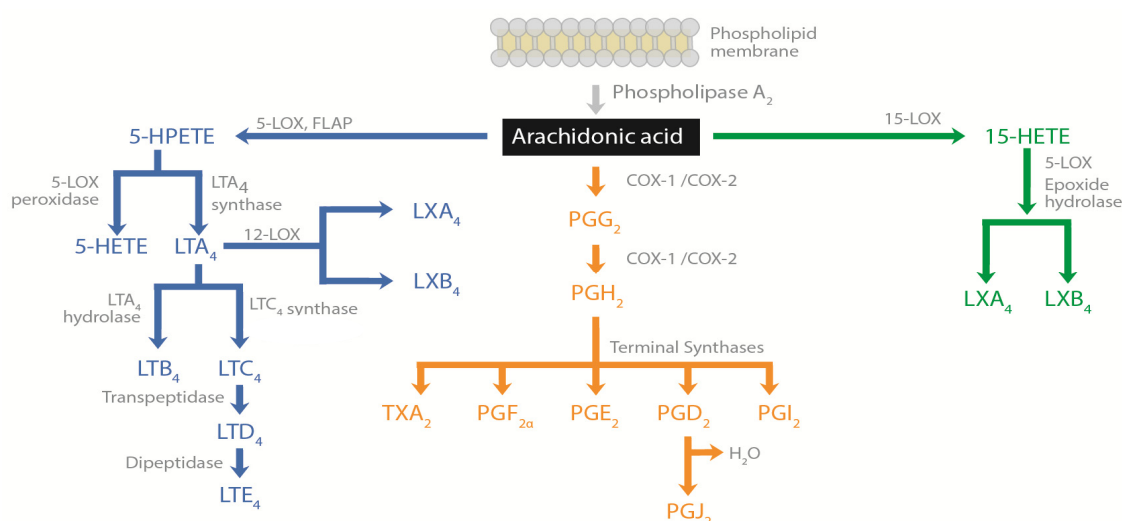


**Figure 4** - Schematic representation of eicosanoids' formation through the arachidonic acid metabolic pathways.

##### 4.2.1.1. Prostaglandins

In the inflammation context there are two enzymes with primordial functions: COX-1 and COX-2. COXs are responsible for the incorporation of oxygen into the carbon-11 of AA, leading to the formation of the five-carbon ring common to all PGs, and the production of the unstable PGG<sub>2</sub>. The PGG<sub>2</sub> is reduced to PGH<sub>2</sub> by COXs, with a PGH synthases function. The PGH<sub>2</sub> is then converted in different prostanoids by the action of specific

terminal synthases, depending on the tissue; different cells produce different PGs in abundance.<sup>4,34</sup> The PGs' level is usually very low in normal tissues, but it rapidly increase in acute inflammation, well before leukocyte recruitment. In chronic inflammation the prostanoids levels are truly augmented due to COX-2 induction with inflammatory stimuli.<sup>5</sup> PGs are not stored in cells, but are produced on demand and seem to exert their effects on the cell of origin or nearby structures. They are rapidly degraded *in vivo* by 15-hydroxyprostaglandin dehydrogenase (PGDH) during one passage through the lungs. COX-1 is expressed constitutively in a variety of tissues, including endothelium, monocytes, platelets, renal collecting tubules and seminal vesicles.<sup>33</sup> Its mediators are relevant in various homeostatic processes, namely in the regulation of renal perfusion and salt and water handling, modulation of platelet aggregation and preservation of gastrointestinal mucosal integrity. Conversely, COX-2 is an inducible enzyme and it is expressed primarily in cells involved in inflammation, such as macrophages, fibroblasts and endothelial cells. COX-2's expression is induced by various stimuli, including, growth factors [PDGF and epidermal growth factor (EGF)] and pro-inflammatory cytokines (IL-1 $\beta$  and TNF- $\alpha$ ). During the inflammatory stimuli it is thought that activation of COX-2 is mediated by peroxynitrite anion (ONOO<sup>-</sup>).<sup>4,34,35</sup> The bioactive prostanoids synthesized after the PGH<sub>2</sub> include: PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2</sub>, PGI<sub>2</sub>, and TXA<sub>2</sub>. The formation of PGJ<sub>2</sub> results from the dehydration of PGD<sub>2</sub>, a non-enzymatic process.<sup>4,34</sup> To exert their effects, PGs need to bind to receptors. G protein-coupled receptors (GPCRs) and peroxisome proliferator-activated receptors (PPARs) are known to be activated *in vitro*, but it is not clear that PPARs mediate PGs effects *in vivo*. Generally the receptors where the COX products exert their effects are designated as P receptors, depending on the affinity to each prostanoid, namely PGD receptors (DP), four subtypes of the PGE receptor (EP1 through EP4), PGF receptor (FP) and PGI<sub>2</sub> receptor (IP). The receptors DP, EP2, EP4 and IP induce the increase of cellular cAMP, while EP1 and FP induce Ca<sup>2+</sup> mobilization. EP2, EP4 and IP regulate the macrophages' cytokines production. Studies on the PGE<sub>2</sub> signalling propose the participation of phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and Wnt pathways in EP2R/EP4R regulation of cell growth, migration and apoptosis. Unlike conventional signalling via GPCRs, PGJ<sub>2</sub> is actively transported into cells, where it acts on the intra-nuclear PPAR- $\gamma$ . Consequently it inhibits the NF- $\kappa$ B activation, compromising the transcription of pro-inflammatory genes. In some cell types, PGJ<sub>2</sub> may well directly inhibit NF- $\kappa$ B.<sup>5,33</sup> The functions exerted by PGs are summarized in table 2, but they depend on the stimulus to inflammation, the predominant prostaglandin produced at a particular time and the profile of prostaglandin-receptor expression. Recent studies have shown that PGs activity is linked with cytokines, PAMPs and damage-associated molecular pattern molecules (DAMPs). So, PGs are also

able to convert short-lived inflammatory responses to long-term gene expression-dependent processes. As such, PGs act as 'cytokine amplifier', explaining the importance of their roles in chronic inflammation.<sup>36</sup>

#### **4.2.1.2. Prostacyclin**

PGI<sub>2</sub> is one of the prostanoids products of the AA metabolism, through the action of COX-2 and subsequently of the prostacyclin synthase (PGIS). The production of PGI<sub>2</sub> can be stimulated by thrombin or it can be generated by transfer of PGH<sub>2</sub> from platelets, contact with activated leukocytes or stretching of the arterial wall. Vascular endothelium lacks thromboxane synthase but possesses PGIS, which leads to the formation of PGI<sub>2</sub>. It has a short half-life, and is rapidly metabolized to the more stable and less biologically active, 6-keto-PGF<sub>1α</sub>. The biological functions of prostacyclin are described in table 2.<sup>3,33,37</sup>

#### **4.2.1.3. Leukotrienes**

The key enzyme for LTs production is 5-LOX. During the inflammatory process, i.e. cell activation, 5-LOX translocates from the cytosol to the nuclear membrane where it meets 5-LOX-activating protein (FLAP). The AA, released from the lipid membrane by cPLA<sub>2</sub>, is also translocated to the nuclear membrane, where FLAP presents it to 5-LOX. 5-LOX oxygenates AA at the C-5, generating 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which in turn is reduced to the more stable 5-hydroxyeicosatetraenoic acid (5-HETE), by a peroxidase, or is converted to LTA<sub>4</sub>, by LTA<sub>4</sub> synthase. LTA<sub>4</sub> can be converted in LTB<sub>4</sub>, by LTA<sub>4</sub> hydrolase (in neutrophils and macrophages) or in LTC<sub>4</sub>, by conjugation with reduced glutathione (in eosinophils, mast cells, endothelial cells and macrophages). The LTC<sub>4</sub>, in turn, migrates from the nuclear envelope to out of the cell, using transporters such as the multidrug resistance-associated protein (MRP), and can be metabolized extracellularly to LTD<sub>4</sub> by the action of transpeptidases, being the latter the precursor of LTE<sub>4</sub>. The designation cysteinyl LTs (Cys LTs) comprises LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>, which correspond to the "slow-reacting substance of anaphylaxis" for their slow and sustained smooth muscle contracting abilities. Each type of LTs has its specific activities, which are enumerated in table 2.<sup>4,5,33</sup> LTs exert their effects via surface receptors. LTB<sub>4</sub> has two identified receptors, BLT1 and BLT2; the first ones are over-expressed during inflammation. The high-affinity LTB<sub>4</sub> receptors, BLT1, transduce chemotaxis and adhesion responses; whereas low-affinity receptors, BLT2, are responsible for secretion of granule contents and superoxide anion radical (O<sub>2</sub><sup>•-</sup>) generation. The Cys LTs also have two receptors, CysLT1 and CysLT2, promoting bronchial smooth muscle contraction through increased Ca<sup>2+</sup> fluxes and kinase activation [(protein kinase (PK)C, MAPK].<sup>33,38</sup>

#### **4.2.1.4. Lipoxins**

Lipoxins (LXs), i.e. lipoxygenase interaction products, can be generated at mucosal surfaces via leukocyte-epithelial cell interactions and within the vascular lumen during platelet-leukocyte interactions.<sup>33</sup> The human neutrophil predominantly produce 5-HPETE from the 5-LOX action; though, when high concentrations of AA are added, 15-LOX can be expressed and also actuate in AA. The AA generated by epithelial cells may be converted by 15-LOX into 15-HETE, which is subsequently converted into lipoxin (LX)A<sub>4</sub> and LXB<sub>4</sub>.<sup>19</sup> In platelets, the LOX expressed is 12-LOX. It originates 12-HPETE, which yields 12-HETE by reduction of the hydroperoxy group; which in turn renders LXA<sub>4</sub> and LXB<sub>4</sub>, from neutrophils' LTA<sub>4</sub>.<sup>33</sup> A third mechanism of LXs production is also described. It produces LXs of different stereochemistry epi-lipoxins, also named as the aspirin/triggered lipoxins (ATL); because the reaction is initiated by aspirin and requires COX2 in the first step.<sup>39</sup> LXs act through their own specific receptors for LXA<sub>4</sub> and LXB<sub>4</sub>, but LXA<sub>4</sub> can also interact with a subtype of LTD<sub>4</sub> receptors. LXs are also able to act as intracellular targets, in their cells of origin or in other cells, after LXs uptake. Overall, LXs may play a role in the resolution of inflammation, as anti-inflammatory lipids.<sup>33</sup> The specific effects of LXA<sub>4</sub> and LXB<sub>4</sub> are explained in table 2. However it is worth to notice that LXA<sub>4</sub> activates PKC, being more potent than diacylglycerol (DAG) or AA. LXA<sub>4</sub> appears to be specific for the  $\gamma$  subspecies of PKC and may be an important modulator of the intracellular signal transduction.

#### **4.2.1.5. Thromboxanes**

TXA<sub>2</sub> is synthesized in platelets and it is converted from PGH<sub>2</sub> via the terminal enzyme thromboxane synthase and unlike PGs, it contains a six-member carbon ring. Even though TXA<sub>2</sub> is known as a platelet product, it can be up-regulated in other cell types in response to cytokines. The TXA<sub>2</sub>'s production is COX-1 dependent. However, TXA<sub>2</sub> is unstable and is rapidly converted to its inactive and stable form TXB<sub>2</sub>.<sup>33</sup> Table 2 summarizes the functions of TXA<sub>2</sub>.

#### **4.2.1.6 Isoprostanes**

All the isoprostanes have in common the top ( $\alpha$ ) and the bottom ( $\omega$ ) side chains, which are always syn-, i. e. crowded together on the same face of the cyclopentane ring. This characteristic is also what distinguishes them from PGs. Isoprostanes are generated from three contiguous methylene-interrupted double bonds, which are present in various naturally occurring polyunsaturated fatty acids. Isoprostanes are released pre-formed, so their production is not blocked by non-steroidal anti-inflammatory drugs (NSAIDs). Thus, it is possible that inflammation unresponsive to NSAIDs may harvest to inhibition of

isoprostanes. Isoprostanes may exert their functions via PPAR $\gamma$  and NF- $\kappa$ B; those functions are summarized in table 2.<sup>33</sup>

**Table 2** - Biological functions of eicosanoids.

| Eicosanoid                                               | Functions                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | References |
|----------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>PGE<sub>2</sub></b>                                   | Vasodilation and vascular hyperpermeability at early inflammation (along with PGI <sub>2</sub> ); pain; fever; induction of smooth muscle contraction.<br>Inhibition of T-lymphocyte activation and proliferation; down-regulation antibody production; variable effects on secretion of MMPs; reduction of the production of several inflammatory cytokines (TNF, IFN- $\gamma$ , and IL-12).                                                                                                    | 4,33,34    |
| <b>PGD<sub>2</sub></b>                                   | Vasodilation; mediation of histamine release from mast cells exposed to anti-IgE antibody; increase of the permeability of post-capillary venules; chemoattraction for neutrophils.                                                                                                                                                                                                                                                                                                               | 4,34       |
| <b>PGF<sub>2</sub></b>                                   | Contraction of uterine and bronchial smooth muscle and small arterioles; it is not known to participate in inflammatory events.                                                                                                                                                                                                                                                                                                                                                                   | 4,34       |
| <b>PGJ<sub>2</sub></b>                                   | Reduction of macrophage activation; reduction of NO production.                                                                                                                                                                                                                                                                                                                                                                                                                                   | 4,34       |
| <b>PGI<sub>2</sub></b>                                   | Vasodilation, via smooth muscle relaxation; inhibition of platelet aggregation; reduced cell proliferation; modulation of the function of dendritic cells, macrophages, monocytes, endothelial cells and eosinophils.                                                                                                                                                                                                                                                                             | 33,37      |
| <b>5-HPETE</b>                                           | Increase of intracellular Ca <sup>2+</sup> levels, facilitating PKC-dependent activation of a O <sub>2</sub> <sup>-</sup> generating system of neutrophils.                                                                                                                                                                                                                                                                                                                                       | 33         |
| <b>5-HETE</b>                                            | Chemotaxis; leukocyte adhesion; increase of intracellular Ca <sup>2+</sup> levels, facilitating PKC-dependent activation of a O <sub>2</sub> <sup>-</sup> generating system of neutrophils.                                                                                                                                                                                                                                                                                                       | 33         |
| <b>LTB<sub>4</sub></b>                                   | Chemotaxis; neutrophil and macrophages chemoattraction; stimulation of leukocyte adhesion to endothelial cells, by up-regulating leukocyte integrin expression; generation of ROS; release of lysosomal enzymes.                                                                                                                                                                                                                                                                                  | 3,5        |
| <b>LTC<sub>4</sub>; LTD<sub>4</sub>; LTE<sub>4</sub></b> | Vasoconstriction; increased vascular permeability; bronchoconstriction; LTs are much more potent than histamine in increasing vascular permeability and causing bronchospasm.                                                                                                                                                                                                                                                                                                                     | 8          |
| <b>LXA<sub>4</sub></b>                                   | Antagonism of the LTD <sub>4</sub> -induced vasoconstriction; blockage of the binding of LTD <sub>4</sub> to its receptors on mesangial cells; suppression of LTB <sub>4</sub> -induced plasma leakage and leukocyte migration; blockage of LTB <sub>4</sub> -induced neutrophil inositol triphosphate generation and Ca <sup>2+</sup> mobilization, but not O <sub>2</sub> <sup>-</sup> generation; activation of monocytes and stimulation of macrophage phagocytosis of apoptotic neutrophils. | 33,39      |
| <b>LXB<sub>4</sub></b>                                   | Stimulation of human peripheral blood monocytes; enhancing growth of myeloid progenitor cells; inhibition of human neutrophil transmigration and adherence.                                                                                                                                                                                                                                                                                                                                       | 40         |
| <b>TXA<sub>2</sub></b>                                   | Vasoconstriction; platelet activation and aggregation; smooth muscle contraction.                                                                                                                                                                                                                                                                                                                                                                                                                 | 3,4        |
| <b>Isoprostane F<sub>2<math>\alpha</math></sub> III</b>  | Vasoconstriction; suppression of the release of mediators of inflammation from macrophages; suppression of the IL-12 production by activated dendritic cells; reduction of cytokine production by Th1 and Th2 cells.                                                                                                                                                                                                                                                                              | 33         |

#### **4.2.2. Effects of Flavonoids on Eicosanoids Production**

Flavonoids have proven to succeed in the inhibition of eicosanoids' production through different pathways. They can inhibit the release of AA, interfering with cPLA2 and inhibit the eicosanoid biosynthesis pathway.<sup>11,15</sup> However, the mechanisms involved in this enzymatic inhibition are not fully elucidated.<sup>29</sup> Quercetin was the first flavonoid described as a PLA2 inhibitor from human neutrophils.<sup>16</sup> 7-O-Methylaromadendrin was shown to inhibit isolated sPLA2.<sup>41</sup> It is believed that the co-planarity of the molecules helps the interaction between the flavonoid and the enzyme, highlighting the importance of the C2=C3 double bond in the C ring of flavonoids, which grants its co-planarity.<sup>29</sup> The majority of studies about the flavonoids effects in the eicosanoids synthesis are done for PGs and LTs.

##### **4.2.2.1. Effects of Flavonoids in the synthesis of Prostaglandins**

Flavonoids may inhibit the production of PGs, interfering with COX-2, by direct activity inhibition or by the reduction of COX-2 expression. Durga et al. reviewed the immunomodulatory effects of flavonoids. They reported that in LPS-induced macrophages, luteolin-induced lowering of mRNA and protein levels of COX-2 and increased iNOS activity; apigenin, kaempferol, catechin, myricetin, and genistein were found to inhibit COX-2.<sup>11</sup> The overall anti-inflammatory effects of genistein were previously reviewed.<sup>42,43</sup> Other studies using the gene-reporter assay to express COX-2 showed that some flavones and flavonols were active suppressors, but epigallocatechin-3-gallate (EGCG), catechin, and myricetin were not.<sup>16</sup> Wang and co-workers demonstrated that apigenin decreased COX-2 expression and inhibited other inflammatory mediators in adenocarcinoma cells.<sup>44</sup> Wogonin was found to inhibit COX-2 activity as well as COX-2 expression in LPS-induced macrophages; and this may be a selective effect, since this flavonoid inhibited PGE<sub>2</sub> production, but not LTB<sub>4</sub> from IL-1 $\beta$  induced gingival fibroblasts.<sup>16</sup> Hämäläinen et al. studied the effect of 26 flavonoids from different classes in LPS-induced PGE<sub>2</sub> production, COX-2 mRNA and protein expression on murine J774 macrophages. They concluded that flavone, luteolin-7-glucoside, kaempferol, isorhamnetin, morin, quercetin, naringenin, taxifolin, pelargonidin, daidzein, genistein, and genistin inhibited the production of PGE<sub>2</sub>. Flavone, isorhamnetin, daidzein, and genistein efficiently inhibited the expression of COX-2. Two flavonols, kaempferol and isorhamnetin, significantly inhibited inducible microsomal prostaglandin E synthase-1 (mPGES-1) accumulation; COX-2 and mPGES-1 are functionally coupled to result in increased PGE<sub>2</sub> formation in macrophages and tissue cells. As so, the modulation of COX-2 pathway by these flavonoids seemed to be independent from the flavonoids' structure, as no relation has been established

between their activity and their flavonoid class.<sup>45</sup> An interesting study about inflammation in human gestational tissues demonstrated the anti-inflammatory effects of the co-administration of luteolin and kaempferol, in the foetal membrane. These flavonoids significantly inhibited the LPS-induced expression of COX-2 and PGE<sub>2</sub> concentration. These two flavonoids only share one structural feature, the OH groups in positions 5 and 7 in A ring, but no structure-activity relationship has been established, probably due to the reduced number of flavonoids studied.<sup>46</sup> Hou and co-workers undertook a study with LPS-activated RAW264 macrophage cells and investigated the effect of anthocyanidins in COX-2 expression. They concluded that an ortho-dihydroxyphenyl structure on the B ring suppressed this expression and that the number of OH groups on the B ring appeared to be related to a molecular conformation that influences the interactions between flavonoids and enzymes such as tyrosine kinase and protein kinase C, which are involved in the transcriptional activity of COX-2.<sup>47</sup> Indeed, more recently, a docking study with luteolin, chrysin, apigenin and quercetin indicated that the catechol group of B ring of luteolin was orientated towards the hydrophobic pocket of COX-2. On the other hand, the A ring of quercetin was orientated towards the pocket, enabling the 3-OH group of C ring to interact; therefore, they concluded that quercetin may not be a classical COX inhibitor.<sup>48</sup> In spite of the general results that have been obtained by these studies, in which COX-2 expression is inhibited by flavonoids, López-Posadas and co-workers developed a study in an intestinal epithelial cell line (IEC18) with some controversial results. They studied 9 flavonoids, among them flavonols, flavones, isoflavones and flavanones and they established the structure-activity relationship (SAR). They concluded that the effect of flavonoids on COX-2 expression was dependent on the experimental conditions tested, in non-stimulated or LPS-stimulated cells. They observed that flavonoids caused an increase in COX-2 expression under basal conditions. However, under LPS stimulation, they decreased or did not affect COX-2 levels, depending on the specific type. To explain these results, and because c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK)1/2 and NF-κB are described as involved in LPS activation, COX-2 regulation and signal transduction, they further investigated these indicators. Once more, they verified that flavonoids show structure-dependent effects on ERK/p38/JNK phosphorylation, as several of them prevented IκB phosphorylation completely, none of the flavonoids prevented p50/p65 nuclear translocation, and genistein actually increased it; flavonoids generally augmented NF-κB dependent gene transcription. They explained these results in the basis that IEC18 cells do not depend on the classical pathway for the regulation of NF-κB target genes and that alternative routes have been long known to exist in some cell types. They also showed that quercetin augment translocation of p50/p50 homodimers, which is commonly associated with repression of transcription, but

it has also been reported to activate transcription. Adding to this, quercetin and possibly other flavonoids can exert these effects not only by affecting I $\kappa$ B $\alpha$  phosphorylation but also Akt and perhaps other targets.<sup>49</sup>

#### **4.2.2.3. Effects of Flavonoids in the synthesis of Leukotrienes**

Another way to modulate the production of eicosanoids is the interference in LTs production. The isoflavones ability to inhibit PMNs' 5-LOX has been studied and it was stated that they act as redox inhibitors, by preventing 5-LOX activation from its resting form (ferrous state) to its reactive state (ferric). Genistein was more potent than daidzein; they only differ in one OH group in A ring.<sup>16</sup> Flavonols (kaemferol, quercetin, morin and myricetin) were found to be better LOX inhibitors than flavones. Flavanones such as naringenin were ineffective.<sup>50</sup> These findings may indicate a relation between flavonoids' structure and their activity, evidencing the importance of the 3-OH in C ring and of the double bond in C2=C3, explaining the weaker performance of flavones and flavanones.<sup>51,52</sup> A group of trihydroxyflavones demonstrated to inhibit the production of LTB<sub>4</sub> by human 5-LOX. Moreover, they inhibited COX -1 and -2 activity, but not in a selective way. These flavonoids also presented a good antioxidant activity as we will see further ahead in this manuscript, corroborating the theory of the redox inhibitors.<sup>53</sup> Notwithstanding the redox inhibition of 5-LOX explanation, sakuranetin, a flavonoid from *Inula viscosa*, inhibited the LTB<sub>4</sub> synthesis in leukocyte homogenates. The authors defend this result may indeed be obtained by the direct inhibition of 5-LOX, but also by interacting with other proteins, such FLAP, PKA, PKC, tyrosine kinases and by MAPK, that can modulate the 5-LOX activity.<sup>41</sup>

Flavocoxid is a recent product composed by baicalin, extracted from *Scutellaria baicalensis*, and catechin, from *Acacia catechu*, concentrated to greater than 90% purity. It has demonstrated great efficacy in the simultaneous inhibition of PLA<sub>2</sub>, COX-2 and 5-LOX. Flavocoxid demonstrated promising results when applied in osteoarthritis and in neuro-inflammatory diseases (e.g. ischaemic stroke, Parkinson's disease, Alzheimer's disease, muscular necrosis, etc.).<sup>54,55</sup>

#### **4.3. Production of Pro-Oxidant Reactive Species in Inflammatory Processes**

During inflammatory process various pro-oxidant reactive species (PORS) are overproduced and released by the activated immune cells. The term PORS may include radical or non-radical species. The immune cells involved in this production are mostly macrophages and neutrophils. The PORS produced are able to kill pathogens, but also function as determinant signal transduction molecules that regulate inflammatory genes'

expression. The problem resides in the association of the overproduction and release with the extraordinary reactivity of these species, which result in oxidative stress and non-specific oxidative reactions between these species and the biological components, including nucleic acids, lipids, carbohydrates, proteins, and matrix components. This may lead to cell death and tissue damage. Therefore, this process, referred as *respiratory* or oxidative burst, is extremely potent and requires tight regulation to prevent phagocytes auto-destruction. The PORS can be subdivided in the ones derived from oxygen, ROS and the ones derived from nitrogen, RNS.<sup>4,5</sup> The production of PORS in neutrophils is summarized in figure 5.

#### **4.3.1. Reactive Oxygen Species**

The enzyme that triggers the PORS production is NADPH oxidase and it needs to be activated to exert its activity. There are several activation stimuli, namely: microbial products (e.g. LPS and lipoproteins), LTB<sub>4</sub>, PAF, cytokines (e.g. IFN- $\gamma$  and IL-8) and engagement of Fc receptors by IgG. These leads to the translocation of various cytosolic proteins including Ras-related C3 botulinum toxin substrate 2 (Rac2) and Rho-family guanosine triphosphatase (GTPase) to the membrane-bound complex carrying cytochrome b<sub>558</sub>, with subsequent activation of reduced NADPH oxidase.<sup>5</sup>

NADPH oxidase is formed by various components that are distributed in cytosol and in the membranes of various intracellular organelles, in resting macrophages and neutrophils. Upon stimulation those components assemble on a membrane-bound vesicle, which merges with cytoplasm.<sup>35</sup> The central component of NADPH oxidase is cytochrome b<sub>558</sub>, composed by two polypeptides: p22<sup>phagocyte oxidase (phox)</sup> and gp91<sup>phox</sup> (thought to act as H<sup>+</sup> channel), which is localized in the plasma membrane and in the membranes of specific granules. Three cytosolic proteins are also required: p47<sup>phox</sup>, p67<sup>phox</sup>, and p21rac. Upon neutrophil stimulation, the p47<sup>phox</sup> and p67<sup>phox</sup> components translocate to membranes to form an active complex with the cytochrome b<sub>558</sub>. The p21rac also translocates in response to stimuli, but its significance is controversial. The protein p40<sup>phox</sup> has also been reported to be associated with p47/p67 in the cytosol. It is reported that p40<sup>phox</sup> may regulate the oxidase system in a positive and a negative manner and may play an important role in phagocytosis-induced O<sub>2</sub><sup>-</sup> production via a phox homology domain that binds to phosphatidylinositol (3,4,5)-trisphosphate (PIP3).<sup>19</sup> However, NADPH oxidases are of great importance in phagocytic cells, they are ubiquitous in other cells, where they are called non-phagocytic oxidases (NOXs). They are involved in a number of cellular events, namely cell proliferation and apoptosis. They also participate in the post-receptor signalling pathways (e.g. PDGF and angiotensin).<sup>4</sup>

When assembled at the phagolysosome membrane, NADPH oxidase catalyzes a one-electron reduction of  $O_2$  to form  $O_2^{\cdot-}$ . The enzyme superoxide dismutase (SOD), contained in phagocytosed microorganisms, catalyzes the dismutation of  $O_2^{\cdot-}$ , originating  $O_2$  and hydrogen peroxide ( $H_2O_2$ ), but this reaction can be also spontaneous, especially at low pH.  $H_2O_2$  originates hydroxyl radical ( $\cdot OH$ ) by the Haber–Weiss/Fenton reaction. So, in the presence of  $Fe^{2+}$  or  $Cu^+$  (free ionized and in a low oxidation state), the  $H_2O_2$  reacts to form  $\cdot OH$  and hydroxyl anion ( $OH^-$ ). Endogenous reducing agents (e.g. ascorbic acid) regenerate a lower oxidation state of the transition metal cation, reinitiating the cycle.  $H_2O_2$  can also originate hypohalous acids, by the action of MPO. MPO is present in the phagocytes and it catalyzes the oxidation of the halide ions chloride ( $Cl^-$ ), bromide ( $Br^-$ ), and iodide ( $I^-$ ) by  $H_2O_2$  to form hypochlorous acid (HOCl), hypobromous acid (HOBr) and hypoiodous acid (HOI), respectively. In neutrophils, most of the  $H_2O_2$  produced is consumed by MPO to catalyze the formation of HOCl. Because MPO has the specialized capacity of oxidize  $Cl^-$  and this last exists in high concentration in the body, it is generally accepted that the main function of MPO is to generate HOCl. This reaction, catalyzed by MPO, is important for the production of other bioreactive molecules, e.g. chloramines, which result from the reaction of HOCl with amines, and that are liposoluble and more toxic than HOCl itself.<sup>56</sup> From the reaction of the oxidized halogen ( $OCl^-$ ), from HOCl, with  $H_2O_2$  results singlet oxygen ( $^1O_2$ ).  $^1O_2$  is an excited state of  $O_2$  with its two paired electrons and it is responsible of various deleterious effects, namely the initiation of lipid peroxidation by the interaction with the membrane lipids. From this reaction other radicals derived from the  $O_2$  can be formed, such as peroxy radicals ( $ROO\cdot$ ), the most common being the protonated form of  $O_2^{\cdot-}$ , which is usually termed hydroperoxyl radical or perhydroxyl radical ( $HOO\cdot$ ). There are another sources of ROS, one of them being the oxidation of xanthine (or hypoxanthine) by xanthine oxidase, using  $O_2$  as co-substrate, to form uric acid and  $O_2^{\cdot-}$ .<sup>35</sup> Another source of ROS is the mitochondrial respiratory chain, where they are produced as by-products.  $O_2^{\cdot-}$  results from the leakage of electrons from the mitochondrial electron transport chain, which is quantitatively the most important mechanism that leads to the production of other ROS in the cell.<sup>35</sup> In terms of stability,  $O_2^{\cdot-}$  is relatively unreactive toward most of the biological substrates. From all the ROS formed,  $\cdot OH$  is the less stable of all, with high reactivity. Followed by  $H_2O_2$ , which, in turn, is rather unreactive. HOCl is the most bactericidal oxidant known to be produced by neutrophils.<sup>4,56</sup> ROS actively participate in the inflammation; activator protein 1 (AP-1) and NF- $\kappa$ B are activated by ROS, displaying the interference of ROS on the production of pro-inflammatory mediators as collagenase, IL-1 $\beta$  and TNF- $\alpha$ . ROS have also demonstrated to up-regulate adhesion molecules and to increase cytokines and chemokines production.<sup>4</sup>

More recently, some studies have defying the affirmation that HOCl is the most powerful tool on pathogens killing produced by neutrophils. This new thesis states that the  $O_2^{\cdot-}$  produced inside the phagolissosome causes a pH increase and a consequent influx of  $K^+$ , liberating cationic proteases from the anionic proteoglycan matrix, freeing them to kill bacteria. Moreover, this model is supported by the fact that MPO deficiency is common (1:2000), yet surprisingly benign. As such, oxidants are not primarily destructive to microbes, but necessary to promote the proteolytic damage.<sup>19</sup>

#### **4.3.2 Reactive Nitrogen Species**

The formation of  $\cdot NO$  is catalyzed by the enzyme NOS. There are three isoforms of NOS: a) neuronal NOS (nNOS or NOS1) and b) endothelial cell NOS (eNOS or NOS3) are both constitutively expressed, with their activity regulated by intracellular  $Ca^{2+}$  levels and the calcium-binding protein calmodulin, and are present in both the spinal cord and brain; and c) iNOS, macNOS, or NOS2,  $Ca^{2+}$  independent, which is normally expressed in macrophages, neutrophils, inflammatory cells and glia. The activity of iNOS is induced by a series of stimuli: IL- $1\beta$ , TNF- $\alpha$ , IFN- $\alpha$ , products of viruses, bacteria (e.g. LPS), protozoa, and fungi and by low oxygen tension and low environmental pH. Thus, iNOS promotes the conversion of L-arginine and  $O_2$  to L-citrulline and  $\cdot NO$ .<sup>4,5</sup>  $\cdot NO$  diffuses to its site of action and there it stimulates guanylate cyclase (GC) to produce cyclic guanosine monophosphate (cGMP). cGMP modifies the activation of protein kinases, ion channels and phosphodiesterases.<sup>34</sup>  $\cdot NO$  is a really important RNS, displaying innumerable functions: through the activation of the enzyme soluble GC, it induces vascular hyperpermeability and inhibits platelet aggregation;<sup>35</sup> bactericidal; induces vasodilation, edema, cytotoxicity; mediates the cytokine-dependent processes that can lead to tissue destruction;<sup>4,8</sup> activates the COXs enzymes and S-nitrosylates proteins;<sup>34</sup> presents an anti-inflammatory function by preventing the adhesion and release of oxidants by activated neutrophils in the microvasculature. Overall,  $\cdot NO$  regulates physiologic processes, host defense, inflammation and immunity.<sup>4</sup> Additionally,  $\cdot NO$  reacts with  $O_2^{\cdot-}$ , producing  $ONOO^-$ , which is very cytotoxic and mutagenic, with a reactivity close to  $\cdot OH$ , rapidly damaging biomolecules. As  $\cdot NO$  diffuses really quickly through cytoplasm and membranes, the formation of  $ONOO^-$  occurs close to the site of production of  $O_2^{\cdot-}$ . The half-life of  $ONOO^-$  is short and it is rapidly converted in its protonated form, peroxyntrous acid ( $ONOOH$ ).  $ONOOH$  becomes activated through a *cis/trans* isomerization, producing *trans*- $ONOOH$ , which is a powerful oxidant, highly reactive and yields oxidizing and nitrating species. Therefore, it is thought to be responsible for many of the toxic effects of  $\cdot NO$ .<sup>35,56</sup> Another fast reaction is the one between  $ONOO^-$  and  $CO_2$ , rendering the formation of nitrosoperoxy carbonate anion ( $ONOCO_2^-$ ). From the decomposition of

$\text{ONOOCO}_2^-$  results  $\text{CO}_2$  and  $\text{NO}_3^-$  (about 70 %) and also the free radicals  $\cdot\text{NO}_2$  and carbonate radical anion ( $\text{CO}_3^{\cdot-}$ ) (about 30 %). Noteworthy, MPO are able to oxidize  $\text{NO}_2^-$  in the presence of  $\text{H}_2\text{O}_2$  or  $\text{H}_2\text{O}_2$ -generating systems, leading to the production of nitrogen dioxide radical ( $\cdot\text{NO}_2$ ). The  $\cdot\text{NO}_2$  can also be formed by auto-oxidation of  $\cdot\text{NO}$  in the presence of  $\text{O}_2$ . In turn,  $\cdot\text{NO}$  can also react with  $\cdot\text{NO}_2$ , producing dinitrogen trioxide ( $\text{N}_2\text{O}_3$ ), a potent nitrosating agent.<sup>56</sup>

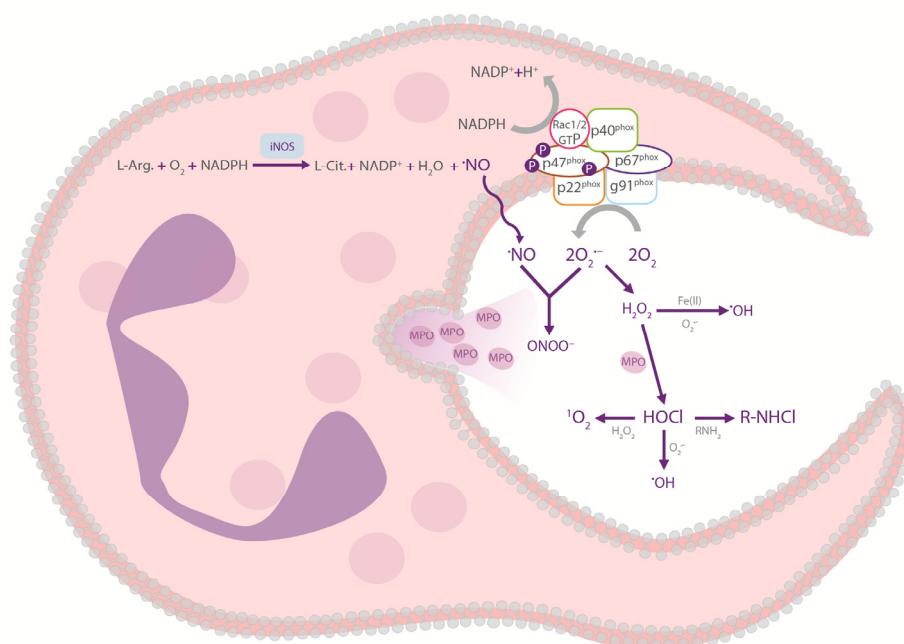


**Figure 5** - Production of PORS in the activated neutrophil.

#### **4.3.3. Antioxidants**

*In vivo*, several endogenous antioxidants are responsible for the task of inactivating pro-oxidant intermediates, namely ROS and RNS, and protect normal tissues from their deleterious effects. Antioxidants can exert this effect by: a) sequestration of transition metal ions into complexes; b) scavenging or quenching of ROS and RNS; c) breaking chain reactions initiated by free radicals; and d) repairing damaged molecules.<sup>57</sup> Transition metal-binding proteins, namely ceruloplasmin, ferritin, transferrin and lactoferrin are responsible for sequestering cationic iron and copper, inhibiting this way the generation of  $\cdot\text{OH}$ . Chain-breaking antioxidants are small molecules that can receive or donate an electron and thereby form a stable by-product with a radical. They are usually classified as aqueous phase [ascorbic acid (vitamin C), albumin, reduced glutathione] or lipid phase [ $\alpha$ -tocopherol (vitamin E), ubiquinol-10, and carotenoids]. SOD and catalase (CAT) enzymes are part of the group of molecules responsible to counter the activity of PORS. As said above, SOD catalyzes the dismutation of  $\text{O}_2^{\cdot-}$  to  $\text{H}_2\text{O}_2$ , which is then removed by CAT or glutathione peroxidase (GPx). CAT is a peroxisomal enzyme that

catalyzes the conversion of  $\text{H}_2\text{O}_2$  to water and  $\text{O}_2$ . Enzymes such as GPx and glutathione reductase (GR) are additional mechanisms for maintaining redox balance and removal of toxic metabolites.<sup>5</sup> This antioxidant system of defense is usually enough to maintain a reducing milieu in cells under physiological conditions. The problem appears when the production of ROS increase abruptly, overwhelming this antioxidant defenses, leading to *oxidative stress*.<sup>35</sup>

#### **4.4. Antioxidant Activity of Flavonoids**

As it was already described, the overproduction of ROS and RNS is deleterious for human tissues and it leads to the progression of the inflammatory response in an endless cycle. In this way molecules capable of arresting this cycle are of great value for the resolution of inflammation. The antioxidant activity of flavonoids is one for their best studied mechanisms of action, and therefore the processes affected by them and the best structure-activity relationship is well established. In general, the antioxidant activity of flavonoids may be undertaken by: a) direct scavenging of ROS or RNS by hydrogen atom donation; b) inhibition of oxidases responsible for  $\text{O}_2^{\cdot-}$  production, namely xanthine oxidase, COX, LOX, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase and NADPH oxidase; c) activation of antioxidant enzymes involving the induction of phase II detoxifying enzymes, namely NAD(P)H-quinone oxidoreductase, glutathione S-transferase, and UDP-glucuronosyltransferase; d) chelating trace metals; and e) mitigation of oxidative stress caused by  $\cdot\text{NO}$ .<sup>12,58,59</sup> Flavonoids can directly act upon ROS and RNS and stabilize them; this is the general equation (equation 1) that summarizes this reaction:



In 2008, Gomes et al. reviewed the essential structural features for the scavenging of ROS and RNS: a) HOCl - a 5,7-OH group in A ring and a OH in C3 in C ring; b)  $^1\text{O}_2$  - a OH in C3 in C ring, a  $\text{C}_2=\text{C}_3$  double bond, the existence of a 3',4'-OH in B ring favours de activity; and c)  $\text{ONOO}^{\cdot-}$  - the total number of OH, including a catechol group in B ring and a OH in C3 in C ring, a  $\text{C}_2=\text{C}_3$  double bond.<sup>15</sup> Thus, the search for the structural features that favour the most the antioxidant activity of flavonoids led to the following conclusions: the B ring 3',4' - OH configuration determine an excellent scavenging of ROS and RNS because it donates hydrogen and an electron to  $\cdot\text{OH}$ ,  $\text{ROO}^{\cdot}$ , and  $\text{ONOO}^{\cdot-}$ , stabilizing them and yielding relatively stable flavonoid radicals. On the other hand, the occurrence of three OH groups in B ring does not increase the efficiency of an antioxidant molecule.

Additionally, it is of general *consensus* the heterocycle contributes to antioxidant activity allowing the conjugation between the aromatic rings and the presence of a free 3-OH in C ring, contributing for the co-planarity of the rings. The existing OH groups in B ring are able to form hydrogen bonds with 3-OH, aligning the three rings. The absence of the C2=C3 double bond decreases the antioxidant activity (naringenin in contrast with apigenin). The combination of the 3- and 5-OH groups with the 4-oxo group in C ring allows electron delocalisation from the 4-oxo group to both substituents.<sup>12,15,29</sup> Recently published articles revised flavones and their antioxidant, and anti-inflammatory properties, with important insights on SAR.<sup>60,61</sup> More recently, a QSAR study using density functional theory (DFT) concluded that decreased electron-donating and electron-accepting capacities in rings A and B, respectively, should be achieved in order to design new flavonoids with antioxidant properties.<sup>14</sup>

#### **4.4.1. Effects of Flavonoids on Reactive Oxygen Species**

In an *in vitro* study, the flavonoids quercetin, luteolin, apigenin, chrysin, 5- and 7-hydroxyflavones scavenged the ROS  $O_2^{\cdot-}$ ,  $H_2O_2$ , HOCl,  $^1O_2$  and  $ROO^{\cdot}$ ; and the features designated above as essential for this activity were confirmed.<sup>62,63</sup> Flavonoids already proved to inhibit NADPH oxidase and in this way they disable the progression of ROS and RNS production. Quercetin, in a human neutrophils study, inhibited the MPO activity, the  $O_2^{\cdot-}$  production, the phosphorylation of some proteins, and even diminished neutrophils degranulation. Kaempferol, even though not having the catechol group in B ring, but just a 4'-OH, has also demonstrated good scavenging activity, maybe because of the combination of the other structural features (C2=C3 double bond, 3-OH group, or 4-oxo group on C ring). Catechins from Chinese green tea also scavenged  $H_2O_2$  and  $O_2^{\cdot-}$  produced by the same system.<sup>14</sup> 5-Hydroxy-3,7,4'-trimethoxyflavone, the cat's whiskers flavonoid, was able to effectively scavenge  $O_2^{\cdot-}$ ,  $OH^{\cdot}$  and  $\cdot NO$  from lymphocytes of patients with rheumatoid arthritis.<sup>64</sup> Eriodictyol, hesperetin, taxifolin and luteolin proved to inhibit MPO release, while eriodictyol and taxifolin also strongly inhibited MPO activity, interfering in HOCl production. In this study they also accept that these results are directly related to the inhibition of  $O_2^{\cdot-}$  production because of the OH groups in B ring. Interestingly, the two flavonoids described in this study as MPO inhibitors lack the C2=C3 double bond that has been considered crucial for this activity, by *in vitro* and by docking studies.<sup>14</sup> In fact, in 2008, Shiba and co-workers studied quercetin and their metabolites as MPO inhibitors and concluded that the OH groups in positions 3, 5, and 4' and the C2=C3 double bond were determinant for the inhibitory effect. Through a computer docking simulation they also suggested the binding of quercetin and the metabolites to a hydrophobic region at the entrance to the distal heme pocket of MPO.<sup>65</sup> Quercetin, silibin

and luteolin have been described as the most potent inhibitors of xanthine oxidase.<sup>29</sup> As mentioned above, xanthine oxidase is one of the enzymes responsible for the  $O_2^{\cdot-}$  production, following the conversion of xanthine dehydrogenase into xanthine oxidase, under stressful conditions. Durga et al. described silibin along with quercetin as inhibitors of the activity of xanthine oxidase, thereby preventing oxidative damage by the release of free radicals.<sup>11</sup> Rutin and epicatechin have also been described as potential radical scavengers. They have a powerful  $\cdot OH$  scavenging effect and also inhibited the generation of  $O_2^{\cdot-}$  in a hypoxanthine - xanthine oxidase system.<sup>11,16</sup> The assessment of the antioxidant properties of flavonoids may be undertaken *in vitro* in non-cellular or cellular systems. When studies are conducted in cellular systems it allows the prediction of the flavonoids activity in an environment that resembles more the physiological conditions, namely their effect in the modulation of the oxidative burst phenomenon. Trihydroxyflavones proved to be good scavengers of ROS *in vitro*, and these results were confirmed by *in vitro* cellular assays.<sup>53</sup> Also in human neutrophils, Freitas et al. studied four chlorinated flavonoids and their non-chlorinated parent flavonoids and concluded the chlorinated ones were more efficient, the more active being 8-chloro-3',4',5,7-tetrahydroxyflavone. These results are of major interest because it has been already described that flavonoids react with HOCl, becoming chlorinated, and these products that will act in the inflammatory site may present different properties from the initial flavonoids, as it was proven.<sup>66</sup>

It is generally accepted that flavonoids are capable of chelating metals. The structural features for this property have already been established. The 3'-OH and the 4'-OH groups in B ring, known as catechol group, are very active in chelating metals. The 3-OH-4-carbonyl and the 5-OH-4-carbonyl groups also contribute to metal chelation.<sup>67</sup> Quercetin, which owns the described features, was found to chelate Fe and hence prevent lipid peroxidation.<sup>11,16</sup> Moreover, a molecular modelling indicated that the preferred site of chelation is located on the 3-OH-4-carbonyl group, followed by the 5-OH-4-carbonyl and the 3', 4'-OH groups. In this sense, Fe can produce complexes with one, two or three molecules of quercetin.<sup>68</sup> Hydroxylated flavones, C-glycosyl flavones, methoxyflavones and flavonols show their inhibitory properties of lipid peroxidation induced by  $FeSO_4$  plus cysteine, in rat liver microsomes. This effect was also observed for hydroxyethyl rutosides and 7,3',4'-trihydroxyethyl quercetin.<sup>14</sup> Assini and co-workers revised the biological properties of citrus flavonoids in what refers to lipid metabolism.<sup>69</sup>

#### **4.4.2. Effects of Flavonoids on Reactive Nitrogen Species**

Flavonoids already proved to inhibit LPS - induced iNOS gene expression and iNOS activity in cultured macrophages, reducing  $\cdot NO$  production.<sup>16,29</sup> Also, quercetin, luteolin

and apigenin inhibited  $\cdot\text{NO}$  production in LPS-induced macrophages and macrophages cell lines. Catechins and flavanones were active, but less potent. Further evidence proved that apigenin, as genistein and kaempferol, effectively lowered the  $\cdot\text{NO}$  production, by the iNOS down-regulation.<sup>50</sup> Isoflavones (genistein, daidzein and glycitein) suppressed  $\cdot\text{NO}$  production in LPS-activated murine macrophages by three different mechanisms: scavenging of  $\cdot\text{NO}$ , inhibition of iNOS enzyme activity and inhibition of iNOS gene expression. Flavones, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin were already described as being able to inhibit LPS-induced iNOS expression. Prenilated flavonoids and bioflavonoids inhibited the production of  $\cdot\text{NO}$  in LPS - stimulated RAW 264.7 cell line, by the suppression of iNOS induction, but not by the inhibition of its activity. Echinoisoflavone was the only one that simultaneously suppressed iNOS enzyme induction and its activity.<sup>16</sup> Jaceosidin, a naturally occurring flavone, reduced LPS-induced  $\cdot\text{NO}$  production and iNOS expression in BV-2 mouse microglial cells, in a concentration-dependent manner, and, similarly, in microglia, astroglia and macrophages.<sup>70</sup> Silibinin inhibits  $\cdot\text{NO}$  activity, in a dose dependent manner.<sup>11</sup>

A study employing 16 flavonoids and flavonoids glycosides confirmed that the total number of OH groups, the number of phenolic groups, the presence of vicinal OH groups and their position in the B ring are determinant for the flavonoid protection against  $\text{ONOO}^-$  reactions.<sup>61</sup> Genistein and daidzein demonstrated to scavenge the highly reactive  $\text{ONOO}^-$ . In an in vitro assay these soybean isoflavones increased the LDL resistance to  $\text{ONOO}^-$  - mediated oxidation, in a concentration – dependent manner.<sup>16</sup>

#### **4.5. Cytokines**

Cytokines are soluble, low-molecular-weight proteins that play an important role in the inflammatory response, in its regulation and propagation. Generally, cytokines can be produced and secreted by leukocyte, endothelial cells, epithelial cells, and fibroblasts. The production and secretion of cytokines are transcriptionally regulated. Cytokines exert a variety of functions in a variety of cells. They are characterized by their redundancy and pleiotropy, various cytokines can target the same receptor and a single cytokine can have multiple, even contradictory, effects.<sup>9</sup> Their major role is the regulation of the intensity and duration of the inflammatory response.<sup>1,35</sup>

Cytokine expression may be triggered by different factors: trauma, stress, ischemia, radiation, ultraviolet light, extracellular matrix components, microbial species and their soluble products (particularly via TLR and nucleotide oligomerization domain [NOD]-like receptors [NLRs]), DNA (mammalian or microbial), heat shock proteins, cell-cell contact, immune complexes/autoantibodies, local complement activation, ROS, RNS, electrolytes

(e.g.  $K^+$  via P2X7 receptors) and cytokines themselves working in autocrine loops.<sup>71</sup> In fact there are well documented examples of this latter regulation: IL-10, along with IL-4 and IL-13, is considered an anti-inflammatory cytokine; they down-regulate pro-inflammatory signals.<sup>1</sup> IL-4 and IL-10 inhibit IL-12 - induced IFN- $\gamma$  secretion. Moreover, IL-10 antagonizes many of the pro-inflammatory effects of TNF- $\alpha$  and IL-1 $\beta$  (table 3).<sup>71</sup> However, the production of IL-4 and IL-10 by monocytes can be down-regulated by IFN- $\gamma$ , another cytokine.<sup>35</sup> On the other hand, IL-6, IL-1 and TNF- $\alpha$  all considered pluripotent cytokines inter-regulate their production; in fact, the IL-6 production may be triggered by TNF- $\alpha$  and IL-1 $\beta$ ; these last two cytokines work synergistically.<sup>72</sup> The IL-11 secretion is enhanced by IL-1 and TGF- $\beta$ .<sup>35</sup>

Cytokines are synthesized in Golgi apparatus and then circulate through the endoplasmatic reticulum; they may remain bound to the membrane or released as soluble mediators or even be processed and migrate intracellularly, returning to the nucleus and act as transcriptional regulators. Cytokines can mediate autocrine or paracrine functions; autocrine, through release or membrane expression and immediate receptor binding on the source cell or intracellularly within the source cell; paracrine, allowing cellular communication beyond that assisted by local cell-cell contact.<sup>71</sup>

The cells' surface displays cytokine receptors to answer cytokine specific messages. So, through these receptors, cells process the information of substrate-bound cytokines, according to the density and state of activation of the surface receptors.<sup>30</sup> Cytokine receptors comprise structurally related superfamilies and high-affinity molecular signalling complexes that mediate the cytokine's communication. In the group of cytokine receptors we may find: heterodimeric or homodimeric receptors - a type I/II cytokine receptor; trimeric proteins usually associated with cell surface - a TNF receptor; receptor serine / threonine kinase - a TGF- $\beta$  receptor; single-pass membrane receptors with Toll-IL-1 receptor domains - a toll/IL-1 receptor; GPCRs family - a chemokine receptor and enzyme-linked receptors that phosphorylate tyrosine residues - a tyrosine kinase receptor.<sup>71</sup>

After receptor coupling, there are several intracellular signal pathways through which cytokines mediate their effects. One of them is the Janus kinases (JAK) and their downstream transcriptional factors [e.g. signal transducers and activators of transcription (STATs)], mediating signals serving those cytokines that bind to common gamma chain, or to glycoprotein 130 (gp130) receptor. Similarly, the MAPK signalling cascade mediates signals in response to stress, cytokines, and proliferation factors. Another one is the spleen tyrosine kinase (Syk) that mediates the effector function of IgFc receptor and the B cell receptor and cooperates with other downstream cytokine-mediated signal pathways. PI3K signalling cascade is another signal pathway, which is involved in the formation and

migration of second messengers. Finally, the NF- $\kappa$ B pathway is central to the pathways that mediate signals of TLRs, IL-1 superfamily members and to the function of TNF receptor. Once initiated the signalling cascade, several transcription factors can be recruited [e.g. NF- $\kappa$ B, AP-1, nuclear factor of activated T-cells (NFAT)] to the cytokine promoter region (figure 6).<sup>71</sup>

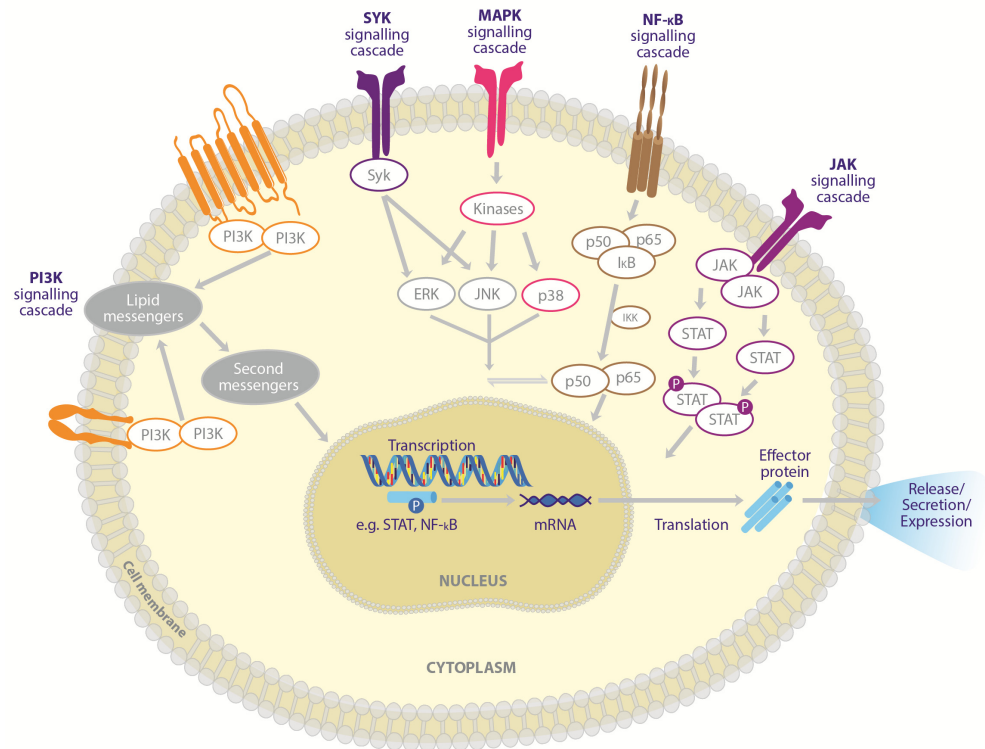


**Figure 6** - Cytokine signalling pathways and regulation. Adapted from 71

There are a myriad of cytokines of diverse cellular sources that play decisive biological functions in inflammation. The most common and reported ones are summarized table 2.

**Table 3** - Principal cellular sources and actions in inflammation of selected cytokines.

| Cytokine                      | Principal sources                                                                                                                             | Actions in inflammation                                                                                                                                                                                                                                                                                                  | References |
|-------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>IL-1<math>\beta</math></b> | Macrophages (main source); monocytes; neutrophils; B lymphocytes; T lymphocytes; NK cells; keratinocytes; dendritic cells; endothelial cells. | Induce transcriptional activation of various genes, encoding cytokines, chemokines, adhesion molecules, iNOS, proteases and COX-2; recruit inflammatory cells at the site of inflammation, by inducing the expression of adhesion molecules on endothelial cells and through the release of chemokines by stromal cells. | 1,3,8,35   |
| <b>IL-2</b>                   | Th1 cells.                                                                                                                                    | Promote proliferation of activated B lymphocytes and T lymphocytes; stimulate secretion of cytokines by T cells; increase cytotoxicity of NK cells (in combination with other stimuli).                                                                                                                                  | 35         |

**Table 3** - Principal cellular sources and actions in inflammation of selected cytokines.(cont.)

| Cytokine     | Principal sources                                                                          | Actions in inflammation                                                                                                                                                                                                                                                                                                                                                                                                                   | References  |
|--------------|--------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| <b>IL-3</b>  | T lymphocytes; NK cells.                                                                   | Stimulate pluripotent bone marrow stem cells to increase production of leukocytes, erythrocytes and platelets.                                                                                                                                                                                                                                                                                                                            | 35          |
| <b>IL-4</b>  | Th2 cells.                                                                                 | Induce the differentiation of CD4+ T cells into Th2 cells; down-regulate the differentiation of CD4+ T cells into Th1 cells; promote the growth and differentiation of B cells; inhibit the production of TNF, IL-1, IL-8 and PGE <sub>2</sub> by stimulated monocytes or macrophages; down-regulate endothelial cell activation induced by TNF; promote expression of the class II major histocompatibility complex (MHC), on B cells.   | 35          |
| <b>IL-5</b>  | T lymphocytes; mast cells; monocytes; macrophages.                                         | Induce production of eosinophils from myeloid precursor cells.                                                                                                                                                                                                                                                                                                                                                                            | 35          |
| <b>IL-6</b>  | Monocytes; macrophages, lymphocytes; endothelial cells; intestinal epithelial cells.       | Stimulate the synthesis of CRP; induce pyrexia; increase the hepatic synthesis of acute phase reactants, such as ferritin, coagulation factors (e.g. fibrinogen) and CRP; cause the differentiation of T cells into Th17 cells; activate the NF- $\kappa$ B and MAPK pathway, specifically, the ERK,                                                                                                                                      | 1,3,8,72,73 |
| <b>IL-8</b>  | Monocytes; macrophages, endothelial cells, enterocytes.                                    | Stimulate chemotaxis oxidative burst by neutrophils.                                                                                                                                                                                                                                                                                                                                                                                      | 35          |
| <b>IL-9</b>  | Th2 cells.                                                                                 | Promote proliferation of activated T cells and Igs secretion by B cells.                                                                                                                                                                                                                                                                                                                                                                  | 35          |
| <b>IL-10</b> | Monocytes; B lymphocytes; T lymphocytes; dendritic cells; epithelial cells; keratinocytes. | Inhibit the production of IL-1, TNF, IL-6, IL-8, IL-12, and GM-CSF, by monocytes and macrophages; inhibit lymphocytic and phagocytic function; increases synthesis IL-1RA, by activated monocytes; down-regulate the proliferation and secretion of IFN- $\gamma$ and IL-2 by activated Th1 cells; potently activate B cell activation and immunoglobulin secretion; reduce adhesion molecule expression, MHC expression and MMP release. | 1,71        |
| <b>IL-11</b> | Dendritic cells; fibroblasts; epithelial cells.                                            | Increase production of platelets; inhibit proliferation of fibroblast.                                                                                                                                                                                                                                                                                                                                                                    | 35          |
| <b>IL-12</b> | Antigen-presenting cells.                                                                  | Promote Th1 responses by helper T lymphocytes; enhance IFN- $\gamma$ secretion by Th1 cells.                                                                                                                                                                                                                                                                                                                                              | 35          |
| <b>IL-13</b> | Th2 cells; undifferentiated CD4+ T cells and CD8+ T cells.                                 | Promote the growth and differentiation of B cells; down-regulate the production of IL-1, TNF, IL-6, IL-8, IL-12, G-CSF, GM-CSF, MIP-1 $\alpha$ and PGE <sub>2</sub> , by activated monocytes and macrophages; increase the production of anti-inflammatory proteins (e.g. IL-1RA and IL-1RII); inhibition of COX-2' induction; induces 15-LOX.                                                                                            | 35          |
| <b>IL-17</b> | Th17 cells; neutrophils.                                                                   | Amplify the neutrophil migration and recruitment; stimulate the production of pro-inflammatory cytokines; induce IL-6 and IL-8 production, from fibroblasts.                                                                                                                                                                                                                                                                              | 19,35       |

**Table 3** - Principal cellular sources and actions in inflammation of selected cytokines.(cont.)

| Cytokine                       | Principal sources                                                                                        | Actions in inflammation                                                                                                                                                                                                                                                                                                                                                                                                                                 | References |
|--------------------------------|----------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>IL-18</b>                   | Mononuclear cells.                                                                                       | Co-stimulation with IL-12 of the production of IFN- $\gamma$ by T lymphocytes and NK cells; induce the production of CC and CXC from human mononuclear cells; activates neutrophils                                                                                                                                                                                                                                                                     | 35         |
| <b>IL-21</b>                   | Th2 and Th17 cells.                                                                                      | Modulate B cell survival; inhibit IgE synthesis and the production of pro-inflammatory cytokine, monocytes and macrophages.                                                                                                                                                                                                                                                                                                                             | 35         |
| <b>IL-23</b>                   | Dendritic cells;<br>monocytes;<br>macrophages.                                                           | Promote differentiation of naïve T cells into Th17 cells, along with TGF- $\beta$ .                                                                                                                                                                                                                                                                                                                                                                     | 35         |
| <b>IL-27</b>                   | Dendritic cells;<br>monocytes;<br>macrophages.                                                           | Suppresses effector functions of lymphocytes, monocytes and macrophages.                                                                                                                                                                                                                                                                                                                                                                                | 35         |
| <b>GM-CSF</b>                  | T lymphocytes;<br>monocytes;<br>macrophages;<br>endothelial cells.                                       | Enhance the production of granulocytes and monocytes by bone marrow and of pro-inflammatory mediators, by monocytes and macrophages.                                                                                                                                                                                                                                                                                                                    | 35         |
| <b>G-CSF</b>                   | Monocytes;<br>macrophages;<br>fibroblasts.                                                               | Enhance the production of granulocytes by bone marrow.                                                                                                                                                                                                                                                                                                                                                                                                  | 35         |
| <b>IFN-<math>\gamma</math></b> | CD4+ Th1 cells;<br>CD8+ Th1 cells; NK cells.                                                             | Promote the differentiation of CD4+ T cells into the Th1 phenotype, along with IL-12; inhibits differentiation of CD4+ T cells into Th2 cells; stimulates the release of pro-inflammatory mediators (e.g. TNF, O <sub>2</sub> <sup>-</sup> , NO).                                                                                                                                                                                                       | 35         |
| <b>MIF</b>                     | Monocytes;<br>macrophages.                                                                               | Up-regulate the expression of TLR4 on macrophages, amplifying the response of the innate immune system to LPS.                                                                                                                                                                                                                                                                                                                                          | 35         |
| <b>TGF-1<math>\beta</math></b> | T lymphocytes;<br>monocytes,<br>macrophages;<br>platelets.                                               | Promote the differentiation of naïve T helper cells into Treg cells; in conjugation with IL-6 or IL-23 promotes the differentiation of naïve helper T cells into Th17cells; stimulates chemotaxis by monocytes and induces synthesis of extracellular proteins by fibroblasts; inhibits Igs secretion by B lymphocytes; down-regulates the activation of NK cells.                                                                                      | 35         |
| <b>TNF-<math>\alpha</math></b> | Macrophages (main source); monocytes;<br>B lymphocytes; T lymphocytes dendritic cells; endothelial cell. | Induce expression of cytokines, chemokines, growth factors and cell surface receptors that recruit hematopoietic cells, neutrophils, T lymphocytes, monocytes and macrophages;<br>Induce the expression of angiogenic cytokines (e.g. VEGF and TGF-b) and plasminogen activator and its receptor;<br>In later stage of inflammation: up-regulate the expression of MMP-9; suppresses the expression of the MMP-9 antagonist, tissue inhibitor of MMP-3. | 1,3,35     |

#### **4.5.1. Modulation of Cytokines Production by Flavonoids**

Various flavonoids were already described as good modulators of cytokines' production. As it was shown for eicosanoids production, the different inflammatory mediators and their functions are all connected; and the same happens for cytokines. The molecular mechanisms involved in their modulation also include the inhibition of transcription factors, NF- $\kappa$ B and AP-1, and reduction of MAPK activity.<sup>50</sup>

The structural features defined until now for a good inhibition of LPS-stimulated TNF- $\alpha$  release are: a double bond at position C2=C3 in C ring with an -oxo function at position C4 of C ring, along with the presence of OH groups at positions 3' and 4' in B ring.<sup>16</sup> Genistein, quercetin, luteolin, apigenin and rutin are capable of inhibiting pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1.<sup>29</sup> Quercetin also affected TNF- $\alpha$  expression in LPS-induced RAW cells by inhibiting MAPK and AP-1 DNA binding.<sup>50</sup> However, in another study, quercetin and luteolin showed a highest degree of inhibition of TNF- $\alpha$  than genistein, kaempferol, apigenin, diosmetin and hesperetin, which have only one hydroxyl group on the B ring, in LPS-stimulated macrophages.<sup>74</sup> In this same study they observed for the first time that luteolin and quercetin stimulate LPS-induced IL-10 secretion. This may be due to post-transcriptional effects of the flavonoids, since it is well established that IL-10 can be regulated by post-transcriptional mechanisms in macrophages. Based on this evidence, they also propose that NF- $\kappa$ B is not the only signalling pathway affected by flavonoids; they may affect p38-MAPK, which is involved in the regulation of IL-10 expression.<sup>74</sup> A metabolite of rutin, 3,4-dihydroxytoluene, also reduced the TNF- $\alpha$  and in a minor extent IL-1 $\beta$  and IL-6, probably through the inhibition of NF- $\kappa$ B signalling in LPS-activated murine macrophage cell line RAW 264.7.<sup>75</sup> This indicates the study of flavonoids' metabolites is also of preponderant interest. Jaceosidin reduced the levels of LPS-induced IL-1 $\beta$ , TNF- $\alpha$  in BV-2 microglial cells.<sup>70</sup> Genistein was found to prevent IL-6, IL-1 $\beta$  and TNF- $\alpha$  formation in LPS induced human monocytes, as in macrophages, gastric epithelial cells and osteoblasts. Reports on LPS-induced macrophage RAW 264.7 cell line revealed that genistein, quercetin, luteolin and luteolin-7-glucoside are effective inhibitors of IL-6 and TNF- $\alpha$  production. In the same cell line, wogonin led to the decrease of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 release. Eriodictyol and hesperetin only inhibited TNF- $\alpha$  release.<sup>11,16</sup> All these works use different cell lineages and they show the same flavonoids having the same positive results in different inflammation related cells, making them even more interesting molecules.

It is also believed that hydroxylation at positions C3, C5 and C7 are compulsory to observe the maximum inhibition of IL-4 production. Scutellarein, 3-hydroxyflavone, kaempferol, quercetin, eriodictyol, fustin and 7-hydroxyflavone share these structural features and demonstrated to be good inhibitors of IL-4 production. However, the detailed

mechanisms by which flavonoids act in the modulation of cytokine synthesis remains unclear.<sup>29</sup> Those are controversial results, because, as it was explained, IL-4, along with IL-10 and IL-13, is considered an anti-inflammatory cytokine. In this sense, the inhibition of its production might not be the best expected result. Luteolin, apigenin and fisetin demonstrated to inhibit the synthesis of IL-14 and IL-13 in an *in vitro* study.<sup>70</sup>

#### **4.6. Chemokines**

Chemokines are a group of small (8 to 12 KD) glycoproteins that, although structurally related to cytokines, are distinct from them as a result of their ability to bind and signal GPCRs. The main biological function of chemokines is the chemoattraction and activation of leukocytes in inflammation; they also control the normal migration of cells through various tissues. They are able to induce chemotaxis in a variety of other cells including eosinophils, fibroblasts and keratinocytes, being both chemotactic and cellular activating factors.<sup>1</sup> Generally, the cellular sources of chemokines are leukocytes and endothelial cells.<sup>8</sup> Numerous pro-inflammatory stimuli can lead to the production of chemokines, including cytokines, such as TNF and IL-1, and PAMPs, such as LPS.<sup>35</sup> The produced chemokines can be stored in granules and are released upon stimulation. They are positively charged, so they can bind to the sulfated proteoglycans present on cell surfaces or they can stay in the extracellular matrix, remaining locally concentrated.<sup>76</sup>

There are two types of chemokines: 1. CC (or  $\beta$ ) chemokines, which include MCP-1, MIP-1 $\alpha$ , RANTES and eotaxin. They act primarily on monocytes, lymphocytes, basophils and eosinophils (eotaxin selectively recruits eosinophils), but not in neutrophils; 2. CXC (or  $\alpha$ ) chemokines, which include IL-8, GRO- $\alpha$ , and epithelial derived neutrophil-activating peptide (ENA-78). They differ from CC chemokines because of the presence of one amino acid between two amino-terminal cysteine (C) residues. CXC chemokines act primarily on neutrophils.<sup>1,5</sup> It is also described the existence of two more families of chemokines: the C (or  $\gamma$ ) chemokines, composed by lymphotactin, which are relatively specific for lymphocytes and the CX3C (or  $\delta$ ) chemokines, with only one member called fractalkine. It exists as a surface-bound protein and as a soluble form, derived by proteolysis of the membrane-bound protein, which has a great chemoattractant activity for monocytes and T cells.<sup>1,3,8,35</sup>

IL-8 is one of the most studied chemokines. It is secreted by activated macrophages, endothelial cells, and other cell types. The IL8's inducers are microbial products and other cytokines, mainly IL-1 and TNF. IL-8 causes chemotaxis and activation of neutrophils. IL-8 leads to neutrophils' degranulation, increased expression of surface adhesion molecules and production of ROS; however, it has limited activity on monocytes and eosinophils.<sup>35</sup>

The chemokines effects are mediated by receptors, in which some receptors are highly specific, whereas others bind multiple chemokines of both CC and CXC families.<sup>1</sup> These receptors are GPCRs and comprise CXC chemokine receptors (CXCR) and CC chemokine receptors (CCR). IL-8, for example, has two receptors, CXCR1 (IL-8R1), predominantly expressed in neutrophils, and CXCR2 (IL-8R2).<sup>35</sup> During the inflammatory process, different leukocytes generally express more than one receptor type, so the type of inflammation will depend on the types of chemokines that are produced and the types of responding leukocytes. Chemokines are essential to determine which of these events will occur: leukocyte extravasation out of the bloodstream and into the tissue, and leukocyte navigation within tissue to the correct anatomic location, however, the exact mechanism of this directional sensing in mammalian cells is not clear.<sup>76</sup>

#### **4.6.1 Modulation of Chemokines Production by Flavonoids**

Noteworthy, chemokines and cell adhesion molecules (CAMs) may not be the more directly studied inflammatory mediators, because their synthesis are intrinsically dependent on cytokines, for example; and once these last are modulated, those other molecules are consequently modulated too. Aromadendrine (dihydrokaempferol) presented anti-inflammatory activity in the keratinocyte cell line NCTC 2544, through the decrease of ICAM-1 expression of membrane and of the IL-8 release induced by IFN- $\gamma$  and histamine.<sup>77</sup> 5,7-Dihydroxy-3,4,6-trimethoxyflavone down-regulated expression of adhesion molecules such as ICAM-1 and VCAM-1 in TNF- $\alpha$  – stimulated human bronchial epithelial cell line BEAS-2B. These effects were a consequence of the inhibition of both phosphorylation of Akt and IKK as well as the activation of NF- $\kappa$ B. Taken together, these led to the weakened adhesion of inflammatory cells such as monocytes and eosinophils to the bronchial epithelial cells.<sup>78</sup> The involvement of the CAMs in inflammation will be discussed further ahead in this review; as well as the effects of flavonoids in chemokines and in CAMs will be further discussed in the *in vivo* section.

#### **4.7. Growth factors**

Growth factors are produced during the inflammation process, but, in general, they exert their effects in the late stages of inflammation, participating in the regulation of cell growth, tissue repair and angiogenesis.<sup>1</sup> The growth factors and respective functions, are: EGF that stimulates keratinocyte migration and granulation tissue formation; fibroblast growth factor (FGF) is chemotactic for fibroblasts, stimulates keratinocyte migration and angiogenesis; insulin-like growth factor 1 (IGF-1) is pro-fibrotic; keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) are pro-inflammatory and fibrotic; platelet

derived growth factor (PDGF) is chemotactic for neutrophils, macrophages, fibroblasts, endothelial cells (angiogenesis) and smooth muscle cells (angiogenesis); TGF- $\beta$  is a mediator of tissue repair and fibrosis, regulates cell growth, death, and apoptosis, cell differentiation and extracellular matrix synthesis, induces vasculogenesis and enhances mesenchymal cell proliferation and VEGF regulates vascular permeability, induces vascular endothelial cell proliferation, upregulates tissue and urokinase plasminogen activator expression. They globally contribute to cellular proliferation and amplification of the inflammatory response.<sup>1,5,8</sup>

#### **4.8. Cell adhesion molecules**

Adhesion molecules are divided into five groups, based on structural homology: cadherins, mucins, integrins, selectins, and immunoglobulin superfamily molecules.

Cadherins are expressed most often on epithelial cells and interact with each other in a  $\text{Ca}^{2+}$ -dependent manner. Mucins are glycosylated proteins that interact mainly with selectins. The integrins family is subdivided in four subfamilies. Overall, they are responsible for a critical physical link between external cell medium components (other cells and the extracellular matrix) and those components within the cell (cytoskeleton, cytosolic molecules and nucleus).<sup>30</sup>

The selectin family is constituted by three homologous proteins: L-selectin, E-selectin and P-selectin. The selectins roles are to mediate cell - cell interaction and to contribute to cell activation by intracytoplasmic signalling L-selectin. During inflammation, L-selectin is responsible for the binding of neutrophils to activated endothelial cells. Its ligands include glycan-bearing cell adhesion molecule-1 (GlyCAM-1), mucosal addressin cell adhesion molecule-1 (MadCAM-1) and CD34.<sup>30</sup> Upon stimulation, L-selectin is constitutively expressed in neutrophils, eosinophils, monocytes, and naïve T and B lymphocytes, as well as on the surface of some activated T cells and memory T cells. E-selectin is not expressed in the surface of non-stimulated endothelium; it is produced after the stimulation with IL-1, TNF- $\alpha$  and bacterial endotoxin. E-selectin functions as a ligand of various myeloid cells and of T lymphocytes. P-selectin is expressed by activated endothelium, but also by platelets. When the endothelial cells are stimulated with agents such as complement C5a, histamine, thrombin, and LTC<sub>4</sub>, the P-selectin preformed stored in intracellular granules is rapidly translocated to the cell surface.<sup>79</sup> The immunoglobulin gene superfamily (IgSF) of CAMs is composed by: ICAM-1, CD54, which is expressed constitutively in low amounts along the luminal, intercellular, and subluminal surface of endothelial cells, in leukocytes, epithelial cells and fibroblasts; its levels greatly increase after stimulation by IL-1, TNF- $\alpha$ , IFN- $\gamma$  or bacterial endotoxin; ICAM-2, CD102, which is

also constitutively expressed on endothelial, mononuclear cells, basophils, mast cells and platelets; its expression appears to be unaffected by cytokines; ICAM-3, CD50, which is constitutively expressed on all leukocytes and on mast cells; its cross-linking results in  $\text{Ca}^{2+}$  mobilization, tyrosine phosphorylation, enhanced adhesion, chemokine secretion and modulation of basophil mediators release; vascular cell adhesion molecule 1 (VCAM-1, CD106), which has been identified on cell types other than endothelium, namely macrophages, dendritic cells, astrocytes, bone marrow stromal cells and respiratory epithelial cell lines; IL-1, TNF- $\alpha$  and bacterial endotoxin induce increased expression of VCAM-1; finally, platelet-endothelial cell adhesion molecule 1 (PECAM-1, CD31), which is expressed on endothelial cells and circulating leukocytes, mediating neutrophil and monocyte transendothelial migration.<sup>79</sup>

#### **4.9. Transcription Factors**

##### **4.9.1. NF- $\kappa$ B**

As it has been becoming clear along this review, NF- $\kappa$ B is involved in almost all of the inflammatory *phenomena*. NF- $\kappa$ B is a crucial transcription factor in the orchestration of the inflammatory response, regulating the transcription of more than 150 genes. NF- $\kappa$ B exists in the form of homo- or heterodimer, the most common is the association of the portions p50 and p65. In resting cells these dimers exist in the cytosol, in an inactive form, because they are bonded to I $\kappa$ B, an inhibitory protein. Five I $\kappa$ B - like proteins have already been identified: I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\epsilon$  and Bcl-3. So, under the stimuli of pro-inflammatory trigger (e.g. TNF, IL-1, or LPS) the I $\kappa$ B is phosphorylated by an enzyme complex called I $\kappa$ B kinase (IKK). IKK contains two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and two copies of a regulatory scaffold protein called NF- $\kappa$ B essential modulator (NEMO or IKK $\gamma$ ).<sup>35</sup> This phosphorylation targets I $\kappa$ B for ubiquitination and subsequent proteosomal degradation. The degradation and removal of I $\kappa$ B allows the translocation of NF- $\kappa$ B active form to the nucleus where it binds to regulatory elements in the promoter regions of various NF- $\kappa$ B-responsive genes. Noteworthy, this system exhibits a self-regulatory behavior, as the activation and migration of NF- $\kappa$ B to the nucleus are associated with the subsequent induction of I $\kappa$ B isoforms. NF- $\kappa$ B has been reported as one of the most remarkable pro-inflammatory gene expression regulators and mediates several cytokines synthesis, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8, as well as COX-2.<sup>38</sup>

##### **4.9.2. cAMP response element-binding protein**

Various receptor systems promote the activation of cAMP response element-binding protein (CREB). CREB is known for its role in cell proliferation, differentiation and survival.

Nevertheless, it simultaneously has demonstrated specific functions in immune responses, including NF- $\kappa$ B inhibition, inducing macrophage survival and promoting the proliferation, survival and regulation of T and B lymphocytes. This signalling pathway is activated in response to various cytokines IL-2, IL-6, IL-10, and TNF- $\alpha$ .<sup>80</sup> The ERK1/2 signalling pathway, also known as the p42/p44 MAPK pathway, is usually associated with pro-survival signalling through mechanisms that may involve activation of the cAMP response CREB.

Upon stimulation, CREB is phosphorylated by serine/ threonine kinases on serine 133 and this activates various signalling cascades, including PKA and PKC. Consequently, CREB seek out the co-factor CREB-binding protein (CBP) and/or p300. This will allow CREB to interact with DNA at cAMP-responsive elements (CREs), leading to the transcription of CREB-responsive genes (e.g., IL-2, COX-2). NF- $\kappa$ B has the ability to bind to CBP and when CREB and NF- $\kappa$ B are both present, CREB act as an NF- $\kappa$ B inhibitor by competing for a restricted pool of CBP.<sup>38</sup>

Noteworthy, ERK belongs to the MAPK family, a distinct signalling cascade in the cell that responds to a variety of extracellular stimuli. MAPKs are implicated in the gene expression associated with the regulation of inflammation, cell survival, proliferation, iNOS and cytokine expression, and collagenase production. p38 is another element of MAPK family; it is associated with cellular responses, including inflammation, cell cycle, development, cell differentiation, senescence, tumorigenesis, and cell death (apoptosis). Finally, JNK is also part of MAPKs and regulates many cellular events, such as growth control, transformation, and programmed cell death (apoptosis). MAPKs also regulate the downstream phosphorylation of the transcription factors, NF- $\kappa$ B and AP-1.<sup>81</sup>

#### **4.9.3. Janus kinase signal transducer and activator of transcription**

The Janus kinase signal transducer and activator of transcription (JAK/STAT) are activated in response to various cytokines, chemoattractants and growth factors. The activity of this transcription factor is mediated via cytokine / hematopoietic receptors that activate specific members of the Janus cytoplasmic tyrosine kinases (JAKs 1 to 3, and TYK2). JAK is consequently dimerized and phosphorylated, creating STATs binding sites. The substrates of the active JAKs are the receptor subunits and members of the STAT family of transcription factors (STATs 1 to 4, 5a/b and 6), which are phosphorylated by JAKs, undergo homo- or heterodimerization and translocate into the nucleus, where they bind to consensus STAT recognition sequences.<sup>38</sup>

#### **4.9.4. Mothers Against Decapentaplegic**

The mothers against decapentaplegic (SMADs) family of transcription factors is the primary signalling cascade initiated by the binding of TGF- $\beta$  superfamily to their type II membrane receptor serine-threonine kinases and transphosphorylate the Gs domains of type I receptors. The receptor - associated SMADs (R-SMADs) are recruited and phosphorylated and they dissociate from the receptors. R-SMADs forms a complex with co-SMAD (e.g. SMAD4) translocate into the nucleus, and bind to SMAD-binding elements (SBEs) in the promoters of target genes, initiating transcription. The R-SMADs are eventually dephosphorylated; they dissociate and the inactive SMADs are exported to the cytoplasm.<sup>38</sup>

#### **4.10. Modulation of Transcription Factors – Gene Expression – by Flavonoids**

Transcription factors have become important subjects of study as they are involved in all the signalling cascades of inflammation, modulating the expression and release of almost all inflammatory mediators. Reversely, their expression is also modulated by these same mediators, creating a cycle, which is essential to interrupt to achieve a successful anti-inflammatory activity.

Flavonoids have been found to modulate PKCs and MAPKs, preventing the transcription of factors as AP-1 or NF- $\kappa$ B.<sup>11,82</sup> The inhibition of kinases is due to the competitive binding of flavonoids with ATP at catalytic sites on these enzymes that are involved in signal transduction and cell activation processes involving cells of the immune system.<sup>12</sup> p38-MAPK positively regulates a number of cytokine genes in vitro including TNF- $\alpha$ , IL-6 and iNOS.<sup>16</sup> Luteolin and quercetin already demonstrate their inhibition of LPS-induced promotion of p38 and ERK 1/2 pathways preventing the release and production of TNF- $\alpha$ .<sup>11</sup> Naringenin's activity in the modulation of TLR2, TLR4, and CD86 receptors and downstream via the p38-MAPK pathway was confirmed in *C. trachomatis* infected J774 macrophages.<sup>83</sup> Rutin decreased PMA-stimulated phosphorylation of p38, ERK1/2, and c-Jun.<sup>84</sup> Flavone jaceosidin suppressed LPS-induced phosphorylation of p38-MAPK and ERK in primary microglial cells. These effects, along with the inhibition of COX-2 expression, make this flavonoid a promising molecule for the resolution of neuro-inflammation.<sup>70</sup>

Luteolin also inhibited the gene expression and the cytokines production by blocking protein tyrosine phosphorylation and NF- $\kappa$ B activation in murine macrophages RAW 264.7; this activity was also described in LPS-induced epithelial and dendritic cells through the inhibition of IKKs and in mouse alveolar macrophages by blocking the NF- $\kappa$ B

and AP-1 activation pathways.<sup>16</sup> However, in another study, luteolin suppressed NF- $\kappa$ B activation and TNF- $\alpha$  production in co-cultured intestinal epithelial Caco-2 and macrophage RAW 264.7 cells.<sup>85</sup> These studies, with co-cultured cell lines, emphasize the importance of luteolin and its promising use in inflammation treatment as it is active in more than one type of cells possibly involved in this process. More recently, also in RAW 264.7 cell line, luteolin inhibited TNF- $\alpha$  production, but did not suppress LPS-induced NF- $\kappa$ B and MAPKs signalling. On the other hand, it markedly attenuated LPS-activated phosphorylation of the JNK downstream c-Jun and Jun-2 luciferase reporter activity.<sup>86</sup> Likewise, quercetin proved to modulate the transcription pathways via suppression of TLR4 signalling, through the up-regulation of Toll-interacting protein expression, once more in RAW 264.7 macrophages.<sup>87</sup> Moreover, in the same LPS-induced cell line, quercetin arrested Src and Syk-mediated PI3K and Akt activations, and it disrupted LPS-induced p85 association to TLR4/MyD88 complex and limited interleukin-1 receptor-associated kinase 1 (IRAK1), TNF receptor associated factor 6 (TRAF6) and TGF- $\beta$  activated kinase-1 (TAK1) with a subsequent suppression in IKK $\alpha\beta$ -mediated I $\kappa$ B phosphorylation. In addition, it attenuated TAK1-mediated JNK1/2 and p38-MAPK phosphorylations but stimulated HO-1 induction, leading to NF- $\kappa$ B and AP-1 expression inhibition.<sup>88</sup> Overall, quercetin and luteolin proved to affect various transcription signalling pathways, in their different levels, in different cell lines, and generally the results obtained are accordant.

Genistein reduced the LPS-induced activation of NF- $\kappa$ B in monocytes, illustrating a potential flavonoid-gene interaction.<sup>16</sup> Genistein also attenuated the inflammatory response via inhibition of NF- $\kappa$ B activation following adenosine monophosphate-activated protein kinase (AMPK) stimulation, which is connected to metabolic syndrome-related diseases, in LPS-stimulated RAW 264.7 macrophages.<sup>89</sup> Galangin, another flavonol, decreased the degradation of I $\kappa$ B $\alpha$  and nuclear translocation of p65 NF- $\kappa$ B, regulating the decrease in the elevated gene expression of TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-8 in mast cells, and consequently in allergic inflammation.<sup>90</sup> In a study with  $\beta$ -amyloid peptide 25-35-stimulated BV-2 cell line, used as a microglial model, genistein was able to inhibit the TLR4 and NF- $\kappa$ B transcription and NF- $\kappa$ B activity. This means that genistein may be used not only to attenuate the common inflammatory processes, but also neuro-inflammation, a process involved in neurodegenerative diseases.<sup>91,92</sup>

Flavones, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin were already described as inhibitors of LPS-activation of NF- $\kappa$ B. However, only genistein, kaempferol, quercetin and daidzein inhibited simultaneously the STAT-1 activation.<sup>16</sup> Kaempferol, in turn, shortened the LPS-induced production of pro-inflammatory mediators through the down-regulation of p38, JNK, TLR4 and NF- $\kappa$ B.<sup>81</sup>

Moreover, kaempferol showed to inhibit the phosphorylation of ERK-1/2, p38 and JNK, as well as the activation of NF- $\kappa$ B by IL-1 $\beta$ , in rheumatoid arthritis fibroblasts. In this same study kaempferol also inhibited the production of COX-2, PGE<sub>2</sub> and MMP-1 and 3.<sup>93</sup>

Wogonin led to the reduction in phosphorylation and activation of STAT1 and STAT3 through the blockade of the nonreceptor tyrosine kinase Jak-1 and Jak-3 in LPS/IFN- $\gamma$ -activated BV2 microglial cells; and the consequently decrease in IL-6 and TNF- $\alpha$  production, in a concentration-dependent manner.<sup>94</sup>

In an inflammation-related colon cancer intervention, oroxylin A suppressed NF- $\kappa$ B activation, through the inhibition of phosphorylation-dependent IKK activation in LPS stimulated HCT116 cells and THP-1 cells.<sup>95</sup> It has already been reported that flavonoids not only directly inhibit NF- $\kappa$ B, but are also able to activate the nuclear factor E2-related factor 2 (Nrf2) systems, which in turn, increase the activity of antioxidant enzymes and inhibit NF- $\kappa$ B. Catechins, quercetin and isoflavones were described as exerting these activities.<sup>96</sup> Quercetin was recently identified as preventing the oxidative damage by down-regulating p38, ERK1/2 and MAPK and modulating the redox cross talk between NF- $\kappa$ B and Nrf-2, in a HepG2 (human hepatoma) cell line exposed to ochratoxin A (mycotoxin).<sup>97</sup> As so, it has been proved that quercetin modulates the NF- $\kappa$ B transcription by more than one signalling pathway.

A study with trimethoxyflavones revealed that 4'-bromo-5,6,7-trimethoxyflavone was the best suppressor of NF- $\kappa$ B, iNOS and COX-2 expression, and consequently the best inhibitor of NO, PGE<sub>2</sub>, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  production, in LPS-treated RAW 264.7 cells. This, taken together, shows the halogenation of flavonoids as a promising feature to enhance their anti-inflammatory activity.<sup>98</sup> The potential of chlorinated flavonoids was also described by Freitas et al. as they efficiently modulated neutrophils' oxidative burst and lifespan, by driving neutrophil apoptosis in a caspase-dependent fashion.<sup>66</sup>

Finally, in a recent study, a biflavonoid, kolaviron, tested in a RAW 264.7 macrophage cell line, showed no inhibition on LPS-induced production of IL-1 $\alpha$ , IL-33, IL-1 $\beta$ , IFN $\beta$ 1-1 and TNF- $\alpha$ . However, it inhibited the IL-6 production, and probably via inhibition of ERK1/2, p38, Akt, p-c-JUN and JNK signalling pathways.<sup>99,100</sup> Also an epicatechin trimer, procyanidin C1, inhibited LPS - induced MAPK and NF- $\kappa$ B signalling through TLR4 in RAW 264.7 macrophages.<sup>101</sup> Other associations of flavonoids have been studied.<sup>102</sup> The review of the flavonoids' effects on gene expression allowed the comprehension of the numerous studies that have been handled to understand the different action pathways. However, it was also clear that one flavonoid can affect more than one pathway at the same time, and sometimes the effects produced may be antagonistic. Nevertheless, the recent studies using flavonoids were undoubtedly focused in gene expression and transcription pathways.

### **4.11. Other Inflammatory mediators**

#### **4.11.1. Platelet-activating factor**

The platelet-activating factor (PAF) precursor, lyso-PAF, is released from the cell membrane by the action of cPLA2 on phosphatidylcholin. PAF, a lipid-inflammation mediator, is synthesized by activated neutrophils, eosinophils, monocytes, platelets, endothelial cells, mast cells and fibroblasts. PAF acts via GPCRs and platelet-activating factor receptor (PAFR), in a paracrine and autocrine manner, on eosinophils, neutrophils, macrophages, platelets, endothelial cells and lymphocytes.<sup>3,4</sup> Activation of PAFR results in inhibition of cAMP, mobilization of  $\text{Ca}^{2+}$ , and activation of MAPKs; the long-term responses depend on intracellular, most likely on nuclear receptor activation. The PAF's functions comprise: activation of platelets, chemoattraction and activation of neutrophils, macrophages and eosinophils, bronchoconstriction, and increase of vascular permeability and vasodilation.<sup>33</sup>

#### **4.11.2. Kinins**

The kinin-generating system is responsible for the generation of peptides important to the inflammatory response. The major proteins of this system are Hageman factor, clotting factor XI, prekallikrein and high molecular weight kininogen. Bradykinin is the most important biologically active peptide generated by kinin-generating system. Bradykinin holds a variety of functions, namely: increasing vascular permeability, vasodilation, hypotension, induction of pain, contraction of many types of smooth muscle cells and activation of PLA2.<sup>30</sup> Additionally, kinins can increase IL-1 production through initial stimulation of TNF. The kinins effects are associated with the production of other inflammatory mediators, such as NO, IL-6, IL-8 and mast cells-derived products.<sup>5</sup>

#### **4.11.3. The inflammasome**

The inflammasome is an intracellular multi-protein complex, composed by 22-family-member human Nod-like receptor (NLR) family of cytoplasmic proteins. The inflammasome is an important regulator of inflammation. The activated NLR proteins recruit additional proteins to form a complex with caspase-1 and with adaptor molecule apoptosis-associated speck like protein (ASC).<sup>5</sup> The activation of caspase-1 is a critical step that leads to the cleavage and activation pro-IL-1 $\beta$  and pro-IL-18.<sup>103</sup>

#### **4.11.4. C-reactive protein**

The exact function of CRP is unknown, but it has an important role in the recognition of phosphocholine, phospholipids, fibronectin, chromatin and histones, all exposed at sites of tissue damage and by apoptotic cells; so, CRP may target them for clearance.

Additionally, CRP activates the classical complement pathway and interacts with cells of the immune system. It is also responsible for the induction of inflammatory cytokines, tissue factors, and shedding of the IL-6 receptor, all of which result in a complement-dependent increase in tissue damage. Nevertheless, CRP presents also anti-inflammatory effects, namely the non-inflammatory clearance of apoptotic cells and preventing neutrophil adhesion to the endothelium. In chronic inflammation the levels of CRP are always raised.<sup>2</sup>

#### **4.11.5. Serum amyloid A**

The precise function of serum amyloid A (SAA) is not known, but it is considered pro-inflammatory. SAA acts via TLR2, activating the NF- $\kappa$ B pathway and binding to the G protein-coupled formyl peptide receptor like-1 (FPR1). It consequently induces various cytokines, acts as chemotactic for leukocytes and stimulates angiogenesis, TF and matrix MMPs' expression.<sup>2</sup>

#### **4.11.6. Vasoactive amines**

These inflammatory mediators comprise two molecules: histamine and serotonin. Histamine is the decarboxylation product of the amino acid histidine and is stored in mast cells and basophils granules. Histamine is released when cells are subjected to certain stimuli, namely: physical injury, such as, trauma, cold or heat; binding of antibodies to mast cells, which underlies allergic reaction, IgE; fragments of complement, anaphylatoxins (C3a and C5a); histamine-releasing proteins derived from leukocytes; neuropeptides (e.g. substance P) and cytokines (IL-1, IL-8).<sup>3</sup> The biological effects produced by histamine are performed via specific receptors, H1 and H4, which are GPCRs, widely distributed across tissues. Its vasoactive effects are mediated mainly by H1 receptors on microvascular endothelial cells, via stimulation of inositol 1,4,5-trisphosphate (IP3) production and Ca<sup>2+</sup> fluxes/PKC activation, increasing NO generation and LTs production. H1 receptors also promote NF- $\kappa$ B activation and inflammatory cytokine/chemokine production.<sup>38</sup> Histamine's main functions are: vasodilation, increase venular permeability, enhance leukocyte rolling and firm adhesion, induction of gaps in the endothelial cell lining, and enhance leukocyte extravasation.<sup>5,8</sup>

Serotonin (5-hydroxytryptamine) is a preformed vasoactive mediator, stored in the dense body granules of platelets.<sup>4</sup> Serotonin is released when platelets aggregate after the interaction with collagen, thrombin, adenosine diphosphate and antigen-antibody complexes.<sup>3</sup> Serotonin actions are similar to those of histamine. Serotonin biological effects comprise: promotion of fibrosis, by enhancing the synthesis of collagen by fibroblasts, and also neurotransmission.<sup>4</sup>

#### **4.11.7. Proteases**

Proteases include cysteine proteases, serine proteases, as well as MMPs and related enzymes [A disintegrin and metalloproteinases (ADAMs) and A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs)].<sup>4</sup> They are vital for the degradation of the extracellular matrix, a key mechanism of tissue turnover in inflammation; preparing the tissue for the ingrowth of new blood vessels and adherence and retention of newly recruited cells.<sup>5</sup>

Serine proteases, such as, trypsin, chymotrypsin, and elastase, are found in high levels and released by infiltrating neutrophils at the inflammation sites. They can directly digest the extracellular matrix or activate the pro-enzyme forms of secreted MMPs. Indeed, the large family of proteases is the MMPs family. They are a family of more than twenty extracellular endopeptidases, including, collagenases, entactin or nidogen-1, gelatinases, matrilysins and stromelysins. MMPs are produced as pro-enzymes and depend on metal ions (zinc/metzincin superfamily) to be active. The gene expression of MMPs is induced by multiple pro-inflammatory cytokines (TNF, IL-1, IL-17 and IL-18), through MAPK signal transduction pathways. They participate in the degradation of the extracellular matrix, in the cleavage of cell surface molecules and other pericellular non-matrix proteins, regulating cell behavior and in the digestion of matrix proteins associated with growth factors. The function of MMPs is endogenously regulated by tissue inhibitor of metalloproteinases (TIMPs). In the presence of cytokines inhibitors and growth factors, matrix protein production increases, and TIMPs inhibit MMPs activity.<sup>5</sup>

The effects of flavonoids in these “Other Inflammatory Mediators” are less studied than in the other mediators referred; however, some of those effects will be described in the next points of this article.

### **5. Flavonoids Conjugates / Complexes in Inflammation**

#### **5.1. Glycosylated flavonoids**

Glycosides of flavonoids are commonly found in nature and their bioavailability is higher compared to their aglycones. Noteworthy, the colonic microflora is considered to be a key hydrolase source for hydrolysis of flavonoid glycosides. In the digestive tract, by the action of  $\beta$ -glucosidases, the flavonoid glycosides suffer hydrolysis and they are absorbed as aglycones. The flavonoid glycosides are too water-soluble to diffuse across the cellular membrane, but their aglycones are more hydrophobic and can be easily absorbed by the epithelial cells through passive diffusion.<sup>104</sup> As so, it has been of interest to study if the biological effects of flavonoid glycosides differ from their aglycones. Generally, the

aglycones are more potent antioxidants than their glycosides. This assumption was ascertained by a study where the antioxidant properties of flavonol glycosides from tea declined as the number of glycosidic moieties increased.<sup>12</sup> When studying the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging potential of O-glycosylated flavonoids, it was concluded that the glycosylation of flavonol on both OCH<sub>3</sub> and OH reduced this potential. The inhibition of xanthine oxidase showed naringenin as having stronger chelating action with metallic ions than its glycoside. The glycosylation of flavonols, kaempferol and quercetin, obviously reduced the ONOO<sup>-</sup> scavenging potential. All these studies corroborate the loss of the antioxidant properties when flavonoids are glycosylated. However, flavonoids may be O-glycosylated and C-glycosylated and several reports showed different results when comparing these two substitutions. The O-glycosylation of flavanones at the C-7 position reduced the radical scavenging potential of flavanones, which may be caused by the steric effect and the ability to delocalize electrons. This behaviour of O-glycosylated flavonoids may be different in a cellular environment; and so far the related data is not sufficient to draw a conclusion on the influence of O-glycosylation of flavonoids on their cellular antioxidant potential. Studies undertaken to discover the antioxidant effects of C-glycosylated flavonoids have concluded that this glycosylation enhances this effect. In a trolox equivalent antioxidant capacity (TEAC) assay, the antioxidant activity of luteolin 6-C-glucopyranoside was lowered when more glycosides were added.<sup>104</sup> Naturally occurring glycosylated flavonoids were not capable of inhibit the production of 'NO in LPS-activated RAW 264.7 cells, regardless the aglycones types. Quercetin showed to have more potential to inhibit 'NO production, iNOS expression and NF-κB activation than its glycosides and the same happened with cirsimaritin and its glycosides in LPS-induced RAW 264.7 macrophages. Similarly, apigenin showed inhibition of 'NO production and iNOS and COX-2 expression while vitexin and isovitexin (apigenin C-glycosides) were inactive, in LPS-stimulated RAW 264.7 cells.<sup>105</sup> Myricetin and its 3-O-glycosides significantly inhibited the LPS-stimulated production of 'NO and pro-inflammatory cytokines and the increasing of iNOS and COX-2 levels in the same cell line as the apigenin study. Icariin and one of its metabolites also significantly inhibited LPS-induced inflammatory response, *in vitro*, in RAW 264.7 cells, and *in vivo*, in C57BL/6J mice.<sup>106,107</sup> All in all, no clear structure-activity relationships, depending on the positions or types of sugar substitution, was found in the anti-inflammatory activity for flavonoid glycosides. These differences between O- and C-glycosylated flavonoids may be simply explained by the mechanism of action of antioxidant flavonoids. As it was already explained, the free OH groups of flavonoids are determinant for the co-planarity of the rings, by the establishment of hydrogen bonds and

the donation of hydrogen and an electron to the PORS and metal chelating, determining the potent antioxidant effects of these flavonoids.<sup>104</sup>

Recently Kim and co-workers undertook a study in RAW 264.7 macrophages with myricetin, myricitrin, myricetin 3-O-(2''-O-galloyl)- $\alpha$ -L-rhamnopyranoside, myricetin 3-O-(2''-O-galloyl)- $\beta$ -D-galactopyranoside and quercetin 3-O-(2''-O-galloyl)- $\beta$ -D-galactopyranoside. Where they found that galloyl flavonol glycosides, the last three, were clearly good inhibitors of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 production, they inhibited the expression of iNOS and COX-2 proteins, indicating that the presence of a galloyl group in the sugar moiety played a key role in O<sub>2</sub><sup>-</sup> radical-scavenging and anti-inflammatory activity.<sup>108</sup> Kaempferol-3-O-rutinoside and kaempferol-3-O-glucoside were effective inhibitor of neuro-inflammation by the modulation of NF- $\kappa$ B and STAT3 activation.<sup>109</sup> Another study with isoorientin (luteolin-6-C-glucoside), in the same neuro-inflammation model, but now induced by LPS, inhibited the translocation of NF- $\kappa$ B from cytosol to the nucleus, by I $\kappa$ B degradation, and decreased the production of TNF- $\alpha$ , IL-1 $\beta$  and 'NO. It was also proved that scavenging of PORS and attenuation of ERK1/2, JNK and p38-MAPK phosphorylation may be involved on the inhibition of the production of pro-inflammatory mediators. In general, it was concluded that the anti-inflammatory effect of isoorientin was mediated through the inhibition of MAPK/NF- $\kappa$ B signalling pathways.<sup>110</sup> Orientin and isoorientin also inhibited high glucose-induced vascular inflammation via the inhibition of NF- $\kappa$ B in primary human endothelial cells. Moreover, they attenuated H<sub>2</sub>O<sub>2</sub> generation, up-regulation of adhesion molecules, monocyte endothelial cell adhesion and disruption of the endothelial barrier function.<sup>111</sup> Furthermore, orientin was able to suppress TNF- $\alpha$ , IL-6 production and the activation of NF- $\kappa$ B or ERK1/2 by LPS and protect the vascular barrier integrity by inhibiting CAMs expression.<sup>112</sup>

Xiao and co-workers revised the flavonoids glycosylation and their biological benefits and stated that, *in vivo* (oral treatment), flavonoid glycosides and their aglycones showed similar anti-inflammatory activity or even higher for the glycosides.<sup>104</sup> However, in another *in vivo* study, carrageenan-induced oedema in the mouse paw (intravenous treatment), aglycones (quercetin and hesperetin) gave positive results, while the glycosylated flavonoids showed no significant activity (rutin and hesperidin) (table 4).<sup>29</sup> These two different results may be attributed to the importance of the administration via of the flavonoids, because they imply different metabolism pathways. It should be noticed that after the intestinal modifications of flavonoids they suffer further metabolization and the non-conjugated aglycones are scarcely detected in the human plasma. Quercetin is an example of that: quercetin-3-O-glucuronide and quercetin-3'-O-sulfate are the major quercetin conjugates in rat and human plasma, while the aglycone could not be detected. However, when macrophage cell lines (RAW 264.7 and/or THP-1) *in vitro* were analysed,

it was clear that quercetin-3-O-glucuronide could bind to the cell surface but not accumulate intracellularly, while the aglycones were incorporated into cells, presumably via simple diffusion. These evidences once more the effects of flavonoids are mostly attributable to their aglycones; and in this case, the  $\beta$ -glucuronidase - mediated conversion of quercetin-3-O-glucuronide into the aglycone is essential for the anti-inflammatory effects. On the other hand, it is also known that the conjugation (glucuronidation and/or sulfation) of OH groups on polyphenols promotes their rapid excretion and attenuates their anti-inflammatory activities. It is why the health benefits of conjugated flavonoids *in vivo* still continue to be controversial.<sup>113</sup> The EGCG effects in inflammation were reviewed in<sup>114</sup>. An increasing number of *in vitro* and *in vivo* studies that describe the anti-inflammatory effects of flavonoids glycosides has being published, showing the rising interest in these molecules.<sup>106,115-124</sup>

Another flavonoid derivative, a flavoalkaloid, named lilaline (3,4',5,7-tetrahydroxy-8-(4-methyl-5-oxo-2-pyrrolidinyl)-flavone), isolated from the flowers of *Lilium candidum* L., was described as having anti-inflammatory properties through the inhibition of COX enzyme.<sup>125</sup>

## **5.2. Flavonoid-metal ion complexes**

As stated above, flavonoids may chelate metals and in this way contribute to the antioxidant resolution of inflammation. Additionally, flavonoid-metal ion complexes have appeared as a novel class of therapeutic agents. Their anti-inflammatory activities have been studied using carrageenan paw oedema and xylene-induced ear oedema mice models. Complexes of luteolin and La(III), Ho(III), Yb(III), Lu(III) and Y(III) showed better anti-inflammatory effect compared to luteolin *per si*. This result was associated to their ability to resist telangiectasia, a condition characterized by development of small new dilated capillaries near the surface of the skin, which retard vascular permeability and prevent tissue oedema caused by the acute inflammation. Similarly, complexes of quercetin and Zn and Mn have also been found to exhibit higher anti-inflammatory activity than quercetin. It is believed that these complexes act in COX and LOX pathways, but more studies are needed to better understand the therapeutic properties of these complexes.<sup>126</sup>

## **6. In vivo studies**

### **6.1. Non-human studies**

There have been an increasing number of *in vivo* studies where the anti-inflammatory properties of flavonoids are evaluated. Table 4 summarizes the *in vivo* – inflammation mediators related studies. The major problem found when we review this topic is the variety of models for the same disease, hindering the possibility of establishing a comparison between them, or even take a conclusion of different results for the same flavonoid obtained in different model diseases. Likewise, there are some diseases for which a model does not exist or is very rare to find a study about it (e.g. multiple sclerosis). Inflammatory bowel disease is one of the diseases for which there are several models. However, they differ in their immune and pathological characteristics. For instance, dietary rutin supplementation failed to ameliorate colitis in MDR1A knockout mice, but it was beneficial in acetic acid, trinitrobenzene sulfonic acid (TNBS) and dextrane sulfate sodium (DSS) induced colitis. As so, it is difficult to predict the implications of these discrepancies, if there are any.<sup>17</sup> The administration form of the flavonoids is also crucial for its effects and it was been a goal to study how the bioavailability of flavonoids could be enhanced. Quercetin has been described as presenting several solubility problems in the administration media. Liposomal quercetin was viewed as an alternative. In fact, liposomal quercetin proved to attenuate bleomycin-induced pulmonary fibrosis in Sprague-Dawley rats, by the suppression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 production.<sup>127</sup> Recently, a study in male Sprague Dawley rats, LPS-induced inflammation model, showed gold-nanoparticles loaded with puerarin or curcumin or both were more potent inflammatory inhibitors than the isolated flavonoids. This demonstrated that the effectiveness of these flavonoids *in vivo* may be conditioned by their bioavailability.<sup>128</sup> It is also very common to find *in vivo* studies about the anti-neoplastic properties of flavonoids through the modulation of the immune response,<sup>17</sup> but in this article we just discuss the anti-inflammatory effects.

**Table 4** - Inflammation mediators related *in vivo* studies.

| Flavonoid/s                                                                                                                 | Model                                                                                                                             | Effect                                                                                                                                                                                                                                                                                                   | Reference             |
|-----------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Fisetin, flavone, tricetin                                                                                                  | Pulmonary inflammation model - LPS – induced, in male C57BL/6 mice                                                                | Fisetin reduced MPO levels, and gene expression of IL-6, TNF- $\alpha$ , MIP-1 $\alpha$ , MIP-2, IL-1 $\beta$ , and I $\kappa$ B $\alpha$ transcription; flavone significantly reduced TNF- $\alpha$ and MIP-2 gene transcription; tricetin reduced IL-1 $\beta$ and I $\kappa$ B $\alpha$ transcription | 129                   |
| Apigenin, fisetin, kaempferol, luteolin, patuletin, patulitrin, quercetin                                                   | Asthma models (BALB/c mice, cats, guinea pigs)                                                                                    | Reduced asthma signals, like IL-4, IL-5, IL-13, TNF- $\alpha$ production, eotaxin levels, IgE levels, NF- $\kappa$ B expression, EPO activity, etc.                                                                                                                                                      | Revised in 17 and 130 |
| Catechin, EGCG, flavopiridol, genistein, hesperidin, nobiletin, quercetin, resveratrol, rutin, $\alpha$ -glucosylhesperidin | Rheumatoid arthritis models (collagen- and adjuvant induced arthritis)                                                            | Inhibition of osteoclast / macrophage differentiation and function, estrogen modulation; down-regulated NFAT (disintegrin-like and metalloprotease) expression in synovial fibroblasts and chondrocytes                                                                                                  | Revised in 17         |
| Baicalin, genistein, resveratrol, silibinin                                                                                 | Multiple sclerosis model - injection of Freund's adjuvant and a myelin glycoprotein - induced allergic encephalomyelitis, in mice | Resveratrol induced apoptosis of preferentially activated but also quiescent T cells; however, in various other cases, the mechanism was largely undetermined                                                                                                                                            | Revised in 17         |
| Diosmin, EGCG, genistein, hesperidin, quercetin glycosides, morin, quercitrin, rutin                                        | Inflammatory bowel disease models                                                                                                 | Diverse intestinal anti-inflammatory activities, through immunomodulatory effects at the epithelial level                                                                                                                                                                                                | Revised in 17         |
| Quercitrin, rutin                                                                                                           | Inflammatory bowel disease models                                                                                                 | As quercetin pro-drugs, prevented premature absorption of the aglycone in the small intestine and releasing it in the colon; this is based on the inability of quercetin to ameliorate DSS colitis and suggests a local action of the flavonoid                                                          | Revised in 17         |
| Fisetin, morin, myricetin                                                                                                   | Atherosclerosis models                                                                                                            | Reduced foam cell formation, inhibited LDL oxidation by neutrophil MPO blockage                                                                                                                                                                                                                          | Revised in 17         |
| EGCG, tea catechins, theaflavin, 3-methoxypuerarin                                                                          | Ischaemia-reperfusion models                                                                                                      | Protection against post-ischemic myocardial remodelling via anti-inflammatory actions; inhibition of PI3K - the structure-activity relationship has been defined                                                                                                                                         | Revised in 17         |
| Chalcone, hesperetin, kaempferol, naringenin, quercetin                                                                     | Metabolic syndrome models                                                                                                         | Decrease of TNF and iNOS expression in visceral fat; partial PPAR- $\gamma$ agonists in fat cells, but it has been argued that the antagonist component may be the predominant one <i>in vivo</i> ; reduction of inflammatory cytokines' production                                                      | Revised in 17         |
| Luteolin, quercetin                                                                                                         | Traumatic brain damage – brain inflammation models                                                                                | Various anti-inflammatory actions in myeloid cells; however quercetin might be toxic to neurons in micromolar concentrations in basal conditions                                                                                                                                                         | Revised in 17         |

**Table 4** - Inflammation mediators related *in vivo* studies. (cont.)

| Flavonoid/s                                                                | Model                                                                                                                               | Effect                                                                                                                                                                                                                                                                                                                                                                                      | Reference      |
|----------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| <b>ECGC, eupatilin, jaceosidin, myricitrin</b>                             | Skin inflammation models                                                                                                            | diminished the skin cells response to IgE-antigen complexes                                                                                                                                                                                                                                                                                                                                 | Revised in 17  |
| <b>Apigenin, flavone, flavonol, kaempferol, luteolin, quercetin, rutin</b> | Carrageenan – induced inflammation model, in male Wistar rats                                                                       | Luteolin, kaempferol, chrysin and hesperidin did not inhibit the total cell migration; quercetin, rutin, flavone, apigenin and flavonol reduced the number of rolling, adherent and migrated leukocytes, except apigenin; quercetin inhibited the cleavage of L-selectin and the expression of $\beta 2$ -integrin; rutin and flavonol only inhibited the expression of $\beta 2$ -integrin | Revised in 131 |
| <b>Hesperidin, naringin</b>                                                | Inflammation model - injection of sterile air followed by carrageenan – induced, in healthy colony-bred Wistar albino male rats     | Increased SOD and GSH, normalized the total count of leukocytes, neutrophils and lymphocyte and TNF- $\alpha$ concentration; hesperidin was more effective than naringin                                                                                                                                                                                                                    | 132            |
| <b>Daidzein, genistein</b>                                                 | Cutaneous inflammatory response model – TPA – induced, in female Swiss albino                                                       | Reduced 'NO production, increased GSH levels, restored SOD and CAT activities, inhibited TNF- $\alpha$ , IL-6 and IL1- $\beta$ production, reduced expression of NF- $\kappa$ B and COX-2                                                                                                                                                                                                   | 133            |
| <b>Curcumin and Quercetin</b>                                              | Acute inflammation model - carrageenan - induced, in male albino rats                                                               | Inhibited the 'NO levels, augmented GSH levels, HO-1 mRNA expression, reduced TNF- $\alpha$ release. Both flavonoids moderately lowered inflammation, while their combination was more effective                                                                                                                                                                                            | 134            |
| <b>Quercetin, rutin</b>                                                    | Hepatotoxic and inflammatory liver model - high cholesterol diet – induced, in Swiss albino mice                                    | Scavenged DPPH radical, chelated Fe, restored the SOD, catalase and glutathione levels, lowered the expression of p65 NF- $\kappa$ B, iNOS and C- reactive protein and the production of TNF- $\alpha$ and IL-6                                                                                                                                                                             | 135            |
| <b>7, 3'-dimethoxy hesperetin</b>                                          | Adjuvant-induced arthritis model, in male Sprague-Dawley rats                                                                       | Suppressed the levels and expression of IL-6; apparently decreased mRNA expression of JAK2 and STAT3 as well as protein expression of p-JAK2 and p-STAT3 in the rats synovium                                                                                                                                                                                                               | 136            |
| <b>7, 3'-dimethoxy hesperetin</b>                                          | Adjuvant-induced arthritis model, in male Sprague-Dawley rats                                                                       | Decreased the serum concentrations of TNF- $\alpha$ and IL-1 $\beta$ , in a dose-dependent manner                                                                                                                                                                                                                                                                                           | 137            |
| <b>Alpinetin</b>                                                           | Lung injury model – LPS - induced, in male BALB/c mice -                                                                            | Inhibited TNF- $\alpha$ , IL-6 and IL-1 $\beta$ production, reduced the total cohort of macrophages and neutrophils (also blocked I $\kappa$ B $\alpha$ and p65 NF- $\kappa$ B phosphorylation and activation of ERK and p38 in RAW 264.7 cells)                                                                                                                                            | 138            |
| <b>Baicalin (baicalein 7-O-glucuronide)</b>                                | Renal ischemia-reperfusion injury model - clamping of the left renal artery plus a right nephrectomy - induced, in male Wistar rats | Slightly down-regulated SOD activity, inhibited the increased expression of TLR2/4, MyD88, p-NF- $\kappa$ B and p- I $\kappa$ B, increase the expression of I $\kappa$ B protein, inhibited mitochondria - mediated cell apoptosis pathway                                                                                                                                                  | 139            |

**Table 4** - Inflammation mediators related *in vivo* studies. (cont.)

| Flavonoid/s      | Model                                                                                                         | Effect                                                                                                                                                                                                                                          | Reference |
|------------------|---------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>Baicalin</b>  | Chronic obstructive pulmonary disease model - cigarette smoke exposure - induced, in male Sprague-Dawley rats | Inhibited IL8, IL6 and TNF- $\alpha$ release, suppressed p65 NF- $\kappa$ B expression                                                                                                                                                          | 140       |
| <b>Baicalin</b>  | Age inflammation model, in young and old specific pathogen free male Fischer 344 rats                         | Old rats showed high levels of PPAR $\gamma$ , but decreased levels of phospho-PPAR $\gamma$ , down-regulated levels of VCAM-1, IL-1 $\beta$ and IL-6 genes                                                                                     | 141       |
| <b>Baicalin</b>  | Mastitis model - <i>S. aureus</i> - induced, in female BALB/c mice                                            | Inhibited TNF- $\alpha$ , IL-1 $\beta$ and IL-6 production, suppressed NF- $\kappa$ B expression and the phosphorylated I $\kappa$ B levels in the mammary gland, slightly reduced the phosphorylated JNK and ERK proteins                      | 142       |
| <b>Baicalin</b>  | Adjuvant-induced arthritis model, in male C57BL/6 (B6) mice                                                   | Inhibited the up-regulated IL-17 mRNA expression                                                                                                                                                                                                | 123       |
| <b>Baicalein</b> | Parkinson's disease model - MPTP - induced, in male C57B/6 mice                                               | Inhibited of NF- $\kappa$ B nuclear translocation, attenuated ERK and JNK phosphorylation in astrocytes                                                                                                                                         | 143       |
| <b>Baicalein</b> | Asthma model - ovalbumin or IL-13 - induced, in male Balb/c mice                                              | Modulated the Th1/Th2 response by increasing IFN- $\gamma$ and decreasing IL-4 and IL-13, reduced TLR2 levels without affecting TLR-4 levels, reduced the 15-LOX metabolites levels                                                             | 144       |
| <b>Baicalein</b> | Acute lung injury model - LPS - induced, in male Sprague-Dawley rats                                          | Diminished iNOS / 'NO cascade and O $_2^{\cdot -}$ formation and nitrotyrosine expression, induced HO-1 due to Nrf2 activation, inhibited p65 NF- $\kappa$ B translocation in lung tissues                                                      | 145       |
| <b>Butein</b>    | Spinal cord injury model - T12 segment - induced, in female Sprague Dawley rats                               | Attenuated the expression of p65 NF- $\kappa$ B, inhibited the production of phosphorylated I $\kappa$ B $\alpha$ , reduced MPO activity                                                                                                        | 146       |
| <b>Catechin</b>  | Cardiotoxicity model - Adriamycin - induced, in male Wistar rats                                              | Increased CAT, GSH-Px and SOD activities, significantly decreased the expression levels of TNF- $\alpha$ and NF- $\kappa$ B                                                                                                                     | 147       |
| <b>Chrysin</b>   | Colitis model - DSS - induced, in BALB/c mice                                                                 | Reduced 'NO and PGE $_2$ production, reduced MPO activity, prevented the elevation of IL-1 $\beta$ and IL-6 levels, had no effect on TNF- $\alpha$ , inhibited I $\kappa$ -B $\alpha$ degradation and NF- $\kappa$ B translocation              | 148       |
| <b>Chrysin</b>   | Colitis model mice - DSS or TNBS - induced, in female C57BL/6                                                 | Inhibited NF- $\kappa$ B, reduced the production of TNF- $\alpha$ and IL-6, the activity of MPO, down-regulated iNOS, Cox-2 and ICAM-1, limited the inflammatory (histologic) response, probably through PXR-mediated NF- $\kappa$ B inhibition | 149       |
| <b>Chrysin</b>   | Obesity model - high-fat - induced, in male C57BL/6J mice                                                     | Attenuated the increase of IL-1 $\beta$ , TNF- $\alpha$ and the reduction of IL-10( also studied murine RAW 264.7 cells - mechanism of action involves activation of PPAR- $\gamma$ functions)                                                  | 150       |

**Table 4** - Inflammation mediators related *in vivo* studies. (cont.)

| Flavonoid/s                         | Model                                                                                                                       | Effect                                                                                                                                                                                                                                                                                         | Reference     |
|-------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| <b>Chrysin</b>                      | Spinal cord injury model - modified weight-drop method - induced, in adult Wistar rats                                      | Dose-dependently suppressed the activities of p65 NF- $\kappa$ B subunit, TNF- $\alpha$ , IL-1 $\beta$ and IL-6, suppressed iNOS activity and decreased 'NO bioavailability                                                                                                                    | 151           |
| <b>Chrysin</b>                      | Lung fibrosis model - bleomycin – induced, in female Wistar Albino rats                                                     | Increased the levels of SOD and CAT, reversed GSH depletion                                                                                                                                                                                                                                    | 152           |
| <b>Chrysin</b>                      | Autoimmune neuritis - PO peptide 180-199 and <i>Mycobacterium tuberculosis</i> (strain H37RA) - induced, in male Lewis rats | Down-regulated the expression of iNOS and COX-2, via inhibition of the NF- $\kappa$ B pathway, inhibited IL-1 $\beta$ , IL-2, IL-6, IL-12, IFN- $\gamma$ and TNF- $\alpha$ , the level of IL-4 was up-regulated; IL-1 $\alpha$ , IL-10, IL-13, GM-CSF and RANTES showed no significant changes | 153           |
| <b>Diosmetin (diosmin aglycone)</b> | Acute pancreatitis model - cerulean – induced, in male C57BL/6 mice                                                         | Lowered the plasma levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, reduced MPO activity, iNOS expression and p65 NF- $\kappa$ B expression and nuclear translocations in pancreas tissue                                                                                                        | 154           |
| <b>Diosmin</b>                      | Hepatic toxicity model - ethanol – induced, in female Wistar rats                                                           | Restored the XO level, reduced the TNF- $\alpha$ levels, suppressed NF- $\kappa$ B, COX-2 and iNOS expression                                                                                                                                                                                  | 155           |
| <b>EGCG</b>                         | Atherosclerosis models                                                                                                      | LDL protection / down-regulation of CD36 mechanism and appears to exert it specifically in atherosclerotic lesions                                                                                                                                                                             | Revised in 17 |
| <b>Genistein</b>                    | Asthma model mice - ovalbumin – induced, in female specific-pathogen-free ICR                                               | Inhibited the up-regulation of eotaxin, IL-4, IL-5 and MMP-9 mRNA expression and the down-regulation of IFN- $\gamma$ and TIMP-1 mRNA expression, induced MPO and SOD activities                                                                                                               | 156           |
| <b>Hesperidin</b>                   | Rheumatoid arthritis – collagen – induced, in male Wistar rats                                                              | Decreased TBARS and 'NO levels, significantly replenished GSH and SOD level, probably by scavenging RS                                                                                                                                                                                         | 157           |
| <b>Isorhamnetin</b>                 | Acute inflammation models - carrageenan – induce, in Sprague-Dawley rats                                                    | Decreased TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and iNOS (in macrophages RAW 264.7 cell line, attenuated JNK and AKT/IKK $\alpha$ $\beta$ , which resulted in decrease of nuclear translocation of the p65 subunit)                                                                              | 158           |
| <b>Kaempferol</b>                   | Air pouch inflammation model rats - carrageenan – induced, in male Wistar rats                                              | Decreased the 'NO and PGE <sub>2</sub> levels of exudates in a dose-dependent manner                                                                                                                                                                                                           | 159           |
| <b>Kaempferol</b>                   | Colitis model - DSS – induced, in female C57BL/6J mice                                                                      | Decreased MPO activity and the plasma levels of LTB <sub>4</sub> , PGE <sub>2</sub> and 'NO, down-regulated the expression of COX-2 and iNOS, suppressed TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 expression                                                                                    | 160           |
| <b>Kaempferol</b>                   | Atherosclerosis model - high cholesterol diet – induced, in male New Zealand white rabbits                                  | Decreased levels of TNF- $\alpha$ and IL-1 $\beta$ , increased serum SOD activity, decreased gene and protein expressions of E-selectin, ICAM-1, VCAM-1, and MCP-1                                                                                                                             | 161           |
| <b>Luteolin</b>                     | Colitis model - DSS – induced, in mice                                                                                      | Blocked epithelial protective NF- $\kappa$ B proteins, because DSS elicits colitis primarily by disruption of the epithelial layer                                                                                                                                                             | Revised in 17 |

**Table 4** - Inflammation mediators related *in vivo* studies. (cont.)

| Flavonoid/s                                      | Model                                                                                                           | Effect                                                                                                                                                                                                                                                                             | Reference     |
|--------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| <b>Luteolin</b>                                  | Colitis, in IL-10 KO mice                                                                                       | Ameliorated colitis, because colonic inflammation develops in a more progressive and subtle fashion originating from immunological imbalance                                                                                                                                       | Revised in 17 |
| <b>Malvidin-3-O-<math>\beta</math> glucoside</b> | Arthritis model - <i>Mycobacterium butyricum</i> - induced, in young female Lewis rats                          | Diminished inflammatory cachexia and arthritic paw scores, down-regulated NO generation and peritoneal macrophages                                                                                                                                                                 | 162           |
| <b>Morin</b>                                     | Diabetes model - citrate buffer - induced, in male Wistar albino rats                                           | Reimbursed the levels of TBARS, GSH and SOD and CAT activities in the brain towards their control values, reduced TNF- $\alpha$ , IL-1 $\beta$ and IL-6                                                                                                                            | 163           |
| <b>Myricetin</b>                                 | Acute inflammation models - carrageenan - induced, in male Wistar rats                                          | Increased the serum levels of SOD                                                                                                                                                                                                                                                  | 164           |
| <b>Naringenin</b>                                | Asthma model - ovalbumin - induced, in female BALB/c mice                                                       | Significantly reduced the level of Th2 cells, IL-4, IL-13, CCL5, CCL11 and iNOS, abolished I $\kappa$ B $\alpha$ degradation, inhibited NF- $\kappa$ B activation                                                                                                                  | 165           |
| <b>Naringenin</b>                                | Colitis model - DSS - induced, in male BALB/c mice                                                              | Reduced expression of IFN- $\gamma$ and IL-6, suppressed IL-17A gene expression, so may directly suppress the differentiation of Th17 cells                                                                                                                                        | 166           |
| <b>Naringenin</b>                                | Colitis model - DSS - induced, in female C57BL/6 mice                                                           | Suppressed TLR4 signalling, reduced phospho-p65 NF- $\kappa$ B expression and the phosphorylation/degradation of I $\kappa$ B $\alpha$ , inhibited p65 NF- $\kappa$ B nuclear translocation, and consequently the expression of iNOS, ICAM-1, MCP-1, Cox-2, TNF- $\alpha$ and IL-6 | 167           |
| <b>Naringin</b>                                  | Chronic pulmonary neutrophilic inflammation - cigarette smoke – induced, in male and female Sprague-Dawley rats | Decreased the release of IL-8 and TNF- $\alpha$ , elevated the release of IL-10, dose-dependently, inhibited MPO activity, inhibited the content of MMP-9, reduced the neutrophils percentage in the bronchoalveolar lavage fluid                                                  | 168           |
| <b>Naringin</b>                                  | Nephrotoxicity model - gentamicin – induced, in male Sprague–Dawley rats                                        | Restored the antioxidant levels (SOD, CAT, GPx, GR, GST, GSH and vitamin C), decreased the expression of NF- $\kappa$ B and NF- $\kappa$ B-DNA binding activities and TNF- $\alpha$ and IL-6 levels, suppressed renal MPO activity                                                 | 169           |
| <b>Puerarin</b>                                  | Kidney inflammation Male ICR mice - carbon tetrachloride – induced                                              | Attenuated the decrease in GSH level and the increase in ROS and TBARS levels, the up-regulation of iNOS, COX-2, phospho-ERK1/2, NF- $\kappa$ B expression in the nuclear fraction and the inhibition of the nuclear translocation of Nrf2                                         | 170           |
| <b>Prunetin</b>                                  | Endotoxemia model - LPS-induced, in male C57BL/6 mice                                                           | Inhibited NO and PGE <sub>2</sub> production, through the suppression of iNOS and COX-2, by modulating I $\kappa$ B $\alpha$ -NF- $\kappa$ B signalling (those effects were also observed in macrophages RAW 264.7 cell line)                                                      | 171           |
| <b>Quercetin</b>                                 | Gastric mucosa inflammation model - <i>H. pylori</i> – induced, in female short-hair guinea pigs                | Decreased neutrophils and mononuclear cells infiltration, decreased lipid peroxidation                                                                                                                                                                                             | 172           |

**Table 4** - Inflammation mediators related *in vivo* studies. (cont.)

| Flavonoid/s                               | Model                                                                                                                    | Effect                                                                                                                                                                                                                                                           | Reference      |
|-------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| <b>Quercetin</b>                          | Chronic obstructive pulmonary disease model - elastase and LPS – induced, in C57BL/6 mice                                | Reduced TBARS, iNOS, the levels of KC/CXCL-1, MIP-2/CXCL-2 and MCP-1/CCL2, IL-1 $\beta$ , IL-12p40 and MIP-1 $\beta$ , the mRNA and activity levels of both MMP9 and MMP12                                                                                       | 173            |
| <b>Quercetin</b>                          | Endotoxaemic condition - LPS – induced, in male ICR mice                                                                 | Enhanced eNOS expression and abolished iNOS expression, decreased NO $_3^-$ / NOO $^-$ levels, reduced the rate of O $_2^-$ production, partially prevented losses of GSH and regained the GSH redox ratio                                                       | 174            |
| <b>Quercetin</b>                          | Renal dysfunction model lead – induced, in adult male Wistar rats                                                        | Inhibited ERK, JNK and p38 phosphorylation and p65 NF- $\kappa$ B translocation, down-regulated IL-1 $\beta$ , IL-6, TNF- $\alpha$ and COX-2 expression                                                                                                          | 175            |
| <b>Quercetin</b>                          | Abdominal aortic aneurysms – CaCl $_2$ - induced, in male C57/BL6 mice                                                   | Reduced TNF- $\alpha$ , IL-1 $\beta$ and MCP-1 levels, NF- $\kappa$ B activation, MMP-2 and MMP-9                                                                                                                                                                | 176            |
| <b>Quercetin</b>                          | Atherosclerotic plaque model - diet induced, in C57BL LDLr-/- mouse                                                      | Decreased MCP-1 and IL-17 $\alpha$ levels                                                                                                                                                                                                                        | 177            |
| <b>Rutin</b>                              | Colitis models - TNBS – induced, in rats                                                                                 | Inhibited the TNF- $\alpha$ – dependent NF- $\kappa$ B activation and suppressed MPO activity                                                                                                                                                                    | Revised in 178 |
| <b>Rutin</b>                              | Colitis model - DSS - induced, in mice                                                                                   | Attenuated the IL-1 $\beta$ , IL-6, GM-CSF production and iNOS activity                                                                                                                                                                                          | Revised in 178 |
| <b>Rutin</b>                              | Gastric mucosal damage - gastric I/R model – induced, in rats                                                            | Inhibited the iNOS and cNOS elevation, in the gastric mucosa                                                                                                                                                                                                     | Revised in 178 |
| <b>Rutin</b>                              | Cholestasis and consequent liver injury model - bile duct ligation and scission – induced, in male Sprague–Dawley rats – | Decreased mitogenic IL-1 $\beta$ and fibrogenic TGF- $\beta$ 1 expression, suppressed inflammatory cell recruitment/accumulation and NF- $\kappa$ B activation, probably via interference with ERK activation and/or enhancement of Nrf2, HO-1 and AMPK activity | 179            |
| <b>Silibinin</b>                          | Asthma model - ovalbumin - induced - pathogen-free inbred female BALB/c mice                                             | Attenuated eosinophils recruitment and inflammatory cells infiltration, reduced TNF- $\alpha$ , IL-1 $\beta$ , IL-4, IL-5 and IL-13 expression, phosphorylation and degradation of I $\kappa$ B $\alpha$ and prevented NF- $\kappa$ B translocation              | 180            |
| <b>Silibinin</b>                          | Nephrotoxicity model - arsenic – induced, in adult male albino rats of Wistar strain                                     | Decreased the TBARS, TNF- $\alpha$ , 'NO, iNOS, NF- $\kappa$ B and caspase-3 levels, increased the GSH, TSH, vitamins C and D, SOD, catalase and GPx levels                                                                                                      | 181            |
| <b>Wogonin</b>                            | Acute lung inflammation model - LPS - induced Female C57BL/6 mice                                                        | Attenuated the release of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , 'NO production and iNOS overexpression, induced PPAR $\gamma$ -mediated attenuation of NF- $\kappa$ B activation                                                                                  | 182            |
| <b>5-Hydroxy-3,7,4'-trimethoxyflavone</b> | Acute inflammation models - carrageenan-induced, in rats                                                                 | Inhibited 'NO production and protected the oxidative DNA damage due to H $_2$ O $_2$ scavenging                                                                                                                                                                  | 64             |

**Table 4** - Inflammation mediators related *in vivo* studies. (cont.)

| Flavonoid/s                                | Model                                                                        | Effect                                                                                                                                                                                         | Reference |
|--------------------------------------------|------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>5,7,3',4',5'-pentamethoxyflav anone</b> | Sepsis model - cecal ligation and puncture – induced, in female C57/BL6 mice | Decreased expression of IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , down-regulated STAT1 (this results were simultaneously assessed in murine RAW 264.7 cells and bone marrow-derived macrophages) | 183       |

MPTP - 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridin; EPO - eosinophil peroxidase; GSH - glutathione; GSH-Px - plasma glutathione peroxidase; TPA - 12-O-tetradecanoylphorbol-13-acetate; PXR - pregnane X receptor; TBARS - thiobarbituric acid reactive substances.

## **6.2. Human studies**

Several studies have been performed to evaluate the modulation of the inflammatory mediators by flavonoids in healthy human volunteers or in patients with coronary artery disease, diabetes, postmenopausal women, etc. The question is that the majority of these studies were conducted in the basis of the administration of a limited number of foods of plant origin such as black and green teas, fruit juices, grape extract and red wine. The studies with isolated flavonoids are scarce, which makes it difficult to understand the true value of each flavonoid for the human pathophysiologic condition. Revised in <sup>50</sup> As an example, quercetin (1 mg/mL) was administered to 20 cyclists (other 20 placebo) for 24 days period. Quercetin effectively attenuated the post-exercise increase in blood IL-8 and IL-10 mRNA and tended to lower plasma levels of IL-8 and TNF- $\alpha$ . However, quercetin did not alter the muscle NF- $\kappa$ B content or the exercise-induced increases in muscle COX-2 mRNA, neither the IL-6, IL-8, IL-1 $\beta$ , and TNF- $\alpha$  expression.<sup>184</sup> The same authors undertook another study with quercetin, this time with runners, where they observed once more the attenuation of IL-8 lowering after exercise; but, one more, the IL-6 levels remained unaffected.<sup>185</sup> Moreover, a 2-month flavonoid quercetin-vitamin C supplementation in non-professional athletes with regular exercise, led to reducing oxidative stress and inflammatory biomarkers, including CRP and IL-6 with little effect on E-selectin.<sup>186</sup> So, quercetin has been revealing promising effects in the modulation of inflammatory mediators, *in vivo*, regardless the particular studies conditions.

Other studies in humans have been developed, and recently published. Landberg and co-workers assessed the relationship between the intake of 6 flavonoid subclasses (flavonols, flavones, flavanones, flavan-3-ols, anthocyanidins, and polymeric flavonoids), and their main sources, and the biomarkers of inflammation, in a cross-sectional study of participants from the Nurses' Health Study cohort. They concluded that, in general, higher intakes of selected flavonoid subclasses (flavanones, flavones, and flavonols) were

associated with a modest decrease in the concentrations of inflammatory biomarkers; also, flavonoids typically found in citrus fruits were modestly associated with lower plasma IL-18 concentrations.<sup>187</sup> Between 2005 and 2007, Filiberto and co-workers, developed a study in healthy regularly menstruating women, to understand whether habitual low isoflavone intake was associated with serum CRP concentration, a factor associated with beneficial effects on inflammation. They concluded that dietary isoflavone intake were associated with decreased serum CRP concentrations, and subsequently may have the potential to improve health status among young women.<sup>188</sup> Another study with isoflavones administered during a year to elderly women concluded that they had produced no effect in the inflammatory mediators, namely IL-6. They also associated the isoflavones to soy protein, however no improvement was shown.<sup>189</sup> Nicastro and co-worker also studied isoflavonoids, but to determine the relationship between excreted urinary isoflavonoids and high serum CRP level or white blood cells count in adults in the United States. However, this sectional analysis only suggested possible inverse associations between soy intake and inflammation; no clear patterns of associations with individual isoflavonoids emerged. The daidzein metabolite, O-desmethylangolensin was the only one whose excretion was inversely associated with circulating levels of both CRP and white blood cells count.<sup>190</sup> Very recently, Jennings and co-workers published a work where they stated that anthocyanidins intake, concentrations readily achieved in the diet, lowered the CRP levels, in a cross-sectional study of 1997 females aged between 18 and 76 years. In reality, they studied the effect of the intake of various flavonoids subclasses (flavanones, anthocyanins, flavan-3-ols, polymeric flavonoids, flavonols, flavones), through a food frequency questionnaire using an extended U. S. Department of Agriculture database; however, no significant associations were observed for total or other flavonoid subclasses.<sup>191</sup> Overall, the results obtained in these studies are truly promising; however, much more needs to be done in this area, because many flaws can be detected, as the majority of the publications point in the studies discussion.

## **7. Conclusions**

Inflammation is a complex process where all the routes are inter-related. The control or modulation of this process requires molecules capable of restrain more than one inflammation route / inflammatory mediator. Flavonoids appear as an alternative class of molecules who proved to be effective from the transcription level to the enzymatic level. Moreover, and besides all the *in vivo* studies already carried out, it is clear that is necessary to establish a disease reference model to compare the effects of different

flavonoids and to enable the comparison of their effects in the all possible inflammatory mediators in the same model. All in all, flavonoids are a great promise in the resolution of the inflammatory processes and more studies should be done to completely characterize all their beneficial mechanisms of action.

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## ***CHAPTER III. Original Research***

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### **III.1. Modulation of human neutrophils' oxidative burst by flavonoids**

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My contribution for this work comprised all the performed studies, except the flavones synthesis.



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## Original article

## Modulation of human neutrophils' oxidative burst by flavonoids



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## ABSTRACT

Inflammation is a normal response towards tissue injury, but may become deleterious to the organism if uncontrolled. The overproduction of reactive species during the inflammatory process may cause or magnify the damage at inflammatory sites. Flavonoids have been suggested as therapeutic agents to avoid such damage, as these compounds exhibit anti-inflammatory activity, through the modulation of oxidative stress and signalling pathways. Both effects may attenuate neutrophils' activities at inflammatory sites. In this study, we investigated the structure/activity relationship of a series of flavonoids on the oxidative burst of human neutrophils *in vitro*, as a measure of its anti-inflammatory potential. Neutrophils were stimulated with phorbol-12-myristate-13-acetate, and fluorescence and chemiluminescence techniques were used to evaluate the generation of reactive oxygen species. All the tested flavonoids revealed the ability to modulate the neutrophil's oxidative burst. From the obtained results, the pivotal role of the catechol group in the B-ring was evidenced as well as the minor importance of the hydroxylations in the A-ring, which did not appear to be determinant for the activity, although clearly influencing the lipophilicity of the tested flavonoids. It is also clarified the importance of the methylation in the OH group at the B-ring catechol moiety. In conclusion, the obtained results uncover new possible strategies for the resolution of inflammatory processes, using flavonoids to modulate neutrophil's oxidative burst.

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## 1. Introduction

The term 'flavonoid' is generally used to describe a broad group of natural products that possess a C<sub>6</sub>–C<sub>3</sub>–C<sub>6</sub> carbon framework, or more specifically an arylbenzopyran skeleton (the benzopyran ring system is also called chroman) [1]. Fig. 1 shows the generic structure of flavonoids and the carbon numbering system. Flavonoids are a group of more than 4000 naturally occurring polyphenolic compounds that are nearly ubiquitous in plants [2]. Apart from their physiological role in plants, flavonoids have been reported to exhibit multiple biological effects, e.g. antioxidant [3–5], antiviral [6], antibacterial [7], anti-inflammatory [5,8,9], vasodilatory [10], anticancer [11], and anti-ischaemic [12] properties. Moreover, these compounds are able to control platelet aggregation and improve

capillary function [13,14]. It has become clear that the mechanisms of action of flavonoids comprise the modulation of oxidative stress and the intracellular signalling pathways, resulting from their interactions with several key enzymes, signalling cascades involving cytokines and regulatory transcription factors, and antioxidant systems, which are vital for the cellular function [15]. These activities of flavonoids may vary significantly, depending on slight variations in their chemical structure or other characteristics such as the spatial conformation or the lipophilicity of the molecule [15,16]. Evaluation of the structure–activity relationship of flavonoids may thus help to choose selected flavonoids or combinations of flavonoids for clinical development.

Inflammation is the first response of the body to infection, irritation or other injuries, and is considered as a non-specific immune response aiming to neutralize the aggressor agents and to repair damaged tissues, assuring this way the survival of the organism. While inflammation is a normal response towards tissue injury, it is often uncontrolled in chronic autoimmune diseases, namely rheumatoid arthritis and Crohn's disease, and when it is linked to an allergic response, like asthma and anaphylactic shock [17]. Due to the permanent affliction, disability, and, many times,

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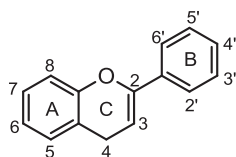


Fig. 1. Generic structure of flavonoids and carbon numbering system.

premature death of millions of patients suffering from these diseases, chronic inflammation is a heavy burden for modern society. Unfortunately, the available treatments, albeit successful in some cases, have numerous and severe side effects. Therefore, there has been a constant pursuit for alternative therapeutic approaches. Flavonoids, in particular, have been considered to possess anti-inflammatory properties, both *in vitro* and *in vivo* [15].

Also known as polymorphonuclear leukocytes, neutrophils are the most common type of white blood cells and constitute the first line of defence of the innate immune system [18]. In the event of an inflammatory response, it occurs an increase in neutrophils' attributes namely in its number, life span, mobility, tissue influx ability, and phagocytic capacity [19]. Neutrophils use an extraordinary array of oxygen-dependent and oxygen-independent microbicidal weapons to destroy and remove infectious agents [18]. The oxygen-dependent mechanisms involve the production of reactive oxygen species (ROS) and oxygen-independent mechanisms include most of the other neutrophil functions, such as chemotaxis, phagocytosis, degranulation, and the release of lytic enzymes and bactericidal peptides [18]. While these factors have important roles in host defence and inflammation, their overproduction, especially when high levels of ROS are attained and sustained, may cause or magnify damage in the inflammatory sites [15,20–22].

The antioxidant and anti-inflammatory effects of flavonoids may contribute to modulate the neutrophils' oxidative burst and therefore be useful for preventing the deleterious effects of sustained or uncontrolled inflammatory processes. For that purpose, much still needs to be investigated to find the most effective compounds. Hence, in this study, we investigated the structure/activity relationship of a series of flavonoids on the oxidative burst of human neutrophils *in vitro*, as a measure of its anti-inflammatory potential. Fig. 2 shows the chemical structures of the studied flavonoids. To study the putative modulatory effect of flavonoids on the stimulation of neutrophils by phorbol-12-myristate-13-acetate (PMA), fluorescence and chemiluminescence techniques were used.

## 2. Materials and methods

### 2.1. Materials

The following reagents were purchased from Sigma–Aldrich Co. LLC (St. Louis, USA): phorbol-12-myristate-13-acetate (PMA), *N*-acetyl-3,7-dihydroxyphenoxazine (amplex red), peroxidase from horseradish (HRP), *N,N'*-dimethyl-9,9'-biacridinium dinitrate (lucigenin), histopaque 1077, histopaque 1119, Dulbecco's Phosphate Buffer saline, without calcium chloride and magnesium chloride (PBS), trypan blue solution 0.4%, dimethylsulfoxide (DMSO), trizma, D-(+)-glucose, superoxide dismutase (SOD), catalase, apigenin, chrysin, luteolin, (±)-naringenin, (±)-eriodictyol, and quercetin. 4-Aminobenzoyl hydrazide (ABAH) was purchased from Calbiochem (San Diego, CA, USA). 2-[6-(4'-Amino)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (APF) was purchased from Invitrogen, Life Technologies Ltd (Paisley, UK). Acacetin,

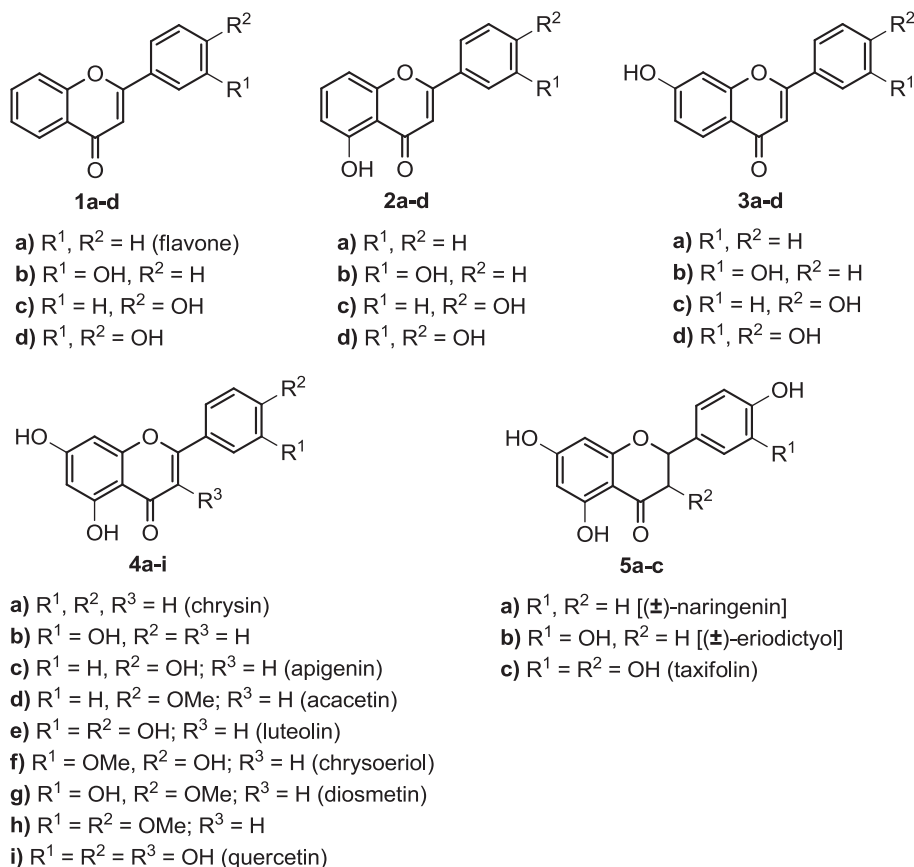


Fig. 2. Chemical structures of the studied flavonoids.

taxifolin and luminol were purchased from Fluka Chemie GmbH (Steinheim, Germany). Calcium chloride dihydrate and magnesium sulphate were purchased from Merck (Darmstadt, Germany). Potassium chloride was purchased from Pronalab (Abrunheira, Portugal) and sodium chloride from Honeywell Riedel-de Haën (Hanover, Germany). The other tested flavonoids were purchased from Indofine Chemical Company, Inc. (Stryker Lane Hillsborough, USA). 3-Methoxybenzaldehyde **7a**, 4-methoxybenzaldehyde **7b** and 3,4-dimethoxybenzaldehyde **7c** were purchased from Sigma–Aldrich (Madrid, Spain). All chemicals and solvents used in the synthesis procedures were obtained from commercial sources and used as received or dried by standard procedures.

## 2.2. General methods

Melting points were measured in a Reichert Thermovar apparatus fitted with a microscope and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 spectrometer (300.13 MHz for  $^1\text{H}$  and 75.47 MHz for  $^{13}\text{C}$ ), in  $\text{CDCl}_3$  as solvent if not stated otherwise. Chemical shifts ( $\delta$ ) are reported in ppm and coupling constants ( $J$ ) in Hz; the internal standard was TMS. Unequivocal  $^{13}\text{C}$  assignments were made with the aid of 2D gHSQC and gHMBC (delays for one-bond and long-range  $J$  C/H couplings were optimised for 145 and 7 Hz, respectively) experiments. Positive-ion ESI mass spectra were acquired with a Q-TOF 2 instrument [dilution of 1  $\mu\text{L}$  of the sample in a chloroform solution (ca. 10–5M) in 200  $\mu\text{L}$  of 0.1% trifluoroacetic acid/methanol solution]. Nitrogen was used as the nebuliser gas and argon as the collision gas. The needle voltage was set at 3000 V, with the ion source at 80 °C and the desolvation temperature at 150 °C. The cone voltage was 30 V. High-resolution mass spectra analyses were performed on a Bruker MicroTOF spectrometer (University of Vigo). Elemental analyses were obtained with a LECO 630-200-200 CHNS analyser (University of Aveiro). Preparative thin-layer chromatography was performed with Merck silica gel (60 DGF254). Column chromatography was performed with Merck silica gel (60, 70–230 mesh).

A microplate reader (Synergy HT, BIO-TEK), for fluorescence and chemiluminescence measurements, plus temperature control capacity, was used for all the assays.

## 2.3. Synthesis of 2'-hydroxyacetophenones **6a**, **b**

2'-Hydroxy-4',6'-dimethoxyacetophenone (95% yield) (**6a**) [23] and 2'-hydroxy-4',6'-bis[(2-methoxyethoxy)methoxy]acetophenone (39% yield) (**6b**) [24] were prepared according to procedures previously described in the literature.

## 2.4. Synthesis of 3-methoxy-4-[(2-methoxyethoxy)methoxy]benzaldehyde (**7c**)

2-Methoxyethoxymethyl chloride (MEMCl) (0.58 mL, 5.10 mmol) was slowly added to a stirring solution of 4-hydroxy-3-methoxybenzaldehyde (0.74 g, 4.85 mmol) and anhydrous  $\text{K}_2\text{CO}_3$  (1.34 g, 9.71 mmol) in acetone (25 mL). The solution was refluxed, under nitrogen atmosphere, for 1 h. The reaction mixture was filtered, evaporated to dryness and purified by silica gel column chromatography using dichloromethane as eluent. 3-Methoxy-4-[(2-methoxyethoxy)methoxy]benzaldehyde (**7c**), a light orange oil, was obtained in good yield (87%, 1.02 g).  $^1\text{H}$  NMR:  $\delta$  3.37 (s, 3H, 4'- $\text{OCH}_3$ ), 3.55–3.58 (m, 2H, H-3'), 3.86–3.90 (m, 2H, H-2'), 3.95 (s, 3H, 3- $\text{OCH}_3$ ), 5.43 (s, 2H, H-1'), 7.33 (d,  $J$  8.7 Hz, 1H, H-5), 7.42–7.45 (m, 2H, H-6 and H-2), 9.88 (s, 1H, CHO) ppm.  $^{13}\text{C}$  NMR:  $\delta$  55.7 (3- $\text{OCH}_3$ ), 59.0 (C-4'), 68.2 (C-2'), 71.4 (C-3'), 94.0 (C-1'), 109.4 (C-2), 114.7 (C-5), 126.4 (C-6), 131.0 (C-1), 150.0 (C-3), 151.9 (C-4), 191.0 (CHO) ppm. MS

(ESI $^+$ )  $m/z$  (rel. int.): 263 ([M + Na] $^+$ , 100). HRMS (ESI $^+$ )  $m/z$ :  $\text{C}_{12}\text{H}_{17}\text{O}_5$ , calcd 241.1071 [M + H] $^+$ , found: 241.1097;  $\text{C}_{12}\text{H}_{16}\text{NaO}_5$ , calcd 263.0890 [M + Na] $^+$ , found: 263.0884.

## 2.5. Synthesis of 2'-hydroxychalcones **8a–d**

An aqueous solution of sodium hydroxide was slowly added (60%, 2.5 mL) to a methanolic solution (2.5 mL) of the appropriate 2'-hydroxycetophenones **6a**, **b** (0.5 mmol). After cooling the reaction mixture to room temperature, the appropriate benzaldehydes **7a–d** (1.0 mmol) were added and the mixture stirred at room temperature for 3 h (for **8a**) to 4 h (for **8b–d**). After this period, the reaction mixture was poured into ice (100 g), water (50 mL) and concentrated hydrochloric acid to adjust pH to 2. The obtained precipitate was filtered and taken in chloroform (100 mL). The organic layer was successively washed with a saturated solution of potassium hydrogen carbonate (2  $\times$  100 mL), water (100 mL) and then dried through anhydrous sodium sulphate. The solvent was evaporated to dryness and the obtained 2'-hydroxychalcones **8a–d** were recrystallized from ethanol (for **8a**) or purified by silica gel column chromatography using dichloromethane as eluent (for **8b–d**). The synthesized compounds were obtained as canary yellow crystals (for **8a**) or bright yellow oils (for **8b–d**) in good yields: **8a** 68%, 0.11 g; **8b** 75%, 0.18 g; **8c** 83%, 0.24 g; **8d** 86%, 0.21 g.

### 2.5.1. 2'-Hydroxy-3,4',6'-trimethoxychalcone (**8a**)

Mp 112–113 °C (lit [25,26], 94 °C).  $^1\text{H}$  NMR:  $\delta$  3.84 (s, 3H, 4'- $\text{OCH}_3$ ), 3.85 (s, 3H, 3- $\text{OCH}_3$ ), 3.92 (s, 3H, 6'- $\text{OCH}_3$ ), 5.96 (d,  $J$  2.4 Hz, 1H, H-5'), 6.11 (d,  $J$  2.4 Hz, 1H, H-3'), 6.94 (ddd,  $J$  8.0, 2.3 and 0.9 Hz, 1H, H-4), 7.12 (t,  $J$  2.3 Hz, 1H, H-2), 7.21 (brd,  $J$  8.0 Hz, 1H, H-6), 7.33 (t,  $J$  8.0 Hz, 1H, H-5), 7.74 (d,  $J$  15.6 Hz, 1H, H- $\beta$ ), 7.88 (d,  $J$  15.6 Hz, 1H, H- $\alpha$ ), 14.28 (s, 1H, 2'-OH) ppm.  $^{13}\text{C}$  NMR:  $\delta$  55.3 (3- $\text{OCH}_3$ ), 55.6 (4'- $\text{OCH}_3$ ), 55.8 (6'- $\text{OCH}_3$ ), 91.3 (C-5'), 93.8 (C-3'), 106.3 (C-1'), 113.6 (C-2), 115.6 (C-4), 120.9 (C-6), 127.9 (C- $\alpha$ ), 129.8 (C-5), 137.0 (C-1), 142.1 (C- $\beta$ ), 159.8 (C-3), 162.5 (C-6'), 166.2 (C-4'), 168.4 (C-2'), 192.6 (C=O) ppm. MS (ESI $^+$ )  $m/z$  (rel. int.): 315 ([M + H] $^+$ , 100), 337 ([M + Na] $^+$ , 11). Anal. Calcd for  $\text{C}_{18}\text{H}_{18}\text{O}_5$  (314.33): C 68.78, H 5.77; found: C 68.74, H 5.87%.

### 2.5.2. 3,2'-Dihydroxy-4-Methoxy-4',6'-di[(2-methoxyethoxy)methoxy]chalcone (**8b**)

$^1\text{H}$  NMR:  $\delta$  3.37 and 3.38 (2s, 2  $\times$  3H, H-4'' and H-4'''), 3.55–3.58 (m, 2H, H-3''), 3.58–3.61 (m, 2H, H-3'''), 3.80–3.83 (m, 2H, H-2'''), 3.87–3.90 (m, 2H, H-2''), 3.91 (s, 3H, 4- $\text{OCH}_3$ ), 5.27 (s, 2H, H-1'''), 5.37 (s, 2H, H-1''), 6.24 (d,  $J$  2.3 Hz, 1H, H-5'), 6.27 (brs, 1H, 3-OH), 6.31 (d,  $J$  2.3 Hz, 1H, H-3'), 6.84 (d,  $J$  8.3 Hz, 1H, H-5), 7.07 (dd,  $J$  8.3 and 1.9 Hz, 1H, H-6), 7.21 (d,  $J$  1.9 Hz, 1H, H-2), 7.70 (d,  $J$  15.5 Hz, 1H, H- $\beta$ ), 7.80 (d,  $J$  15.5 Hz, 1H, H- $\alpha$ ), 14.03 (s, 1H, 2'-OH) ppm.  $^{13}\text{C}$  NMR:  $\delta$  55.8 (4- $\text{OCH}_3$ ), 58.8 (C-4'' and 4'''), 68.0 (C-2'''), 68.5 (C-2''), 71.3 (C-3'' and C-3'''), 92.8 (C-1''), 94.0 (C-1'''), 94.6 (C-5'), 97.3 (C-3'), 107.2 (C-1'), 110.5 (C-5), 112.8 (C-2), 122.6 (C-6), 125.1 (C- $\alpha$ ), 128.7 (C-1), 142.8 (C- $\beta$ ), 145.8 (C-3), 148.8 (C-4), 159.5 (C-6'), 163.1 (C-4'), 167.2 (C-2'), 192.6 (C=O) ppm. MS (ESI $^+$ )  $m/z$  (rel. int.): 501 ([M + Na] $^+$ , 100). HRMS (ESI $^+$ )  $m/z$ :  $\text{C}_{24}\text{H}_{31}\text{O}_{10}$ , calcd 479.1912 [M + H] $^+$ , found: 479.1903;  $\text{C}_{24}\text{H}_{30}\text{NaO}_{10}$ , calcd 501.1731 [M + Na] $^+$ , found: 501.1733.

### 2.5.3. 2'-Hydroxy-3-methoxy-4,4',6'-tri[(2-methoxyethoxy)methoxy]chalcone (**8c**)

$^1\text{H}$  NMR:  $\delta$  3.35, 3.38 and 3.39 (3s, 3  $\times$  3H, H-4'', H-4''' and H-4'''), 3.53–3.58 (m, 6H, H-3'', H-3''' and H-3'''), 3.80–3.83 (m, 2H, H-2''), 3.83–3.87 (m, 2H, H-2'''), 3.87–3.90 (m, 2H, H-2'''), 3.92 (s, 3H, 3- $\text{OCH}_3$ ), 5.28 (s, 2H, H-1''), 5.37 (s, 2H, H-1'''), 5.38 (s, 2H, H-1'''), 6.27 (d,  $J$  2.3 Hz, 1H, H-5'), 6.33 (d,  $J$  2.3 Hz, 1H, H-3'), 7.14 (d,  $J$  1.7 Hz, 1H, H-2), 7.19 (dd,  $J$  8.4 and 1.7 Hz, 1H, H-6), 7.23 (d,  $J$  8.4 Hz,

1H, H-5), 7.74 (d, *J* 15.5 Hz, 1H, H- $\beta$ ), 7.84 (d, *J* 15.5 Hz, 1H, H- $\alpha$ ), 14.87 (s, 1H, 2'-OH) ppm. <sup>13</sup>C NMR:  $\delta$  55.8 (3-OCH<sub>3</sub>), 59.0 (C-4'', C-4''' and C-4'''), 68.0 (C-2'''), 68.1 (C-2''), 68.6 (C-2'''), 71.4 (C-3'', C-3''' and C-3'''), 92.9 (C-1''), 94.1 and 94.2 (C-1''' and 1'''), 94.8 (C-5'), 97.5 (C-3'), 107.4 (C-1'), 111.0 (C-2), 115.8 (C-5), 122.1 (C-6), 125.7 (C- $\alpha$ ), 129.7 (C-1), 142.5 (C- $\beta$ ), 148.4 (C-4), 149.6 (C-3), 159.7 (C-6'), 163.3 (C-4'), 167.2 (C-2'), 192.6 (C=O) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 589 ([M + Na]<sup>+</sup>, 100). HRMS (ESI<sup>+</sup>), *m/z*: C<sub>28</sub>H<sub>39</sub>O<sub>12</sub>, calcd 567.2436 [M + H]<sup>+</sup>, found: 567.2457; C<sub>28</sub>H<sub>38</sub>NaO<sub>12</sub>, calcd 589.2256 [M + Na]<sup>+</sup>, found: 589.2265.

#### 2.5.4. 2'-Hydroxy-3,4-dimethoxy-4',6'-di[(2-methoxyethoxy)methoxy]chalcone (**8d**)

<sup>1</sup>H NMR:  $\delta$  3.34 (s, 3H, H-4'''), 3.39 (s, 3H, H-4''), 3.52–3.55 (m, 2H, H-3'''), 3.55–3.58 (m, 2H, H-3''), 3.80–3.83 (m, 2H, H-2''), 3.83–3.86 (m, 2H, H-2'''), 3.93 (s, 6H, 3,4-OCH<sub>3</sub>), 5.28 (s, 2H, H-1''), 5.38 (s, 2H, H-1'''), 6.26 (d, *J* 2.4 Hz, 1H, H-5'), 6.34 (d, *J* 2.4 Hz, 1H, H-3'), 6.90 (d, *J* 8.3 Hz, 1H, H-5), 7.14 (d, *J* 1.9 Hz, 1H, H-2), 7.22 (dd, *J* 8.3 and 1.9 Hz, 1H, H-6), 7.75 (d, *J* 15.5 Hz, 1H, H- $\beta$ ), 7.83 (d, *J* 15.5 Hz, 1H, H- $\alpha$ ), 13.09 (s, 1H, 2'-OH) ppm. <sup>13</sup>C NMR:  $\delta$  55.8 and 56.0 (3-OCH<sub>3</sub> and 4-OCH<sub>3</sub>), 59.0 (C-4'''), 59.1 (C-4''), 68.2 (C-2''), 68.7 (C-2'''), 71.5 (C-3'' and C-3'''), 93.0 (C-1''), 94.3 (C-1'''), 94.8 (C-5'), 97.6 (C-3'), 107.6 (C-1'), 110.2 (C-2), 111.1 (C-5), 122.8 (C-6), 125.2 (C- $\alpha$ ), 128.4 (C-1), 142.8 (C- $\beta$ ), 149.2 (C-3), 151.1 (C-4), 159.7 (C-6'), 163.3 (C-4'), 167.2 (C-2'), 192.7 (C=O) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 515 ([M + Na]<sup>+</sup>, 100). HRMS (ESI<sup>+</sup>), *m/z*: C<sub>25</sub>H<sub>33</sub>O<sub>10</sub>, calcd 493.2068 [M + H]<sup>+</sup>, found: 493.2084; C<sub>25</sub>H<sub>32</sub>NaO<sub>10</sub>, calcd 515.1888 [M + Na]<sup>+</sup>, found: 515.1880.

#### 2.6. Synthesis of 3',5,7-trimethoxyflavone (**10a**)

A catalytic amount of iodine (6 mg, 5.70 mmol) was added to a solution of 2'-hydroxy-3,4',6'-trimethoxychalcone **8a** (0.30 g, 0.95 mmol) in DMSO (10 mL). The solution was stirred at reflux, under nitrogen atmosphere, for 30 min. After this period the reaction was poured into ice (100 g), water (50 mL) and sodium thiosulfate (~0.5 g). The obtained solid was filtered and taken in chloroform (100 mL). The organic layer was successively washed with a sodium thiosulfate solution (20%, 2  $\times$  100 mL) and water (100 mL) and then dried through anhydrous sodium sulphate. The solvent was evaporated to dryness and the residue was recrystallized in ethanol to give 3',5,7-trimethoxyflavone (**10a**), a white solid, in good yield (71%, 0.21 g). Mp 147–148 °C. <sup>1</sup>H NMR:  $\delta$  3.89 (s, 3H, 3'-OCH<sub>3</sub>), 3.92 (s, 3H, 7-OCH<sub>3</sub>), 3.96 (s, 3H, 5-OCH<sub>3</sub>), 5.38 (d, *J* 2.3 Hz, 1H, H-6), 6.57 (d, *J* 2.3 Hz, 1H, H-8), 6.68 (s, 1H, H-3), 7.05 (ddd, *J* 7.9, 2.6 and 1.4 Hz, 1H, H-4'), 7.40–7.38 (m, 1H, H-2'), 7.40 (t, *J* 7.9 Hz, 1H, H-5'), 7.46 (dt, *J* 7.9 and 1.4 Hz, 1H, H-6'), 13.09 (s, 1H, 2'-OH) ppm. <sup>13</sup>C NMR:  $\delta$  55.4 (5-OCH<sub>3</sub>), 55.7 (7-OCH<sub>3</sub>), 56.4 (3'-OCH<sub>3</sub>), 92.8 (C-8), 96.2 (C-6), 109.2 (C-3 and C-10), 111.3 (C-2'), 116.9 (C-4'), 118.3 (C-6'), 130.0 (C-5'), 132.8 (C-1'), 159.9 (C-3'), 159.9 (C-9), 160.4 (C-2), 160.9 (C-5), 164.0 (C-7), 177.6 (C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 313 ([M + H]<sup>+</sup>, 100), 647 ([2M + Na]<sup>+</sup>, 77). MS (ESI<sup>+</sup>) *m/z* (rel. int.): 301 ([M + H]<sup>+</sup>, 34). HRMS (EI), *m/z* for C<sub>18</sub>H<sub>16</sub>O<sub>5</sub>: calcd 312.0998 [M]<sup>+</sup>, found: 312.0998.

#### 2.7. Synthesis of polyalkoxyflavones **10b–d**

Sodium periodate (2.97 mmol) was added to a suspension of the appropriated 2'-hydroxychalcones **8b–d** (0.59 mmol) in DMSO (10 mL). The reaction mixture was stirred under nitrogen at 100 °C for 8 h (for **8b**), 48 h (for **8c**) or 72 h (for **8d**). Then, it was poured into ice (100 g) and a sodium thiosulfate solution (10%, 50 mL). The obtained precipitate was filtered, taken in ethyl acetate (100 mL), washed with water (2  $\times$  100 mL) and then dried over anhydrous sodium sulphate. The solvent was evaporated to dryness and the obtained oils were purified by preparative thin-layer

chromatography using ethyl acetate as eluent affording polyalkoxyflavones **10b–d** as yellowish oils in moderated yields: **10b**, 36%, 0.10 g; **10c**, 44%, 0.15 g; **10d**, 44%, 0.13 g.

##### 2.7.1. 3'-Hydroxy-4'-methoxy-5,7-di[(2-methoxyethoxy)methoxy]flavone (**10b**)

<sup>1</sup>H NMR:  $\delta$  3.38 and 3.40 (2s, 2  $\times$  3H, H-4'' and H-4'''), 3.56–3.61 (m, 4H, H-3'' and H-3'''), 3.84–3.87 (m, 2H, H-2''), 3.93–3.96 (m, 2H, H-2'''), 3.97 (s, 3H, 4'-OCH<sub>3</sub>), 5.35 (s, 2H, H-1'''), 5.42 (s, 2H, H-1''), 5.92 (brs, 1H, 3'-OH), 6.56 (s, 1H, H-3), 6.77 (d, *J* 2.3 Hz, 1H, H-6), 6.89 (d, *J* 2.3 Hz, 1H, H-8), 6.94 (d, *J* 8.5 Hz, 1H, H-5'), 7.42 (dd, *J* 8.5 and 2.2 Hz, 1H, H-6'), 7.46 (d, *J* 2.2 Hz, 1H, H-2') ppm. <sup>13</sup>C NMR:  $\delta$  56.1 (4'-OCH<sub>3</sub>), 58.9 and 59.1 (C-4'' and C-4'''), 68.1 (C-2''), 68.2 (C-2'''), 71.5 (C-3'' and C-3'''), 93.3 (C-1'''), 94.4 (C-1''), 97.2 (C-8), 102.0 (C-6), 107.7 (C-3), 110.6 (C-10), 110.6 (C-5'), 112.2 (C-7), 118.7 (C-6'), 124.6 (C-1'), 146.0 (C-3'), 149.2 (C-4'), 158.0 (C-2'), 159.3 (C-9), 160.8 (C-2), 161.2 (C-5), 177.4 (C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 477 ([M + H]<sup>+</sup>, 34), 499 ([M + Na]<sup>+</sup>, 100), 975 ([2M + Na]<sup>+</sup>, 15). HRMS (ESI<sup>+</sup>), *m/z*: C<sub>24</sub>H<sub>29</sub>O<sub>10</sub>, calcd 477.1755 [M + H]<sup>+</sup>, found: 477.1753; C<sub>24</sub>H<sub>28</sub>NaO<sub>10</sub>, calcd 499.1575 [M + Na]<sup>+</sup>, found: 499.1573.

##### 2.7.2. 3',5-Dihydroxy-4'-methoxy-7-[(2-methoxyethoxy)methoxy]flavone (**9a**)

Mp 142–143 °C (light yellow powder recrystallized from ethanol). <sup>1</sup>H NMR:  $\delta$  3.40 (s, 3H, H-4''), 3.57–3.60 (m, 2H, H-3''), 3.83–3.87 (m, 2H, H-2''), 4.00 (s, 3H, 4'-OCH<sub>3</sub>), 5.34 (s, 2H, H-1''), 5.75 (s, 1H, 3'-OH), 6.48 (d, *J* 2.2 Hz, 1H, H-6), 6.58 (s, 1H, H-3), 6.69 (d, *J* 2.2 Hz, 1H, H-8), 6.97 (d, *J* 8.0 Hz, 1H, H-5'), 7.44 (d, *J* 2.2 Hz, 1H, H-2'), 7.45 (dd, *J* 8.0 and 2.2 Hz, 1H, H-6'), 12.75 (s, 1H, 5-OH) ppm. <sup>13</sup>C NMR:  $\delta$  56.1 (4'-OCH<sub>3</sub>), 59.1 (C-4''), 68.2 (C-2''), 71.5 (C-3''), 93.3 (C-1''), 94.3 (C-8), 100.1 (C-6), 104.7 (C-3), 106.3 (C-10), 110.7 (C-5'), 112.3 (C-2'), 119.2 (C-6'), 124.4 (C-1'), 146.0 (C-3'), 149.6 (C-4'), 157.6 (C-9), 162.0 (C-5), 162.9 (C-7), 163.9 (C-2), 182.5 (C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 489 ([M + H]<sup>+</sup>, 100). HRMS (ESI<sup>+</sup>), *m/z* for C<sub>20</sub>H<sub>21</sub>O<sub>8</sub>: calcd 389.1231 [M + H]<sup>+</sup>, found: 389.1229.

##### 2.7.3. 3'-Methoxy-4',5,7-tri[(2-methoxyethoxy)methoxy]flavone (**10c**)

<sup>1</sup>H NMR:  $\delta$  3.38, 3.39 and 3.40 (3s, 3  $\times$  3H, H-4'', H-4''' and H-4'''), 3.56–3.61 (m, 6H, H-3'', H-3''' and H-3'''), 3.85–3.88 (m, 2H, H-2''), 3.89–3.91 (m, 2H, H-2'''), 3.93–3.97 (m, 2H, H-2''), 4.00 (s, 3H, 3'-OCH<sub>3</sub>), 5.37 (s, 2H, H-1'''), 5.41 (s, 2H, H-1'''), 5.43 (s, 2H, H-1''), 6.59 (s, 1H, H-3), 6.79 (d, *J* 2.3 Hz, 1H, H-6), 6.92 (d, *J* 2.3 Hz, 1H, H-8), 7.31 (d, *J* 8.5 Hz, 1H, H-5'), 7.36 (d, *J* 2.1 Hz, 1H, H-2'), 7.48 (dd, *J* 8.5 and 2.1 Hz, 1H, H-6') ppm. <sup>13</sup>C NMR:  $\delta$  56.1 (3'-OCH<sub>3</sub>), 58.9 and 59.0 (C-4'', C-4''' and C-4'''), 68.0 and 68.1 (C-2'', C-2''' and C-2'''), 71.4 (C-3'', C-3''' and C-3'''), 93.2 (C-1'''), 94.1 (C-1'''), 94.4 (C-1''), 97.1 (C-8), 102.1 (C-6), 108.0 (C-3), 109.0 (C-2'), 111.6 (C-10), 115.7 (C-5'), 119.5 (C-6'), 125.3 (C-1'), 149.2 (C-4'), 149.7 (C-3'), 158.0 (C-7), 159.2 (C-9), 160.7 (C-2), 161.1 (C-5), 177.3 (C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 565 ([M + H]<sup>+</sup>, 100), 587 ([M + Na]<sup>+</sup>, 94). HRMS (ESI<sup>+</sup>), *m/z*: C<sub>28</sub>H<sub>37</sub>O<sub>12</sub>, calcd 565.2280 [M + H]<sup>+</sup>, found: 565.2264; C<sub>28</sub>H<sub>36</sub>NaO<sub>12</sub>, calcd 587.2099 [M + Na]<sup>+</sup>, found: 587.2077.

##### 2.7.4. 5-Hydroxy-3'-methoxy-4',7-di[(2-methoxyethoxy)methoxy]flavone (**9b**)

Mp 124–125 °C (white powder recrystallized from ethanol). <sup>1</sup>H NMR:  $\delta$  3.38 and 3.39 (2  $\times$  s, 6H, H-4'' and H-4'''), 3.56–3.60 (m, 4H, H-3'' and H-3'''), 3.84–3.91 (m, 4H, H-2'' and H-2'''), 4.00 (s, 3H, 3'-OCH<sub>3</sub>), 5.36 (s, 2H, H-1''), 5.42 (s, 2H, H-1'''), 6.48 (d, *J* 2.2 Hz, 1H, H-6), 6.61 (s, 1H, H-3), 6.73 (d, *J* 2.2 Hz, 1H, H-8), 7.32 (d, *J* 8.6 Hz, 1H, H-5'), 7.38 (d, *J* 2.1 Hz, 1H, H-2'), 7.49 (dd, *J* 8.6 and 2.1 Hz, 1H, H-6'), 12.73 (s, 1H, 5-OH) ppm. <sup>13</sup>C NMR:  $\delta$  56.2 (3'-OCH<sub>3</sub>), 59.0 and 59.1 (C-4'' and C-4'''), 68.1 (C-2''), 68.2 (C-2'''), 71.4 (C-3'' and C-3'''), 93.1 (C-1''), 94.1 (C-1'''), 94.2 (C-8), 100.3 (C-6), 105.0 (C-3 and C-10),

109.2 (C-2'), 115.7 (C-5'), 120.0 (C-1'), 125.0 (C-6'), 149.8 (C-3' and C-4'), 157.6 (C-9), 162.0 (C-5), 163.0 (C-7), 164.0 (C-2 and C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 477 ([M + H]<sup>+</sup>, 100). HRMS (ESI<sup>+</sup>), *m/z*: C<sub>24</sub>H<sub>29</sub>O<sub>10</sub> for calcd 477.1755 [M + H]<sup>+</sup>, found: 477.1748.

#### 2.7.5. 3',4'-Dimethoxy-5,7-di[(2-methoxyethoxy)methoxy]flavone (**10d**)

<sup>1</sup>H NMR:  $\delta$  3.38 and 3.40 (2  $\times$  s, 6H, H-4'' and H-4'''), 3.57–3.61 (m, 4H, H-3'' and H-3'''), 3.85–3.88 (m, 2H, H-2''), 3.94–3.96 (m, 2H, H-2''), 3.97 (s, 3H, 4'-OCH<sub>3</sub>), 4.00 (s, 3H, 3'-OCH<sub>3</sub>), 5.37 (s, 2H, H-1'''), 5.43 (s, 2H, H-1''), 6.59 (s, 1H, H-3), 6.79 (d, *J* 2.4 Hz, 1H, H-6), 6.92 (d, *J* 2.4 Hz, 1H, H-8), 6.97 (d, *J* 8.5 Hz, 1H, H-5'), 7.35 (d, *J* 2.1 Hz, 1H, H-2'), 7.52 (dd, *J* 8.5 and 2.1 Hz, 1H, H-6'), 12.73 (s, 1H, 5-OH) ppm. <sup>13</sup>C NMR:  $\delta$  56.0 (4'-OCH<sub>3</sub>), 56.1 (3'-OCH<sub>3</sub>), 58.9 and 59.0 (C-4'' and C-4'''), 68.2 (C-2'' and C-2'''), 71.4 and 71.5 (C-3'' and C-3'''), 93.2 (C-1'''), 94.5 (C-1''), 97.1 (C-8), 102.1 (C-6), 107.7 (C-3), 108.5 (C-2'), 110.6 (C-10), 111.1 (C-5'), 119.6 (C-6'), 124.0 (C-1'), 149.2 (C-3'), 151.7 (C-4'), 158.0 (C-7), 159.3 (C-9), 160.9 (C-2), 161.1 (C-5), 177.4 (C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 491 ([M + H]<sup>+</sup>, 54), 513 ([M + Na]<sup>+</sup>, 100). HRMS (ESI<sup>+</sup>), *m/z* for C<sub>25</sub>H<sub>30</sub>NaO<sub>10</sub>: calcd 513.1731 [M + Na]<sup>+</sup>, found: 513.1728.

#### 2.7.6. 5-Hydroxy-3',4'-dimethoxy-7-[(2-methoxyethoxy)methoxy]flavone (**9c**)

Mp 136–137 °C (light yellow powder recrystallized from ethanol). <sup>1</sup>H NMR:  $\delta$  3.39 (s, 3H, H-4''), 3.57–3.60 (m, 2H, H-3''), 3.84–3.87 (m, 2H, H-2''), 3.98 (s, 3H, 4'-OCH<sub>3</sub>), 4.00 (s, 3H, 3'-OCH<sub>3</sub>), 5.36 (s, 2H, H-1''), 6.48 (d, *J* 2.2 Hz, 1H, H-6), 6.61 (s, 1H, H-3), 6.73 (d, *J* 2.2 Hz, 1H, H-8), 6.99 (d, *J* 8.5 Hz, 1H, H-5'), 7.36 (d, *J* 2.1 Hz, 1H, H-2'), 7.53 (dd, *J* 8.5 and 2.1 Hz, 1H, H-6'), 12.75 (s, 1H, 5-OH) ppm. <sup>13</sup>C NMR:  $\delta$  56.1 (3'-OCH<sub>3</sub> and 4'-OCH<sub>3</sub>), 59.1 (C-4''), 68.2 (C-2''), 71.4 (C-3''), 93.1 (C-1''), 94.1 (C-8), 100.2 (C-6), 104.7 (C-3 and C-10), 108.7 (C-2'), 111.1 (C-5'), 120.1 (C-6'), 123.7 (C-1'), 149.3 (C-3'), 152.3 (C-4'), 157.6 (C-9), 161.9 (C-5), 162.9 (C-7), 164.1 (C-2 and C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 403 ([M + H]<sup>+</sup>, 100). HRMS (ESI<sup>+</sup>), *m/z* for C<sub>21</sub>H<sub>23</sub>O<sub>8</sub>: calcd 403.1387 [M + H]<sup>+</sup>, found: 403.1383.

#### 2.8. Synthesis of 3',5,7-trihydroxyflavone (**4b**)

A solution of 3',5,7-trimethoxyflavone (**10a**) (81 mg, 0.36 mmol) in freshly distilled dichloromethane (5 mL) was cooled to –78 °C in a propan-2-ol bath. A solution of 1 M boron tribromide in dichloromethane (1.95 mL, 1.95 mmol) was added dropwise (2.5 equiv. for each methyl group to be cleaved). The solution was stirred under nitrogen for 10 days at room temperature. After this period, water (40 mL) was slowly added to the reaction mixture with vigorously stirring until the formation of a yellow precipitate. The solid was removed by filtration and then abundantly washed with water (150 mL) and diethyl ether (50 mL) affording 3',5,7-trihydroxyflavone (**4b**) as a light yellow solid (78.6%, 55 mg). Mp<sub>dec</sub> 290–201 °C. <sup>1</sup>H NMR (CD<sub>3</sub>OD):  $\delta$  6.23 (d, *J* 2.0 Hz, 1H, H-6), 6.46 (d, *J* 2.0 Hz, 1H, H-8), 6.66 (s, 1H, H-3), 7.00 (brdd, *J* 7.6 and 1.5 Hz, 1H, H-4'), 7.33–7.39 (m, 1H, H-2'), 7.36 (t, *J* 7.8 Hz, 1H, H-5'), 7.43 (brd, *J* 7.8 Hz, 1H, H-6') ppm. <sup>13</sup>C NMR (CD<sub>3</sub>OD):  $\delta$  95.1 (C-8), 100.3 (C-6), 105.5 (C-10), 105.9 (C-3), 113.8 (C-2'), 118.5 (C-6'), 120.1 (C-4'), 131.3 (C-5'), 133.7 (C-1'), 159.4 (C-3'), 159.5 (C-9), 163.3 (C-5), 165.7 (C-2), 166.3 (C-7), 183.9 (C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 271 ([M + H]<sup>+</sup>, 100). MS (ESI<sup>+</sup>) *m/z* (rel. int.): 301 ([M + H]<sup>+</sup>, 34). HRMS (EI), *m/z* for C<sub>15</sub>H<sub>10</sub>O<sub>5</sub>: calcd 270.0528 [M]<sup>+</sup>, found: 270.0529.

#### 2.9. Synthesis of 5,7-dihydroxyflavones **4f–h**

A diluted solution of hydrochloric acid (20%, 10 mL) was added to a suspension of the appropriated polyalkoxyflavones **10b–**

**d** (0.27 mmol) in tetrahydrofuran (5 mL). The reaction was stirred under nitrogen at room temperature for 3 h. After this period the reaction was poured into ice (100 g) and water (50 mL). The solid so obtained was filtered, washed abundantly with water (150 mL) and diethyl ether (50 mL) affording the expected 5,7-dihydroxyflavones **4f–h** as light yellow solids in good yields: **4f**, 90%, 73 mg; **4g**, 89%, 70 mg; **4h**, 86%, 73 mg.

##### 2.9.1. 4',5,7-Trihydroxy-3'-methoxyflavone (**4f**)

Mp<sub>dec</sub> 322–323 °C (lit [27], 330–331 °C). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>):  $\delta$  3.90 (s, 3H, 3'-OCH<sub>3</sub>), 6.21 (d, *J* 2.0 Hz, 1H, H-6), 6.52 (d, *J* 2.0 Hz, 1H, H-8), 6.90 (s, 1H, H-3), 6.94 (d, *J* 8.9 Hz, 1H, H-5'), 7.56 (s, 1H, H-2'), 7.56–7.59 (m, 1H, H-6'), 10.02 (brs, 1H, 3'-OH), 10.89 (brs, 1H, 7-OH), 12.98 (s, 1H, 5-OH) ppm. <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>):  $\delta$  56.0 (3'-OCH<sub>3</sub>), 94.1 (C-8), 98.9 (C-6), 103.3 (C-3), 103.8 (C-10), 110.2 (C-2'), 115.8 (C-5'), 120.4 (C-6'), 121.6 (C-1'), 148.1 (C-3'), 150.8 (C-4'), 157.4 (C-9), 161.5 (C-5), 163.7 (C-2), 164.2 (C-7), 181.9 (C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 301 ([M + H]<sup>+</sup>, 100), 323 ([M + Na]<sup>+</sup>, 4). HRMS (EI), *m/z* for C<sub>16</sub>H<sub>12</sub>O<sub>6</sub>: calcd 300.0634 [M]<sup>+</sup>, found: 300.0634.

##### 2.9.2. 3',5,7-Trihydroxy-4'-methoxyflavone (**4g**)

Mp<sub>dec</sub> 252–253 °C (lit [27], 253–254 °C). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>):  $\delta$  3.87 (s, 3H, 4'-OCH<sub>3</sub>), 6.20 (d, *J* 2.1 Hz, 1H, H-6), 6.47 (d, *J* 2.1 Hz, 1H, H-8), 6.76 (s, 1H, H-3), 7.09 (d, *J* 8.6 Hz, 1H, H-5'), 7.43 (d, *J* 2.3 Hz, 1H, H-2'), 7.55 (dd, *J* 8.6 and 2.3 Hz, 1H, H-6'), 9.52 (bs, 1H, 7-OH), 12.94 (s, 1H, 5-OH) ppm. <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>):  $\delta$  55.8 (4'-OCH<sub>3</sub>), 94.0 (C-8), 98.9 (C-6), 103.6 (C-3), 103.8 (C-10), 112.2 (C-5'), 113.0 (C-2'), 118.8 (C-6'), 123.0 (C-1'), 146.8 (C-3'), 151.2 (C-4'), 157.4 (C-9), 161.5 (C-5), 163.6 (C-2), 164.3 (C-7), 181.8 (C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 301 ([M + H]<sup>+</sup>, 34). HRMS (EI), *m/z* for C<sub>16</sub>H<sub>12</sub>O<sub>6</sub>: calcd 300.0634 [M]<sup>+</sup>, found: 300.0632.

##### 2.9.3. 5,7-Dihydroxy-3',4'-dimethoxyflavone (**4h**)

Mp 283–284 °C. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>):  $\delta$  3.86 (s, 3H, 3'-OCH<sub>3</sub>), 3.89 (s, 3H, 4'-OCH<sub>3</sub>), 6.21 (d, *J* 2.0 Hz, 1H, H-6), 6.54 (d, *J* 2.0 Hz, 1H, H-8), 6.98 (s, 1H, H-3), 7.13 (d, *J* 8.5 Hz, 1H, H-5'), 7.57 (d, *J* 1.9 Hz, 1H, H-2'), 7.69 (dd, *J* 8.5 and 1.9 Hz, 1H, H-6'), 10.87 (brs, 1H, 7-OH), 12.93 (s, 1H, 5-OH) ppm. <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>):  $\delta$  55.7 (3'-OCH<sub>3</sub>), 55.8 (4'-OCH<sub>3</sub>), 94.1 (C-8), 98.9 (C-6), 103.8 (C-10), 103.8 (C-3), 109.4 (C-2'), 111.7 (C-5'), 120.0 (C-6'), 122.9 (C-1'), 149.0 (C-3'), 152.1 (C-4'), 157.4 (C-9), 161.4 (C-5), 163.3 (C-2), 164.2 (C-7), 181.8 (C-4) ppm. MS (ESI<sup>+</sup>) *m/z* (rel. int.): 315 ([M + H]<sup>+</sup>, 100). HRMS (EI), *m/z*: C<sub>17</sub>H<sub>14</sub>O<sub>6</sub>: calcd 314.0790 [M]<sup>+</sup>, found: 314.0799.

#### 2.10. Cell viability

Cell viability was determined by the trypan blue exclusion assay. Neutrophils were incubated with all the tested flavonoids (at the maximum concentration tested, 100  $\mu$ M) for 1 h at 37 °C. Twenty  $\mu$ L of neutrophil suspension were added to an equal volume of trypan blue solution 0.4% in a microtube and gently mixed. After 2 min on ice, neutrophil number and viability (viable cells excluding trypan blue) were counted. Assays were performed in triplicate.

#### 2.11. Measurement of oxidative burst in human neutrophils

Neutrophils were isolated from blood donated by healthy human volunteers to whom the design and execution of the experiment were thoroughly explained, and informed consent was obtained. Essentially, the study was based on the stimulation of human neutrophils with PMA and subsequent measurement of the neutrophil-ROS-generating-capacity. For this purpose, chemiluminescence methods, using luminol and lucigenin as probes, and fluorescence methods, using amplex red and APF as probes, were adapted to a microplate reader (Synergy HT, BIO-TEK), as detailed

below. Each study corresponds to, at least, four individual experiments, performed in triplicate in each experiment.

### 2.12. Isolation of neutrophils

Following informed consent, venous blood was collected from healthy human volunteers by antecubital venipuncture, into K<sub>3</sub>EDTA vacuum tubes. The isolation of human neutrophils was performed by the gradient density, as previously reported [28]. Briefly, 3 mL of histopaque 1077 was carefully layered on top of 3 mL of histopaque 1119 in a 15 mL polypropylene tube. Then 4.5 mL of the collected blood was decanted on top of this discontinuous density gradient. The tube was centrifuged at 890 g for 30 min at 20 °C. After centrifugation the neutrophil pellet was removed using a Pasteur pipette and doubled in volume using PBS (this reduces the viscosity of the histopaque–neutrophil suspension so that cells can be centrifuged without the need of high g forces); the neutrophils were then centrifuged at 870 g for 5 min at 4 °C. The supernatant was decanted and a mixture of 1.25 mL of PBS + 5.25 mL of sterile distilled water was added to the neutrophil pellet to lyse any remaining red blood cells. The tube was gently inverted for 2 min, after which isotonicity was re-established by adding 2.2 mL of 3% NaCl. This suspension was then submitted to a new centrifugation at 870 g for 5 min at 4 °C after which the supernatant was decanted and the neutrophil pellet resuspended in Tris–glucose. The obtained cell suspensions contained more than 99% of neutrophils and the control of their viability showed more than 95% of the cells excluding trypan blue solution 0.4%. Isolated neutrophils were kept in ice until use. Tris–glucose (25 mM Tris, 1.26 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 5.37 mM KCl, 0.81 mM MgSO<sub>4</sub>, 140 mM NaCl, and 5.55 mM D-Glucose) was the incubation media used, as previously recommended [29].

### 2.13. Evaluation of neutrophils' oxidative burst

#### 2.13.1. Oxidation of luminol

The chemiluminescent probe luminol has been thoroughly studied and used for monitoring the production of reactive species by neutrophils, namely the superoxide anion radical (O<sub>2</sub><sup>•−</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical (HO<sup>•</sup>), hypochlorous acid (HOCl), nitric oxide (•NO) and peroxynitrite anion (ONOO<sup>−</sup>) [22]. The measurement of neutrophils' oxidative burst was undertaken by chemiluminescence, by monitoring ROS-induced oxidation of luminol, according to a previously described procedure [30]. The reaction mixtures contained neutrophils (1 × 10<sup>6</sup> cells/mL) and the following reagents at the indicated final concentrations (in a final volume of 250 μL): tested compounds at various concentrations (0–100 μM), luminol (500 μM) and PMA (160 nM). Cells were pre-incubated with luminol and the tested compounds for 5 min before the addition of PMA and the measurements were carried out at 37 °C, under continuous soft shaking. Kinetic readings were initiated immediately after cell stimulation. Measurements were taken at the peak of the curve. This peak was observed at around 10 min. Effects are expressed as the percent inhibition of luminol oxidation. ABAH, SOD and catalase were used as positive controls.

#### 2.13.2. Oxidation of lucigenin

The measurement of neutrophils' oxidative burst was undertaken by chemiluminescence, by monitoring O<sub>2</sub><sup>•−</sup>-induced lucigenin oxidation, according to a previously described procedure [30]. The reaction mixtures contained neutrophils (5 × 10<sup>6</sup> cells/mL) and the following reagents at the indicated final concentrations (in a final volume of 250 μL): tested compounds at various concentrations (0–100 μM), lucigenin (50 μM) and PMA (160 nM). Cells were pre-incubated with lucigenin and the tested compounds for 5 min

before the addition of PMA and the measurements were carried out at 37 °C, under continuous soft shaking. Kinetic readings were initiated immediately after cell stimulation. Obtained values correspond to the slope measured between 10 and 30 min. Effects are expressed as the percent inhibition of lucigenin oxidation. SOD was used as positive control.

#### 2.13.3. Oxidation of amplex red

Amplex red is a highly sensitive and chemically stable fluorescent probe for the extracellular detection of H<sub>2</sub>O<sub>2</sub> [22]. The measurement of neutrophil burst was undertaken by fluorescence, by monitoring H<sub>2</sub>O<sub>2</sub>-induced oxidation of amplex red, according to a previously described procedure [31], with modifications. The reaction mixtures contained neutrophils (1 × 10<sup>6</sup> cells/mL) and the following reagents at the indicated final concentrations (in a final volume of 250 μL): tested compounds at various concentrations (0–100 μM), amplex red (25 μM), HRP (0.25 U/mL) and PMA (160 nM). Cells were pre-incubated with amplex red, HRP and the tested compounds for 5 min before the addition of PMA and the measurements were carried out at 37 °C, under continuous soft shaking. Kinetic readings were initiated immediately after cell stimulation. The excitation and emission wavelengths used were 563 and 587 nm, respectively. Obtained values correspond to the slope measured between 5 and 10 min. Effects are expressed as the percent inhibition of amplex red oxidation. Catalase was used as positive control.

#### 2.13.4. Oxidation of APF

APF is a non-fluorescent derivative of fluorescein that is oxidized by HOCl, ONOO<sup>−</sup> and HO<sup>•</sup>, in a concentration-dependent manner. Its selectivity was tested using the myeloperoxidase (MPO) inhibitor, ABAH. The addition of ABAH to human neutrophils, stimulated with PMA, decreased the APF-dependent fluorescence signal to the level of the control assay, ruling out the involvement of HO<sup>•</sup> and ONOO<sup>−</sup> [22]. The measurement of neutrophils' oxidative burst was undertaken by fluorescence, by monitoring HOCl-induced oxidation of APF, according to a previously described procedure [31], with modifications. The reaction mixtures contained neutrophils (2 × 10<sup>6</sup> cells/mL) and the following reagents at the indicated final concentrations (in a final volume of 250 μL): tested compounds at various concentrations (0–100 μM), PMA (160 nM) and APF (1 μM). Cells were pre-incubated with PMA and the tested compounds for 6 min before the addition of APF and the measurements were carried out at 37 °C, under continuous soft shaking. Kinetic readings were initiated immediately after the addition of APF. The excitation and emission wavelengths used were 500 and 520 nm, respectively. Obtained values correspond to the slope measured between 5 and 15 min. Effects are expressed as the percent inhibition of APF oxidation. ABAH was used as positive control.

## 3. Results

### 3.1. Synthesis of flavones

Flavones **10a–d** were obtained by a conventional synthetic route involving the base-catalysed aldol condensation of conveniently protected 2'-hydroxyacetophenones **6a,b** with the appropriate benzaldehydes **7a–d** leading to 2'-hydroxychalcones **8a–d** which, by oxidative cyclization, can be converted to flavones **10a–d** in good yields (Scheme 1). The cyclodehydrogenation of 2'-hydroxychalcone **8a** with a catalytic amount of iodine in refluxing DMSO yielded flavone **10a** in good yield (71%). However, the cyclization step of chalcones **8b–d**, by this method, was inefficient. The cleavage of the protecting methoxyethoxymethyl

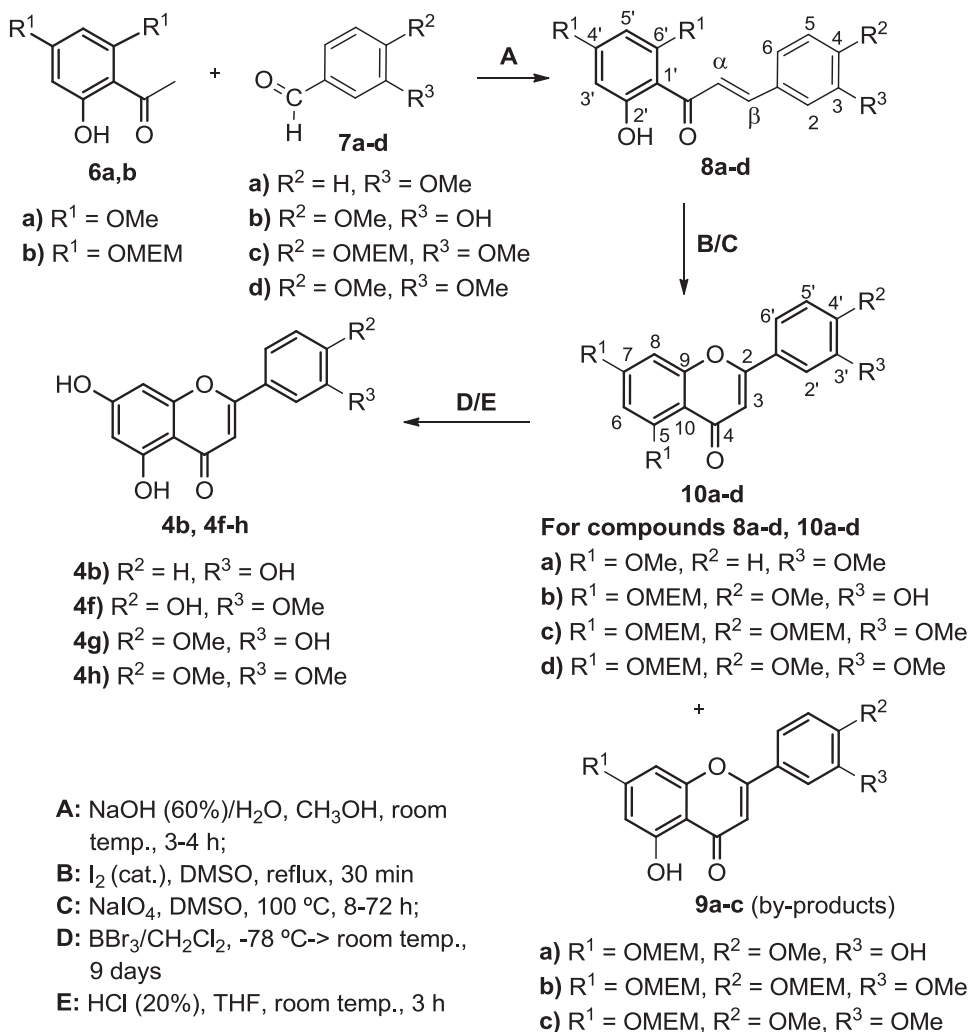
(MEM) groups, probably due to the formation of HI, led to a complex mixture of degradation products (yields of less than 10%). Aiming for better yields milder reaction conditions were used [32], thus 2'-hydroxychalcones **8b–d** were heated at 100 °C for 8–72 h with sodium periodate in DMSO promoting a smooth conversion to the corresponding flavones **10b–d**. Under these conditions, it was obtained approximately 15% of 5-OMEM deprotected flavones **9a–c**, as by-products, due to the markedly leaving character of MEM group that consequently decreased the obtained main flavones **10b–d** yields. The final synthetic step involved the selective cleavage of the protecting groups of flavones **10a–d** affording the expected 5,7-dihydroxyflavones **4b** and **4f–h** in good yields. The cleavage of the methyl groups of flavone **10a** was achieved by treatment with boron tribromide (2.5 equiv per methyl group) in dichloromethane and the cleavage of MEM groups of flavones **10b–d** was performed by stirring in a diluted hydrochloric acid solution.

### 3.2. Cell viability

Neutrophils' viability was assessed by the trypan blue assay after exposure to the tested flavonoids. Cell viability was maintained over 98%, after 1 h of exposure to the different flavonoids, at the maximum concentration tested, 100  $\mu$ M.

### 3.3. Oxidation of luminol by neutrophils – generated ROS

All the tested flavonoids **1a–d**, **2a–d**, **3a–d**, **4a–i** and **5a–c** were effective inhibitors of the neutrophils' oxidative burst, compound **2d** being the most active ( $IC_{50} = 1.6 \pm 0.2 \mu$ M). As shown in Table 1, from all flavones of group 1, flavone **1d** was noticeably more effective than the others, presenting an  $IC_{50}$  of  $3.0 \pm 0.4 \mu$ M, which is around 8 times lower than the second most active flavone of this group **1b**, with an  $IC_{50}$  of  $24.0 \pm 4.0 \mu$ M. From group 2, it is clear that flavone **2d** was much more active than the other flavones from this group. On the other hand, flavone **2a** reached only a  $30.1 \pm 4.1\%$  effect for the concentration of 25  $\mu$ M. In what concerns group 3, **3d** and **3b** were the most active, the first one being considerably more potent than the second one, with  $IC_{50}$ s of  $13.5 \pm 3.3 \mu$ M and  $37.4 \pm 3.0 \mu$ M, respectively. The **3a** and **3c** did not even reach an activity of 50%. For flavonoids **4a–i**, chrysoeriol **4f** was the most potent ( $IC_{50} = 2.8 \pm 0.5 \mu$ M), the obtained  $IC_{50}$  for diosmetin **4g** being slightly higher than **4f** ( $IC_{50} = 3.6 \pm 0.4 \mu$ M). Luteolin **4e** presented an  $IC_{50}$  of  $9.3 \pm 1.2 \mu$ M, close to the one obtained for **4b** ( $IC_{50} = 10.0 \pm 2.0 \mu$ M). The order of potencies found for the other derivatives was: apigenin **4c** ( $IC_{50} = 24.0 \pm 3.0 \mu$ M) > quercetin **4i** > chrysin **4a** > **4h** > acacetin **4d** ( $26.7 \pm 4.3\%$ , inhibition for 25  $\mu$ M). Flavanones **5a–c** appeared to be less effective modulators of neutrophils' oxidative burst than the corresponding flavones **4c**,



Scheme 1.

**Table 1**

Chemical structures and inhibition of PMA-induced neutrophils' oxidative burst by the studied flavonoids, assessed by the chemiluminescent probes, luminol and lucigenin ( $IC_{50}$   $\mu$ M, mean  $\pm$  SEM).

| Compound                        |  | R1  | R2  | R3 | Luminol<br>$IC_{50}$ ( $\mu$ M) | Lucigenin<br>$IC_{50}$ ( $\mu$ M) |
|---------------------------------|--|-----|-----|----|---------------------------------|-----------------------------------|
| <b>1a</b> Flavone               |  | H   | H   | —  | LA                              | 57.0 $\pm$ 7.5                    |
| <b>1b</b>                       |  | OH  | H   | —  | 24.0 $\pm$ 4.0                  | 47.0 $\pm$ 5.0                    |
| <b>1c</b>                       |  | H   | OH  | —  | 40.3 $\pm$ 2.4                  | LA                                |
| <b>1d</b>                       |  | OH  | OH  | —  | 3.0 $\pm$ 0.4                   | 8.1 $\pm$ 0.6                     |
| <b>2a</b>                       |  | H   | H   | —  | LA                              | 96.0 $\pm$ 4.0                    |
| <b>2b</b>                       |  | OH  | H   | —  | 14.5 $\pm$ 2.6                  | 32.0 $\pm$ 3.0                    |
| <b>2c</b>                       |  | H   | OH  | —  | 18.0 $\pm$ 3.0                  | 67.8 $\pm$ 7.3                    |
| <b>2d</b>                       |  | OH  | OH  | —  | 1.6 $\pm$ 0.2                   | 4.9 $\pm$ 0.4                     |
| <b>3a</b>                       |  | H   | H   | —  | LA                              | 33.0 $\pm$ 7.0                    |
| <b>3b</b>                       |  | OH  | H   | —  | 37.4 $\pm$ 3.0                  | 34.8 $\pm$ 5.3                    |
| <b>3c</b>                       |  | H   | OH  | —  | LA                              | 26.9 $\pm$ 5.2                    |
| <b>3d</b>                       |  | OH  | OH  | —  | 13.5 $\pm$ 3.3                  | 14.7 $\pm$ 2.5                    |
| <b>4a</b> Chrysin               |  | H   | H   | H  | 53.0 $\pm$ 4.6                  | 9.59 $\pm$ 0.05                   |
| <b>4b</b>                       |  | OH  | H   | H  | 10.0 $\pm$ 2.0                  | 15.8 $\pm$ 0.8                    |
| <b>4c</b> Apigenin              |  | H   | OH  | H  | 24.0 $\pm$ 3.0                  | 7.7 $\pm$ 2.8                     |
| <b>4d</b> Acacetin              |  | H   | OMe | H  | LA                              | 7.0 $\pm$ 1.5                     |
| <b>4e</b> Luteolin              |  | OH  | OH  | H  | 9.3 $\pm$ 1.2                   | 9.5 $\pm$ 2.2                     |
| <b>4f</b> Chrysoeriol           |  | OMe | OH  | H  | 2.8 $\pm$ 0.5                   | 7.1 $\pm$ 0.3                     |
| <b>4g</b> Diosmetin             |  | OH  | OMe | H  | 3.6 $\pm$ 0.4                   | 15.0 $\pm$ 4.9                    |
| <b>4h</b>                       |  | OMe | OMe | H  | 57.6 $\pm$ 4.02                 | 16.0 $\pm$ 1.0                    |
| <b>4i</b> Quercetin             |  | OH  | OH  | OH | 26.0 $\pm$ 4.2                  | 11.3 $\pm$ 2.3                    |
| <b>5a</b> ( $\pm$ )-Naringenin  |  | H   | H   | —  | 51.4 $\pm$ 4.6                  | 31.0 $\pm$ 5.0                    |
| <b>5b</b> ( $\pm$ )-Eriodictyol |  | OH  | H   | —  | 18.0 $\pm$ 3.0                  | 15.5 $\pm$ 2.6                    |
| <b>5c</b> Taxifolin             |  | OH  | OH  | —  | 53.5 $\pm$ 2.4                  | 15.5 $\pm$ 4.6                    |
| Positive controls               |  |     |     |    |                                 |                                   |
| ABAH                            |  | —   | —   | —  | 43.6 $\pm$ 8.8                  | —                                 |
| Catalase                        |  | —   | —   | —  | 2597 $\pm$ 684 U/mL             | —                                 |
| SOD                             |  | —   | —   | —  | —                               | 48.5 $\pm$ 8.4 U/mL               |

LA - Less than 40% of inhibition neutrophils' oxidative burst, up to the highest tested concentration (100  $\mu$ M).

**e** and flavonol **4i**. ( $\pm$ )-Eriodictyol **5b** was the most potent of this last group ( $IC_{50}$  = 18.0  $\pm$  3.0  $\mu$ M). ABAH and catalase, which were used as positive controls, presented an  $IC_{50}$  of 43.6  $\pm$  8.8  $\mu$ M and 2597  $\pm$  684 U/mL, respectively. SOD, which was also used as a positive control, presented a 49.3  $\pm$  5.1% effect for the concentration of 1000 U/mL.

#### 3.4. Oxidation of lucigenin by neutrophils – generated $O_2^{\bullet-}$

All the studied flavonoids **1a–d**, **2a–d**, **3a–d**, **4a–i** and **5a–c** revealed good activity in modulating the production of the  $O_2^{\bullet-}$  or in its scavenging, compound **1c** being the less active of all the tested

flavonoids, as shown in Table 1. Flavone **2d** was the most effective of all ( $IC_{50}$  = 4.9  $\pm$  0.4  $\mu$ M), as it was for the probe luminol. For compounds **1a–d**, **1d** showed once more the lowest  $IC_{50}$ , 8.1  $\pm$  0.6  $\mu$ M. Flavones **2a–c** were considerably less active than flavone **2d**. In what concerns compounds **3a–d**, **3d** was the most effective ( $IC_{50}$  = 14.7  $\pm$  2.5  $\mu$ M), the other flavones from this group being significantly less potent. Regarding the results of **4a–i**, it is obvious that acacetin **4d**, chrysoeriol **4f** and apigenin **4c** were the most potent, presenting  $IC_{50}$  of 7.0  $\pm$  1.5  $\mu$ M, 7.1  $\pm$  0.3  $\mu$ M and 7.7  $\pm$  2.8  $\mu$ M, respectively. Once again, flavanones **5a–c** revealed to be less effective than the corresponding flavones **4c**, **e** and flavonol **4i**, but this difference is not as obvious as seen before for luminol.

The flavanone ( $\pm$ )-eriodictyol **5b** and taxifolin **5c** were the most potent of this group, with  $IC_{50}$ s of  $15.5 \pm 2.6 \mu M$  and  $15.5 \pm 4.6 \mu M$ , respectively. SOD, which was used as a positive control, presented an  $IC_{50}$  of  $48.5 \pm 8.4 U/mL$ .

### 3.5. Oxidation of amplex red by neutrophils – generated $H_2O_2$

Table 2 shows the results of the amplex red oxidation. It is clear that in group **1a–d** the most potent was compound **1d** ( $IC_{50} = 3.2 \pm 0.9 \mu M$ ). Compound **1b**, for the concentration of  $40 \mu M$ , inhibited the oxidation of amplex red just in  $17.0 \pm 3.4\%$ . The

other two flavonoids showed no activity at concentrations up to  $100 \mu M$ . From flavonoids **2a–d**, **2d** was the one that had the best activity ( $IC_{50} = 3.2 \pm 0.7 \mu M$ ), followed by **2b** and **2c** that presented an activity under 20% for  $40 \mu M$ . 5-Hydroxyflavone **2a** had no activity for the tested concentrations. In group **3a–d**, flavones **3d** and **3b** proved to be the most active, although the first one was considerably more potent than the second one, with  $IC_{50}$ s of  $3.7 \pm 0.5 \mu M$  and  $79.6 \pm 5.5 \mu M$ , respectively. In group **4a–i**, only chrysin **4a** and acacetin **4d** showed no activity at concentrations up to  $100 \mu M$ . The most effective was luteolin **4e** ( $IC_{50} = 4.0 \pm 1.0 \mu M$ ). From the flavanones **5a–c** it is noteworthy that ( $\pm$ )-eriodictyol **5b**

**Table 2**  
Chemical structures and inhibition of PMA-induced neutrophils' oxidative burst by the studied flavonoids, assessed by the fluorescent probes, amplex red and APF ( $IC_{50} \mu M$ , mean  $\pm$  SEM).

| Compound                                 | R1  | R2  | R3 | Amplex red<br>$IC_{50} (\mu M)$ | APF<br>$IC_{50} (\mu M)$ |
|------------------------------------------|-----|-----|----|---------------------------------|--------------------------|
| <b>1a Flavone</b>                        | H   | H   | —  | NA                              | $64.9 \pm 6.2$           |
| <b>1b</b>                                | OH  | H   | —  | LA                              | <sup>a</sup>             |
| <b>1c</b>                                | H   | OH  | —  | NA                              | <sup>a</sup>             |
| <b>1d</b>                                | OH  | OH  | —  | $3.2 \pm 0.9$                   | $0.9 \pm 0.2$            |
| <b>2a</b>                                | H   | H   | —  | NA                              | <sup>a</sup>             |
| <b>2b</b>                                | OH  | H   | —  | LA                              | <sup>a</sup>             |
| <b>2c</b>                                | H   | OH  | —  | LA                              | <sup>a</sup>             |
| <b>2d</b>                                | OH  | OH  | —  | $3.2 \pm 0.7$                   | $0.39 \pm 0.08$          |
| <b>3a</b>                                | H   | H   | —  | LA                              | <sup>a</sup>             |
| <b>3b</b>                                | OH  | H   | —  | $79.6 \pm 5.5$                  | <sup>a</sup>             |
| <b>3c</b>                                | H   | OH  | —  | LA                              | <sup>a</sup>             |
| <b>3d</b>                                | OH  | OH  | —  | $3.7 \pm 0.5$                   | $5.2 \pm 1.4$            |
| <b>4a Chrysin</b>                        | H   | H   | H  | NA                              | NA                       |
| <b>4b</b>                                | OH  | H   | H  | LA                              | $65.4 \pm 4.6$           |
| <b>4c Apigenin</b>                       | H   | OH  | H  | $16.2 \pm 3.3$                  | $12.3 \pm 2.2$           |
| <b>4d Acacetin</b>                       | H   | OMe | H  | NA                              | NA                       |
| <b>4e Luteolin</b>                       | OH  | OH  | H  | $4.0 \pm 1.0$                   | $0.9 \pm 0.1$            |
| <b>4f Chrysoeriol</b>                    | OMe | OH  | H  | $8.6 \pm 1.8$                   | $0.4 \pm 0.2$            |
| <b>4g Diosmetin</b>                      | OH  | OMe | H  | $10.3 \pm 1.2$                  | $0.7 \pm 0.3$            |
| <b>4h</b>                                | OMe | OMe | H  | LA                              | $3.33 \pm 0.09$          |
| <b>4i Quercetin</b>                      | OH  | OH  | OH | $8.0 \pm 3.0$                   | $0.7 \pm 0.1$            |
| <b>5a (<math>\pm</math>)-Naringenin</b>  | H   | H   | —  | LA                              | <sup>a</sup>             |
| <b>5b (<math>\pm</math>)-Eriodictyol</b> | OH  | H   | —  | $4.0 \pm 1.0$                   | $1.4 \pm 0.2$            |
| <b>5c Taxifolin</b>                      | OH  | OH  | —  | $41.3 \pm 11.9$                 | $15.3 \pm 2.7$           |
| Positive controls                        |     |     |    |                                 |                          |
| ABAH                                     | —   | —   | —  | —                               | $30.6 \pm 6.1$           |
| Catalase                                 | —   | —   | —  | $386.9 \pm 6.9 U/mL$            | —                        |

NA – No activity was found up to the highest tested concentration ( $100 \mu M$ ).

LA – Less than 40% of inhibition neutrophils' oxidative burst, up to the highest tested concentration ( $100 \mu M$ ).

<sup>a</sup> Interference with the methodology.

presented an  $IC_{50}$  similar to the corresponding flavone, luteolin **4e**,  $IC_{50} = 4.0 \pm 1.0 \mu M$ . Catalase, which was used as a positive control, presented an  $IC_{50}$  of  $386.9 \pm 6.9 U/mL$ .

### 3.6. Oxidation of APF by neutrophils-generated HOCl

In what concerns APF assays, all the flavonoids that could be tested presented a great capacity of scavenge or preventing the production of HOCl during the oxidative burst (Table 2). From flavonoids **1a–d** it is clear that the most potent was **1d** ( $IC_{50} = 0.9 \pm 0.2 \mu M$ ), followed by **1a** ( $IC_{50} = 64.9 \pm 6.2 \mu M$ ). In group **2a–d**, flavonoid **2d** was the only one that could be tested, showing a potent activity,  $IC_{50} = 0.39 \pm 0.08 \mu M$ . In group **3a–d**, **3d** was the only one that did not interfere with the methodology and presented a  $IC_{50}$  of  $5.2 \pm 1.4 \mu M$ . Regarding the results of the flavonoids **4a–i**, the order of potencies found was: chrysoeriol **4f** ( $IC_{50} = 0.4 \pm 0.2 \mu M$ ) > quercetin **4i** = diosmetin **4g** > luteolin **4e** > **4h** > apigenin **4c** > **4b** ( $IC_{50} = 65.4 \pm 4.6 \mu M$ ). The flavones chrysin **4a** and acacetin **4d** had no activity at concentrations up to  $100 \mu M$ . Analysing the  $IC_{50}$  of the flavanones **5a–c**, (±)-eriodictyol **5b** appeared to be the best neutrophils' oxidative burst modulator, with an  $IC_{50}$  of  $1.4 \pm 0.2 \mu M$ , followed by taxifolin **5c**. It was not possible to evaluate the other tested flavonoids using this screening test because those compounds interfered with the methodology, causing an increase in the fluorescence signal in a concentration-dependent manner, in the presence of the stimulated neutrophils. ABAH, which was used as a positive control, presented an  $IC_{50}$  of  $30.6 \pm 6.1 \mu M$ .

## 4. Discussion

The group of flavonoids evaluated in the present study, **1a–d**, **2a–d**, **3a–d**, **4a–i** and **5a–c**, enabled the establishment of important structure/activity relationships, and presented potent compounds, concerning the modulation of the human neutrophils' oxidative burst. The observed modulation of the oxidative burst may arise from the flavonoids' effects on different processes, namely ROS-scavenging or inhibition of their production, by interfering with the release or the activity of different enzymes, like NADPH-oxidase or MPO, although one should also consider that these activities could be conditioned by the spatial conformation or the lipophilicity of the compounds studied and their possible interaction with cellular macromolecules.

Luminol is a highly sensitive chemiluminescent probe widely used to detect  $O_2^{\cdot -}$ ,  $HO^{\cdot}$ ,  $H_2O_2$ , HOCl,  $\cdot NO$  and  $ONOO^-$ . Due to its chemical structure, small size and lipophilicity, luminol is able to diffuse across biological membranes and consequently allow the detection of both extra and intracellular production of reactive species. Among all the studied flavonoids, **2d** was the one that demonstrated the best inhibition of the chemiluminescence ( $IC_{50} = 1.6 \pm 0.2 \mu M$ ), followed by chrysoeriol **4f**, **1d** and diosmetin **4g**, with  $IC_{50}$ s of  $2.8 \pm 0.5$ ,  $3.0 \pm 0.4$  and  $3.6 \pm 0.4 \mu M$ , respectively. All of these compounds are substituted in the B-ring, **2d** and **1d** have in common a catechol group in the B-ring, and the ones from group **4**, **4f** and **4g**, have a methoxyl group and a hydroxyl group in the same ring. The results obtained indicate that the modulating activity of flavonoids may be determined by the B-ring substitution, the presence of a catechol group being the most important factor for a high activity. Interestingly, methylation of the 3'-hydroxyl group might lead to similarly efficient compounds, though the absence of this hydroxyl group decreased the activity, as ascertained by comparison of chrysoeriol **4f** with apigenin **4c**, with  $IC_{50}$ s of  $2.8 \pm 0.5$  and  $24.0 \pm 3.0 \mu M$ , respectively. These observations are in agreement with the findings of Mladenka and coworkers [12]. The substitution pattern in A-ring did not seem determinant for a

good activity. When a catechol group was present in the B-ring, the 5-OH seemed to be more important than the 7-OH, which apparently contributed to diminish the activity, **2d** vs **3d**, with  $IC_{50}$ s of  $1.6 \pm 0.2$  and  $13.5 \pm 3.3 \mu M$ , respectively. Moreover, the existence of more than one hydroxyl in the A-ring also did not favour the activity, **2d** vs luteolin **4e**, with  $IC_{50}$ s of  $1.6 \pm 0.2$  and  $9.3 \pm 1.2 \mu M$ , respectively. The diffusion of chemical compounds into the cell is a function of their lipophilicity, i.e. their ability to incorporate in the lipids of the cell membrane. Replacement of a hydroxyl by a methoxyl group increases the lipophilicity [33], thus enhancing the inhibitory activity of chrysoeriol **4f** ( $IC_{50} = 2.8 \pm 0.5$ ) and diosmetin **4g** ( $IC_{50} = 3.6 \pm 0.4$ ) comparing with luteolin **4e** ( $IC_{50} = 9.3 \pm 1.2 \mu M$ ). Landi-Librandi and coworkers [34] undertook a study with liposomal flavonoids and concluded that the ability to inhibit the chemiluminescence of luminol was a function of the lipophilicity of the compounds. In spite of this, flavone **4h**, which has two methoxyl groups in the B-ring presented a low activity ( $IC_{50} = 57.6 \pm 4.02 \mu M$ ), which may indicate that the lipophilicity is not by itself determinant for the activity observed. In fact, the more hydrophobic flavonoids can partitionate in the hydrophobic core of the membranes and the more hydrophilic flavonoids interact by hydrogen bonding with the polar head groups at the lipid–water interface of membranes. This type of interaction may provide a level of protection for the bilayer from external and internal aggressors (i.e. oxidants) contributing to preserve the structure and function of biological membranes, in part by inhibiting the lipid oxidation chain reaction. A possible consequence of this interaction is a direct modulation of membrane physical properties on the capacity of flavonoids to efficiently scavenge free radicals [35,36]. It was already stated that luminol detects unspecifically a series of reactive species and the effect observed may be caused by the inhibition of a number of different processes. Thus, to assess the flavonoids activity in more detail it was necessary to use different and more specific probes.

In this sense, lucigenin was used to specifically detect  $O_2^{\cdot -}$ . It has been suggested that  $O_2^{\cdot -}$  production might be artificially overestimated because lucigenin may undergo redox cycling. Notwithstanding this possibility, Afanas'ev and coworkers [37] attested that the redox cycling of lucigenin is negligible, particularly in cellular systems that produce significant amounts of  $O_2^{\cdot -}$ , and for that reason lucigenin-amplified chemiluminescence remains the most sensitive and highly specific test for  $O_2^{\cdot -}$  in biological systems [22].  $O_2^{\cdot -}$  is the first reactive specie produced during the oxidative burst, from which starts the reactive species cascade formation. Therefore its scavenging, or production inhibition, is crucial for the modulation of the oxidative burst. The results obtained revealed that flavonoid **2d** was the most effective in inhibiting the oxidation of lucigenin, with an  $IC_{50}$  of  $4.9 \pm 0.4 \mu M$ . Flavones acacetin **4d**, chrysoeriol **4f** and apigenin **4c** presented almost the same capacity of inhibiting the oxidative burst, with an  $IC_{50}$  of  $7.0 \pm 1.5$ ,  $7.1 \pm 0.3$  and  $7.7 \pm 2.8 \mu M$ , respectively, followed by flavone **1d**, with an  $IC_{50}$  of  $8.1 \pm 0.6 \mu M$ . In a more general approach of the results, when the flavonoids presented none or just one hydroxyl group in the A-ring, it is clear that the hydroxyl groups in the B-ring are essential for a higher activity, **1a** vs **1d**, with  $IC_{50}$ s of  $57.0 \pm 7.5$  and  $8.1 \pm 0.6 \mu M$ , and **2a** vs **2d**, with  $IC_{50}$ s of  $96.0 \pm 4.0$  and  $4.9 \pm 0.4 \mu M$ , respectively. When the flavonoid presented two hydroxyl groups in the A-ring, and a *meta*-methylation of the catechol in the B-ring, a greater activity was registered, luteolin **4e** vs chrysoeriol **4f**, with  $IC_{50}$ s of  $9.5 \pm 2.2$  and  $7.1 \pm 0.3 \mu M$ , respectively. Thus, in this case, the unsubstituted catechol in the B-ring does not seem to be so determinant for a higher activity. In addition, in group **4a–i**, the presence of just one 4'-OH in the B-ring rendered apigenin **4c**, one of the most potent in this group of flavonoids, with an  $IC_{50}$  of  $7.7 \pm 2.8 \mu M$ . Actually, Hu and coworkers

[38] concluded that the presence of hydroxyl groups in the B-ring contributes to a higher  $O_2^{\cdot -}$  scavenging activity. On the other hand, the free radical scavenging activity may not be the main mechanism governing the inhibitory effect of flavonoids [34]. Actually, bibliography indicates that (i) flavonoids containing an unsubstituted catechol in the B-ring (quercetin **4i**, luteolin **4e**) do not inhibit NADPH-oxidase; (ii) methylation of the catechol arrangement in the B-ring (chrysoeriol **4f** vs luteolin **4e**) or the omission of one OH group converts the flavonoid into an NADPH-oxidase inhibitor; (iii) among flavonoids of non-catechol type, those possessing a 4'-OH group in the B-ring are NADPH-oxidase inhibitors (apigenin **4c** vs chrysin **4a**) and (iv) the presence of a vicinal (*ortho*) hydroxy-methoxy arrangement at an aromatic ring generally enhances the inhibitory potency, but is not essential for the inhibitory capacity [12,16]. The results obtained in the present work are in agreement with these statements, so probably the tested flavonoids act as  $O_2^{\cdot -}$  scavengers as well as inhibitors of its production, by interfering with NADPH-oxidase. It should also be clear that here the flavanones **5a–c** were not more active than the corresponding flavones **4c**, **4e** or flavonol **4i**. In fact, the  $IC_{50}$  of quercetin **4i** and taxifolin **5c** are not so different, with  $IC_{50}$ s of  $11.3 \pm 2.3$  and  $15.5 \pm 4.6$   $\mu$ M, respectively, though the same cannot be said about the other four flavonoids (Table 1). Therefore it was not clear if the C<sub>2</sub>–C<sub>3</sub> double bond in the C-ring is or not determinant for the inhibition of the lucigenin reduction.

To detect the extracellular  $H_2O_2$  released by human neutrophils, we used a highly specific and sensitive fluorescent probe, amplex red. Among all the tested flavonoids the most potent inhibitors of the oxidation of amplex red were those that present a catechol moiety in the B-ring, with this order of effect: **2d** ( $IC_{50} = 3.2 \pm 0.7$   $\mu$ M) > **1d** > **3d** > luteolin **4e** = ( $\pm$ )-eriodictyol **5b** ( $IC_{50} = 4.0 \pm 1.0$   $\mu$ M) (Table 2). Hence, this moiety clearly displays a crucial importance for the inhibition in study. Noteworthy, a 7-OH group in the A-ring appeared to be as important as the existence of a 5-OH or even none hydroxyl groups, **3d**, **2d** and **1d**, with  $IC_{50}$ s of  $3.7 \pm 0.5$ ,  $3.2 \pm 0.7$  and  $3.2 \pm 0.9$   $\mu$ M, respectively. As well, the existence of two hydroxyl groups in position 5 and 7 in the A-ring simultaneously did not contribute for a higher activity, **3d**, **2d** or **1d** vs luteolin **4e**, with an  $IC_{50}$  of  $4.0 \pm 1.0$   $\mu$ M. However, the methoxyhydroxylated flavonoids, chrysoeriol **4f** and diosmetin **4g**, and the one with a 3-OH group in the C-ring, quercetin **4i**, presented good activities, with  $IC_{50}$ s of  $8.6 \pm 1.8$ ,  $10.3 \pm 1.2$  and  $8.0 \pm 3.0$   $\mu$ M, respectively, demonstrating that these features have some meaning in the inhibition of amplex red oxidation. Flavonoids that did not possess a catechol moiety in the B-ring displayed very low activities or no activity at all, as for example **1b** or chrysin **4a**. Nevertheless, it should be stated that amplex red only detects the extracellular  $H_2O_2$ , giving information about the ability of the flavonoids to scavenge or inhibit the formation of this reactive specie, but not the total effect because the intracellular  $H_2O_2$  is not taken in account. In what concerns the flavanones **5a–c** tested, ( $\pm$ )-eriodictyol **5b** stood out among all being much more active, with an  $IC_{50}$  of  $4.0 \pm 1.0$   $\mu$ M, demonstrating that even in the absence of a C<sub>2</sub>–C<sub>3</sub> double bond, the 3',4'-(OH)<sub>2</sub> in the B-ring remained with the pivotal role and the 3-OH did not contribute for a good activity. Moreover, ( $\pm$ )-eriodictyol **5b** and luteolin **4e** showed the same activity, which made clear that with this OH's substitution pattern, the C<sub>2</sub>–C<sub>3</sub> is not important for the studied effect. On the other hand, this is not true for quercetin **4i** and taxifolin **5c** (Table 2). For example, the first one is much more active than the second, with  $IC_{50}$ s of  $8.0 \pm 3.0$  and  $41.3 \pm 11.9$   $\mu$ M, respectively. Taxifolin **5c** has been already described as having a low capacity to scavenge  $H_2O_2$  and organic peroxides and a low antioxidant capacity in comparison with quercetin **4i**, in studies undertaken in retinal cells after ascorbate/ $Fe^{2+}$ -induced oxidative stress, and it has been suggested that differences may arise from the

less planar structure of taxifolin which results in a higher ability to undergo inactivation through the formation of strong hydrogen bonds with macromolecules [39,40].

APF is a non-fluorescent derivative of fluorescein that originates fluorescein intracellularly, allowing the selective detection of HOCl, in a concentration-dependent manner, as it was demonstrated by using ABAH. Because of the high concentration of  $Cl^-$  in body fluids, MPO has the specialized ability to oxidize chloride and it is generally considered that its function is to generate HOCl, which represents a critical step in the production of many other bio-reactive molecules, namely chloramines, which are more toxic than HOCl itself [22]. Therefore the capacity of scavenging HOCl or modulating its production is very important for the modulation of neutrophils' oxidative burst. Flavone **2d** kept being the most active ( $IC_{50} = 0.39 \pm 0.08$   $\mu$ M), followed by other flavonoids, in descending order of potencies: chrysoeriol **4f** ( $IC_{50} = 0.4 \pm 0.2$   $\mu$ M) > quercetin **4i** > diosmetin **4g** > luteolin **4e** > **1d** > ( $\pm$ )-eriodictyol **5b** ( $IC_{50} = 1.4 \pm 0.2$   $\mu$ M). Analysing these results, and the chemical structures of the tested compounds, we can infer that the most active flavonoid **2d** has a 5-OH group in the A-ring and a catechol group in the B-ring, though all the others hold a 5,7-OH or a 7-OH in the A-ring. Thus, probably, the presence of two hydroxyl groups in the A-ring is not really important for the activity. Moreover, chrysoeriol **4f** and diosmetin **4g** present a methoxylation in the B-ring which appeared to contribute for the activity, with  $IC_{50}$ s of  $0.4 \pm 0.2$  and  $0.7 \pm 0.3$   $\mu$ M, respectively, as well as the 3-OH in the C-ring, because quercetin **4i** proved to be a little more active than luteolin **4e**, with  $IC_{50}$ s of  $0.7 \pm 0.1$  and  $0.9 \pm 0.1$   $\mu$ M, respectively. The 3-OH substitution has already been described as an important feature for the HOCl scavenging activity, considering that quercetin **4i** showed the strongest effect in a cyclic voltammetric analysis [41]. In this same study, luteolin **4e** and apigenin **4c** also showed a good scavenging activity. In general, excluding those flavonoids not tested due to the interference with the methodology, it became clear that the presence of two substitution groups in positions 3'- and 4'- in the B-ring is essential for a good activity (**1b** vs **1d** and **4a** vs luteolin **4e**). In 1988, Pincemail and coworkers [42] have already stated that quercetin **4i** was an inhibitor of MPO activity, indicating that it behaved as a competitive inhibitor. More recently, a docking study suggested that quercetin **4i** binds to the hydrophobic region at the entrance to the distal haeme pocket of MPO, with the B-ring of quercetin being oriented to the haeme center. The results also indicated that the B-ring's hydroxyl group(s) of flavonoids may be important for entering the pocket, reaching the active site of MPO. Moreover, quantitative structure–activity relationship analysis suggested that the existence of the 3-, 4'-, and 5-OHs, and C<sub>2</sub>–C<sub>3</sub> double bond were required for the inhibitory effect [43]. These results suggested that quercetin **4i** and related flavonoids are effective substrates for the MPO enzyme. Regarding flavanones, they showed to be less active than the corresponding flavone, ( $\pm$ )-eriodictyol **5b** vs luteolin **4e**, with  $IC_{50}$ s of  $1.4 \pm 0.2$  and  $0.9 \pm 0.1$   $\mu$ M, respectively or flavonol, taxifolin **5c** vs quercetin **4i**, with  $IC_{50}$ s of  $15.3 \pm 2.7$  and  $0.7 \pm 0.1$   $\mu$ M, respectively, enlightening the importance of the C<sub>2</sub>–C<sub>3</sub> double bond for the inhibition of the amplex red oxidation. It was also clearly demonstrated that among the flavanones tested **5a–c**, the 3-OH group in the C-ring did not play a pivotal role for a good activity. Hart and coworkers [44] conducted a study on human neutrophils stimulated with serum treated zymosan and concluded that taxifolin **5c** and eriodictyol **5b** inhibited the release of MPO by 50–60% and from those, 40% of the activity were also inhibited. In turn, luteolin **4e** inhibited almost in 100% the release of MPO, though it barely inhibited the MPO activity. Using a combination of biological assays and theoretical calculation studies, Shiba and coworkers [43] suggested this order of inhibitory effect on MPO: quercetin **4i** > luteolin **4e** > taxifolin

**5c**, what is in agreement with our results. According to this order of potencies, they concluded that, the activity of the flavonoids lacking the 3-OH in the C-ring, compared to quercetin **4i**, was relatively weak, showing the significance of the number and position of the hydroxyl group on the inhibitory effect. In addition, taxifolin **5c**, which contains the same number and positions of hydroxyl groups as quercetin **4i**, exhibited weak activity, showing the requirement of a C<sub>2</sub>–C<sub>3</sub> double bond for the activity. These main conclusions are identical to the ones reached in this work for the modulation of the oxidative burst, related to HOCl scavenging or production. It should be noted that MPO enzyme is localized inside of the phagolysosome and that APF detects the intracellular HOCl, thus, the activity of the flavonoid is not just a function of its real activity but also a function of its lipophilicity, i.e., of its capacity to interact with the cell membranes. This may explain why flavone **2d** is the most active, even with less hydroxyl groups, because it is less hydrophobic and therefore can penetrate easily into membranes and reach the active site in higher concentrations. This could also explain why the flavones with a methoxylation in the B-ring **4f–h** had become such good inhibitors of APF oxidation, almost as good as quercetin **4i** or even better.

To the best of our knowledge, some of the flavonoids tested in this work were never studied for this kind of modulation activity, allowing us to do a better integration of the obtained results. Thus, the present study evidences the catechol group in the B-ring as the pivotal characteristic that renders flavonoids good modulators of the neutrophils' oxidative burst. It was also clearly shown that the number of hydroxyl groups really influences the activity, as the flavone **1a** did not present good activity for any of the assays, when compared with the other tested flavonoids. In addition, the current study extends knowledge on the importance of the hydroxylations in the A-ring, which did not appear to be determinant for the activity, although clearly influence the lipophilicity of the tested flavonoids and consequently the capacity to pass through the cell membranes. Another main feature that contributed for higher activity was the presence of a 3'-methoxyl group in the B-ring. On the other hand, the presence of the 3-OH in the C-ring and the C<sub>2</sub>–C<sub>3</sub> double bond just became important features when the H<sub>2</sub>O<sub>2</sub> and HOCl reactive species were studied. Overall, these observations indicate that the studied flavonoids are undoubtedly good modulators of the human neutrophils' oxidative burst, at very low concentrations, which may represent an excellent new strategy for the resolution of inflammatory processes.

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## ***CHAPTER III. Original Research***

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### **III.2. Inhibition of LOX by flavonoids: a structure-activity relationship study**

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## Original article

## Inhibition of LOX by flavonoids: a structure–activity relationship study



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## ABSTRACT

The lipoxygenase (LOX) products have been identified as mediators of a series of inflammatory diseases, namely rheumatoid arthritis, inflammatory bowel disease, psoriasis, allergic rhinitis, atherosclerosis and certain types of cancer. Hence, LOX inhibitors are of interest for the modulation of these phenomena and resolution of the inflammatory processes. During LOX activity, peroxy radical complexes are part of the reaction and may function as sources of free radicals. Thus antioxidants, such as flavonoids, capable of inhibiting lipid peroxidation and scavenging free radicals, may act as LOX inhibitors. The aim of this work was to assess the structure–activity relationship among a series of flavonoids concerning 5-LOX inhibition, through a systematic study of the inhibition of the formation of LTB<sub>4</sub> in human neutrophils. The type of inhibition of the flavonoids was further studied using soybean LOX, type I, and Saturation Transfer Difference <sup>1</sup>H NMR (STD-<sup>1</sup>H NMR) was used to characterize the binding epitopes of the compounds to LOX-1. The obtained results reinforce flavonoids as effective inhibitors of LTB<sub>4</sub> production in human neutrophils. It was also possible to establish a structure/activity relationship for the inhibitory activity and the type of inhibition.

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## 1. Introduction

Lipoxygenases (LOXs) constitute a family of nonheme iron-containing dioxygenases ubiquitously distributed in plants, fungi and animals. In humans there are six functional LOXs: 5-lipoxygenase (5-LOX), 12/15-LOX (15-LOX-1), platelet-type 12-LOX, epidermis-type 12R-LOX, epidermis-type 15-LOX (15-LOX-2) and epidermis-Alox3 [1]. These enzymes catalyze four distinct reactions (5S, 12R, 12S, or 15S oxygenation), using arachidonic acid (AA) as substrate, and producing fatty acid hydroperoxides. [2]

5-LOX is expressed in leukocytes, neutrophils, eosinophils, basophils and monocytes/macrophages [3]. In resting cells, 5-LOX is located either in the cytosol, e.g. in neutrophils, or in a nuclear soluble compartment associated with chromatin. Upon stimulation, in neutrophils, 5-LOX translocates from the cytosol to the nuclear envelope and contiguous perinuclear endoplasmic

reticulum. Simultaneously, cytosolic phospholipase A2 (cPLA2) migrates and liberates AA from phospholipids. Membrane-bound 5-LOX activating protein (FLAP) acts as an AA transfer protein that “presents” the substrate, AA, to 5-LOX on the nuclear membrane [4,5]. The leukotrienes (LTs) biosynthesis begins with the dioxygenation of AA, which originates 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) and leukotriene A<sub>4</sub> (LTA<sub>4</sub>). The latter is converted in LTB<sub>4</sub> by LTA<sub>4</sub>-hydrolase. 5-HPETE can also be reduced by glutathione peroxidases to form the corresponding 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE) [3]. The biosynthesis of these LOX products play an important role in a variety of disorders related with inflammatory processes, through the interaction with the LTB<sub>4</sub> receptors, BLT1 and BLT2, the first one expression is upregulated in inflammation [6]. LTs have been identified as mediators of a series of inflammatory diseases, namely rheumatoid arthritis, inflammatory bowel disease, psoriasis, allergic rhinitis, bronchial asthma, atherosclerosis and certain types of cancer [7,8]. LTB<sub>4</sub> participates in a range of pathophysiological events and exerts its activity by the recruitment of leukocytes to the inflammation sites. It has been demonstrated to

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be a potent neutrophil chemoattractant in humans *in vivo* [9,10]. Additionally, LTB<sub>4</sub> induces neutrophil adhesion, resulting in neutrophil aggregation and stimulates neutrophil generation of reactive oxygen species, release of granular enzymes and phagocytosis of bacteria. LTB<sub>4</sub> also extends neutrophil survival by preventing apoptosis. These effects contribute to the accumulation of effector leukocytes at the invasion sites of pathogen as part of host immune response [6]. It is worth to notice that in other cells, like eosinophils, basophils, mast cells and monocytes, other leukotrienes are produced, LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>. They are also involved in the inflammatory processes being important mediators in bronchoconstriction and in hypersensitivity reactions [7]. Hence, LOX inhibitors might be of interest for the modulation of these phenomena and for the resolution of the inflammatory processes. Presently available LTs inhibitors may be divided in redox-active compounds, iron-ligand inhibitors with weak redox-active properties and non-redox-type inhibitors. Nordihydroguaiaretic acid (NDGA) and coumarins are examples of redox-active compounds. However they are nonselective, occasioning severe side effects like methemoglobin formation or are rapidly metabolized [8]. Iron chelating hydroxamic acid derivatives also proved to inhibit chronic LTB<sub>4</sub> production, though they presented extensive metabolism and catabolite accumulation *in vivo* along with unfavorable pharmacokinetic properties [11]. The classification of non-redox-type inhibitors relates to the lack of redox activity of the drugs themselves but does not exclude that the inhibitory potency of the drugs is affected by the redox state of their target, the 5-LOX. Some of these drugs were already tested by oral administration, though despite their strong potency in several *ex vivo* and *in vitro* models, they failed to toughly inhibit LTs synthesis at sites of chronic

inflammation [8]. During the LOX-mediated metabolism of AA, peroxy radical complexes are part of the reaction, and may also function as sources of free radicals. Thus antioxidants, such as flavonoids, capable of inhibiting lipid peroxidation and scavenging free radicals, may be potential LOX inhibitors [12–16]. Thus, the plethora of beneficial pharmacological effects mediated by flavonoids [17–19] and their anti-inflammatory effects may constitute a solid alternative to the traditional anti-inflammatory molecules, in the search for molecules presenting less side effects and higher efficacy [20]. Still, the precise mechanisms of anti-inflammatory action have not been fully disclosed so far. Nonetheless, this action may comprise the inhibition of inflammatory enzymes of the AA's cascade, including 5-LOX [21,22]. Accordingly, the aim of this work was to assess the structure–activity relationship among a series of flavonoids (Fig. 1) concerning 5-LOX inhibition, through a systematic study of the inhibition of the formation of LTB<sub>4</sub> in human neutrophils. The type of inhibition of the flavonoids was further studied using soybean LOX, type I (LOX-1) and Saturation Transfer Difference 1H NMR (STD-1H NMR) was applied to investigate the interactions between the studied flavonoids and LOX-1 and to determine the binding epitope in the several ligands.

## 2. Materials and methods

### 2.1. Reagents

The following reagents were obtained from Sigma–Aldrich Co. LLC (St. Louis, USA): trypan blue solution 0.4%, dimethylsulfoxide (DMSO), Hank's balanced salt solution (HBSS), nordihydroguaiaretic acid (NDGA), calcium ionophore (A23187), arachidonic acid

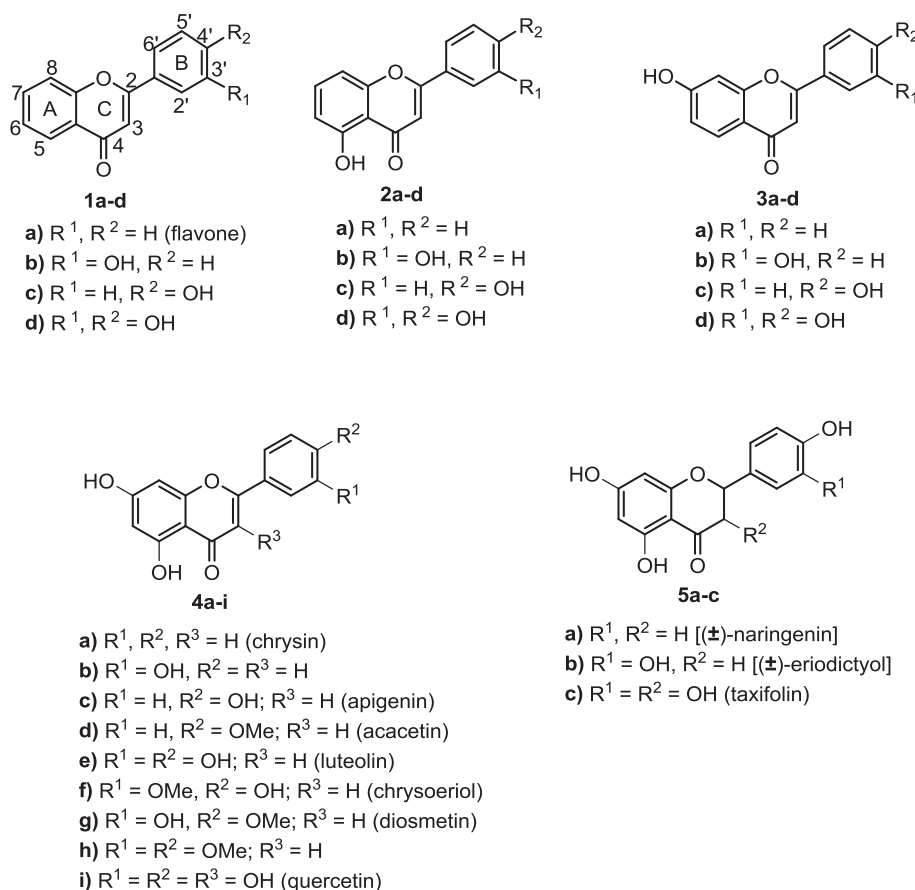


Fig. 1. Chemical structures of the studied flavonoids.

(AA), methanol, soybean LOX-1 type I-B (LOX-1), linoleic acid, magnesium chloride ( $\text{MgCl}_2$ ), apigenin, chrysin, luteolin, ( $\pm$ )-naringenin, ( $\pm$ )-eriodictyol, and quercetin. Boric acid and borax were obtained from Merck (Darmstadt, Germany). Tris base and DL-dithiothreitol (DTT) were obtained from Nzytech (Lisbon, Portugal). Ammonium sulfate  $[(\text{NH}_4)_2\text{SO}_4]$  was obtained from Scharlau (Barcelona, Spain). Deuterium oxide ( $\text{D}_2\text{O}$ ) was obtained from Eurisotop (Gif-sur-Yvette, France). Dimethylsulfoxide- $d_6$  ( $\text{DMSO}-d_6$ ) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, USA). Acacetin and taxifolin were obtained from Fluka Chemie GmbH (Steinheim, Germany). The flavonoids **4b**, **f**, **g** and **h** were synthesized by co-authors of this manuscript, from the Departamento de Química & QOPNA, Universidade de Aveiro [23]. The other tested flavonoids were obtained from Indofine Chemical Company, Inc. (Stryker Lane Hillsborough, USA). The “Leukotriene B4 Enzyme Immunoassay (EIA) Kit” was obtained from Cayman Chemical (Ann Arbor, MI).

## 2.2. Equipment

A multimode microplate reader (Synergy HT, BIO-TEK), with temperature control capacity, was used in the spectrophotometric readings in all the assays. All STD-NMR experiments were acquired at 37 °C in a Bruker Avance III spectrometer operating at 600 MHz, with a 5 mm triple resonance cryogenic probe head.

## 2.3. Isolation and treatment of human neutrophils

Neutrophils were isolated from blood collected from healthy human volunteers following informed consent. Venous blood was collected by antecubital venipuncture, into  $\text{K}_3\text{EDTA}$  vacuum tubes. The isolation of the human neutrophils was performed by the density gradient centrifugation method as previously reported [24]. Cell viability and cell yield were evaluated by the trypan blue exclusion method, using a Neubauer chamber and an optic microscope with the 40 $\times$  magnification. HBSS was used as incubation medium and the isolated neutrophils' suspensions were kept on ice until use. Neutrophil suspensions ( $3.5 \times 10^6$  cells/mL) in HBSS were placed in 96-well microplates (140  $\mu\text{L}$ /well) at 37 °C for 10 min to equilibrate. The tested flavonoids (0.15–40  $\mu\text{M}$ ) were then added, and pre-incubated for 10 min. The cells were subsequently incubated with A23187 (5  $\mu\text{M}$ ) and AA (10  $\mu\text{g}/\text{mL}$ ) for 8 min. The reaction was stopped by the addition of cold methanol. Samples were subsequently centrifuged at 13,000 g for 1 min, at 4 °C [25].

## 2.4. Determination of LTB4 production in human neutrophils

The amount of LTB4 in the samples was measured using the above mentioned commercial EIA kit, according to the manufacturer's instructions. The 5-LOX inhibitor, NDGA (1  $\mu\text{M}$ ), was used as positive control. The results are expressed as the percent inhibition of control LTB4 production. Each study corresponds to at least three experiments.

## 2.5. Soybean LOX-1 inhibition assay

This kinetic study was performed to determine the type of inhibition of the tested compounds. The assay was carried out by monitoring the LOX-1-mediated transformation of linoleic acid into linoleic acid cis,trans-hydroperoxide derivative, at 234 nm, as previously described [26], with modifications. The reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 300  $\mu\text{L}$ ): soybean LOX-1 (2000 U/mL), in borate buffer 0.2 M, pH 9.0, tested compounds at various concentrations (0–100  $\mu\text{M}$ ), in DMSO, and linoleic acid at various

concentrations (0–200  $\mu\text{M}$ ). LOX-1 was pre-incubated with the tested compounds for 5 min before the addition of the substrate, linoleic acid, and the measurements were carried out at 30 °C, subjected to continuous soft shaking. The increase in absorbance was monitored for 10 min, with readings at each 30 s, and the obtained values correspond to the slope measured between 0 and 3 min. The type of inhibition (competitive, uncompetitive, noncompetitive) of the tested compounds was studied by using the nonlinear regression Michaelis–Menten enzyme kinetics and the corresponding Lineweaver–Burk plots for each concentration of the inhibitor (three different concentrations in each case) for three different concentrations of substrate. Each study corresponds to, at least, four individual experiments, performed in triplicate in each experiment.

## 2.6. STD- $^1\text{H}$ NMR studies

### 2.6.1. Samples for STD-NMR

The soybean LOX-1 was supplied lyophilized. Thus a Tris buffer solution was used (20 mM Tris, 7 mM  $(\text{NH}_4)_2\text{SO}_4$ , 3.5 mM  $\text{MgCl}_2$  and 0.3 mM DTT in  $\text{D}_2\text{O}$ ) [27]. For the STD-NMR experiments, the tested compounds stock solutions (16.6 mM) were prepared in  $\text{DMSO}-d_6$ . From these, an amount of 15  $\mu\text{L}$  was added to the LOX solution directly in the NMR tube. Then the Tris buffer solution previously prepared at pH 9.0 was used to adjust the volume to 200  $\mu\text{L}$ . Final concentrations of LOX and ligands were 4  $\mu\text{M}$  and 1248  $\mu\text{M}$ , respectively, and the ratio inhibitors/protein was kept to 300:1.

### 2.6.2. NMR Spectroscopy

The STD-NMR spectra were acquired with 384 transients in a matrix with 64 K data points in  $t_2$  in a spectral window of 12335 Hz centered at 2825 Hz. Excitation sculpting with gradients was employed to suppress the water proton signals.  $n\text{A}$  spin lock filter ( $T_{1\rho}$ ) with a 2 kHz field and a length of 20 ms was applied to suppress protein background. Selective saturation of protein resonances (on resonance spectrum) was performed by irradiating at –600 Hz using a series of 40 Eburp2.1000 shaped 900 pulses (50 ms, 1 ms delay between pulses) for a total saturation time of 2.0 s. For the reference spectrum (off resonance) the samples were irradiated at 20000 Hz. Proper control experiments were performed with the reference samples in order to optimize the frequency for protein saturation (–1.0 ppm) and off-resonance irradiation, to ensure that the ligand signals were not affected. The STD effect was calculated using  $(I_0 - I_{\text{STD}})/I_0$ , in which  $(I_0 - I_{\text{STD}})$  is the peak intensity in the STD spectrum and  $I_0$  is the peak intensity in the off-resonance spectrum. The STD intensity of the largest STD effect was set to 100% as a reference, and the relative intensities were determined [28–30].

## 2.7. Statistical analysis

Statistics were calculated using GraphPad Prism™ (version 6.0; GraphPad Software). Results are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical comparison between groups was estimated using the one-way analysis of variance (ANOVA), followed by the Bonferroni's post-hoc test. In all cases,  $p$ -values lower than 0.05 were considered as statistically significant. The type of inhibition parameters were all calculated with GraphPad Software.

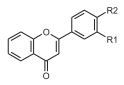
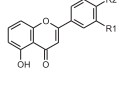
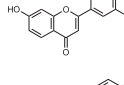
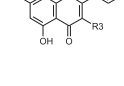
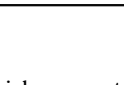
## 3. Results

### 3.1. Determination of LTB4 production by human neutrophils

It is possible to establish three distinct groups among all the studied flavonoids. The first group comprises the most active

**Table 1**

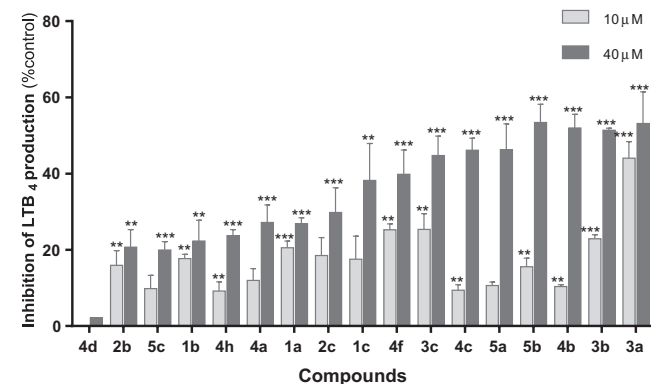
Inhibitory effects ( $IC_{50}$   $\mu$ M, mean  $\pm$  SEM) of flavonoids **1d**, **2d**, **3d**, luteolin **4e** and quercetin **4i** on the production of LTB<sub>4</sub> by human neutrophils. NDGA was used as positive control. Each study corresponds to at least three experiments.

| Compounds           |                                                                                   | R1 | R2 | R3 | $IC_{50}$ ( $\mu$ M) $\pm$ SEM |
|---------------------|-----------------------------------------------------------------------------------|----|----|----|--------------------------------|
| <b>1d</b>           |  | OH | OH | —  | $1.7 \pm 0.1$                  |
| <b>2d</b>           |  | OH | OH | —  | $2.9 \pm 0.8$                  |
| <b>3d</b>           |  | OH | OH | —  | $2.0 \pm 0.7$                  |
| <b>4e</b> Luteolin  |  | OH | OH | H  | $1.6 \pm 0.3$                  |
| <b>4i</b> Quercetin |  | OH | OH | OH | $4.0 \pm 1.2$                  |
| NDGA                | —                                                                                 | —  | —  | —  | $56.6 \pm 2.5^{1\mu M}$        |

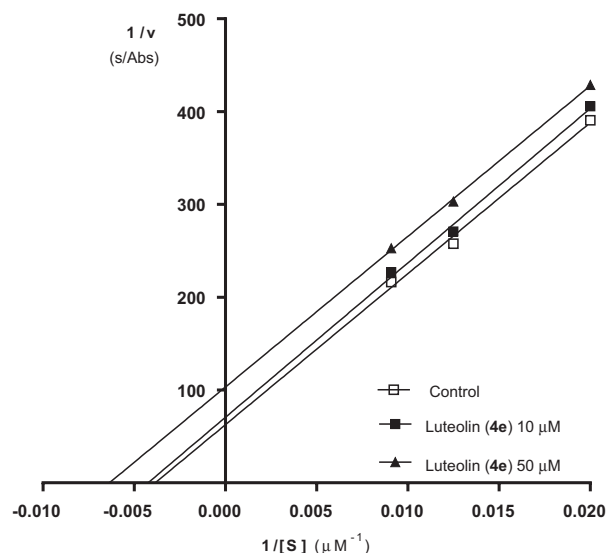
flavonoids, which presented  $IC_{50}$  values below 5  $\mu$ M. The most active ones were luteolin **4e** and 3',4'-dihydroxyflavone **1d** ( $IC_{50}$  of  $1.6 \pm 0.3$   $\mu$ M and  $1.7 \pm 0.1$   $\mu$ M, respectively). Other 3',4'-dihydroxyflavone derivatives **2d** and **3d** showed also a really good inhibition of LTB<sub>4</sub> production ( $IC_{50}$  of  $2.9 \pm 0.8$   $\mu$ M and  $2.0 \pm 0.7$   $\mu$ M, respectively). From this group quercetin **4i** was the less active ( $IC_{50} = 4.0 \pm 1.2$   $\mu$ M) (Table 1). The second group corresponds to the flavonoids that expressed lower activities. Some of them did not even reach an  $IC_{50}$ . When analyzing Graphic 1, flavonoids **5b**, **4b**, **3b** and **3a** appeared as the most actives, yet they only achieved inhibitions of  $53.4 \pm 4.8\%$ ,  $52.0 \pm 3.6\%$ ,  $51.40 \pm 0.5\%$  and  $53.1 \pm 8.3\%$ , respectively, for 40  $\mu$ M. The other flavonoids in this group had activities lower than 50% of LTB<sub>4</sub> production inhibition for 40  $\mu$ M. Finally, in the last group are placed the flavones **2a** and **4g** which did not inhibit the production of LTB<sub>4</sub>, although **4d** barely inhibited it too.

### 3.2. Soybean LOX-1 inhibition assay

The most active flavonoids on the inhibition of LTB<sub>4</sub> production assay were selected to study their type of inhibition. Additionally, four flavonoids with lower activity were also studied, in order to



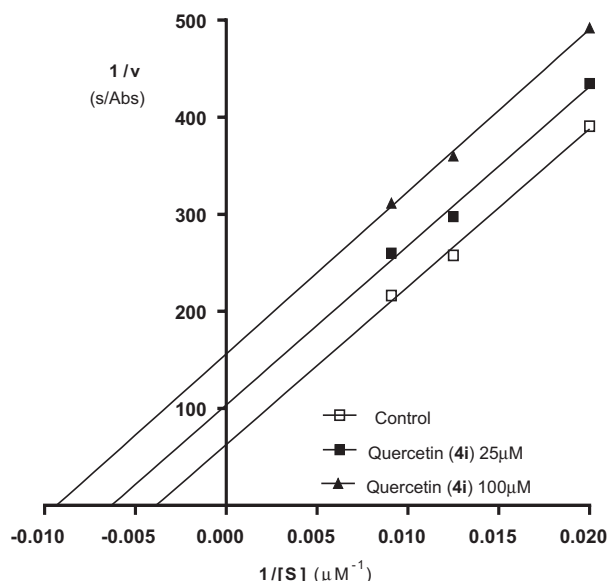
**Graphic 1.** Inhibition of LTB<sub>4</sub> production in human neutrophils by the indicated studied flavonoids determined by EIA. Each value represents mean  $\pm$  SEM of at least three experiments. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , compared to control.



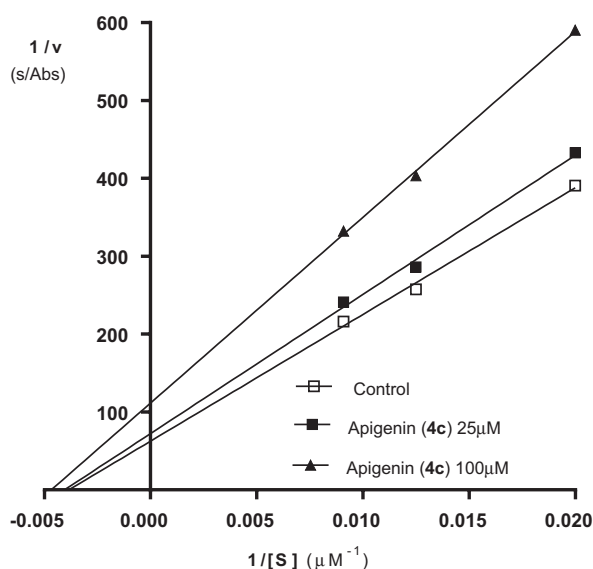
**Graphic 2.** Lineweaver–Burk plot of soybean LOX-1 inhibition by luteolin **4e**.

find a relationship between the flavonoid's structure and the type of LOX-1 inhibition. The studied flavonoids were: **1d**, **2c**, **2d**, **3d**, chrysin **4a**, **4b**, apigenin **4c**, luteolin **4e** and quercetin **4i**. The type of inhibition was deduced from the calculation of  $K_m$  (Michaelis–Menten constant) and  $V_{max}$  (maximum enzyme velocity) parameters obtained by the nonlinear regression Michaelis–Menten enzyme kinetics, and complemented with the construction of the Lineweaver–Burk double reciprocal plots. Parallel lines were obtained for flavones **2d** and **4b**, luteolin **4e** (Graphic 2) and quercetin **4i** (Graphic 3), attesting an uncompetitive inhibition type. In the absence of inhibitors, a  $K_m$  value of 200.4  $\mu$ M and a  $V_{max}$  value of 0.013 Abs/s were observed. For the increasing concentrations of these flavonoids, the  $K_m$  and  $V_{max}$  values decreased.

The other tested flavonoids shared a mixed noncompetitive type of inhibition, though with different variation patterns of  $K_m$  and  $V_{max}$ . Flavonoid **1d** showed  $K_m$  and  $V_{max}$  values almost invariable, when increasing its concentrations. For increasing



**Graphic 3.** Lineweaver–Burk plot of soybean LOX-1 inhibition by quercetin **4i**.



**Graphic 4.** Lineweaver–Burk plot of soybean LOX-1 inhibition by apigenin **4c**.

concentrations of flavone **2c**,  $K_m$  and  $V_{max}$  values decreased. The  $K_m$  and  $V_{max}$  values of flavonoid **3d** suffered an increment when its concentration was increased.  $K_m$  and  $V_{max}$  values of chrysin **4a** decreased with increasing concentrations. The  $K_m$  of apigenin **4c** (Graphic 4) remained constant with increasing concentrations of the flavonoid, however the  $V_{max}$  values were lowered. To calculate the values of inhibition constant ( $K_i$ ) the reciprocal of maximal velocity ( $1/V_{max}$ ) was plotted against the inhibitor concentrations, where the intercept on the inhibitor axis gave the value of  $K_i$  [14]. The obtained  $K_m$ ,  $V_{max}$  and  $K_i$  values are shown in Table 2.

### 3.3. STD- $^1H$ NMR spectroscopy

STD-NMR spectroscopy experiments have become a robust method to detect the binding of ligands to a given receptor [31–33]. We have applied STD to analyze the interaction of the different flavonoids with LOX-1. The flavonoids assayed in the inhibition studies were selected for the STD experiments and all showed STD response. The STD-NMR spectrum is obtained by subtracting a spectrum in which the protein is saturated (on-resonance) from one without protein saturation (off-resonance), the difference spectra contain only signals of the protons that received saturation from the protein. Fig. 2 shows the STD result of luteolin **4e** and of 5,3',4'-trihydroxyflavone **2d**, these ligands have a strong and a weak STD response, respectively.

The first step consisted on the analysis of the  $^1H$  NMR spectra of LOX-1 using the prepared solution of the enzyme in a Tris buffer at 37 °C. The spectra is dominated by a strong signal at 3.8 ppm from the methylene protons of the Tris buffer. However these signals do not overlap with the ligands resonance peaks which appear exclusively in the aromatic region, and thus these conditions are ideal of NMR.

Proper control experiments were performed to optimize the frequency for protein saturation (–1 ppm) and off-resonance irradiation, to ensure that the ligand signals were not affected.

Overall, in the STD-NMR spectra obtained for all the compounds it was possible to integrate the resonances of the resolved proton signals and determine relative STD effects on the A, B and C rings of the studied flavonoids.

The structural assignment of the flavonoids was performed according to the reported data, for flavones **1d**, **2d** and **2c** [34], for flavone **3d** [35], for flavonoids **4a**, **4c**, **4e** and **4i** and [36] for flavone **4b**.

The strongest STD results were obtained for apigenin **4c**, luteolin **4e**, **4b**, **1d**, and quercetin **4i**. The weakest STD response was obtained for flavones **2c**, **2d**, chrysin **4a** and **3d**. The analysis of the STD

**Table 2**  
Inhibitor constant ( $K_i$ ) values ( $\mu M$ ) for the inhibition of soybean LOX-1 by the tested flavonoids.

| Compounds |           | R1 | R2 | R3 | Concentration (μM) |       |       |       |       |       |       |       | Ki (μM) |
|-----------|-----------|----|----|----|--------------------|-------|-------|-------|-------|-------|-------|-------|---------|
|           |           |    |    |    | 10                 |       | 25    |       | 50    |       | 100   |       |         |
|           |           |    |    |    | Km                 | Vmax  | Vm    | Vmax  | Km    | Vmax  | Km    | Vmax  |         |
| 1d        |           | OH | OH | —  | 136.6              | 0.009 | 191.1 | 0.009 | —     | —     | —     | —     | 84.5    |
| 2c        |           | H  | OH | —  | 104.7              | 0.007 | —     | —     | 61.28 | 0.005 | —     | —     | 102.6   |
| 2d        |           | OH | OH | —  | —                  | —     | 103.8 | 0.006 | 46.39 | 0.003 | —     | —     | 18.0    |
| 3d        |           | OH | OH | —  | 97.36              | 0.007 | 190.5 | 0.01  | —     | —     | —     | —     | ND      |
| 4a        | Chrysin   | H  | H  | H  | 70.96              | 0.006 | 55.02 | 0.005 | —     | —     | —     | —     | 19.4    |
| 4b        |           | OH | H  | H  | —                  | —     | —     | —     | 144.7 | 0.009 | 111.4 | 0.006 | 95.4    |
| 4c        | Apigenin  | H  | OH | H  | —                  | —     | 191.4 | 0.01  | —     | —     | 191   | 0.008 | 178.3   |
| 4e        | Luteolin  | OH | OH | —  | 189.6              | 0.012 | —     | —     | 148.5 | 0.009 | —     | —     | 121.7   |
| 4i        | Quercetin | OH | OH | OH | —                  | —     | 129.2 | 0.008 | —     | —     | 100.4 | 0.006 | 106.7   |

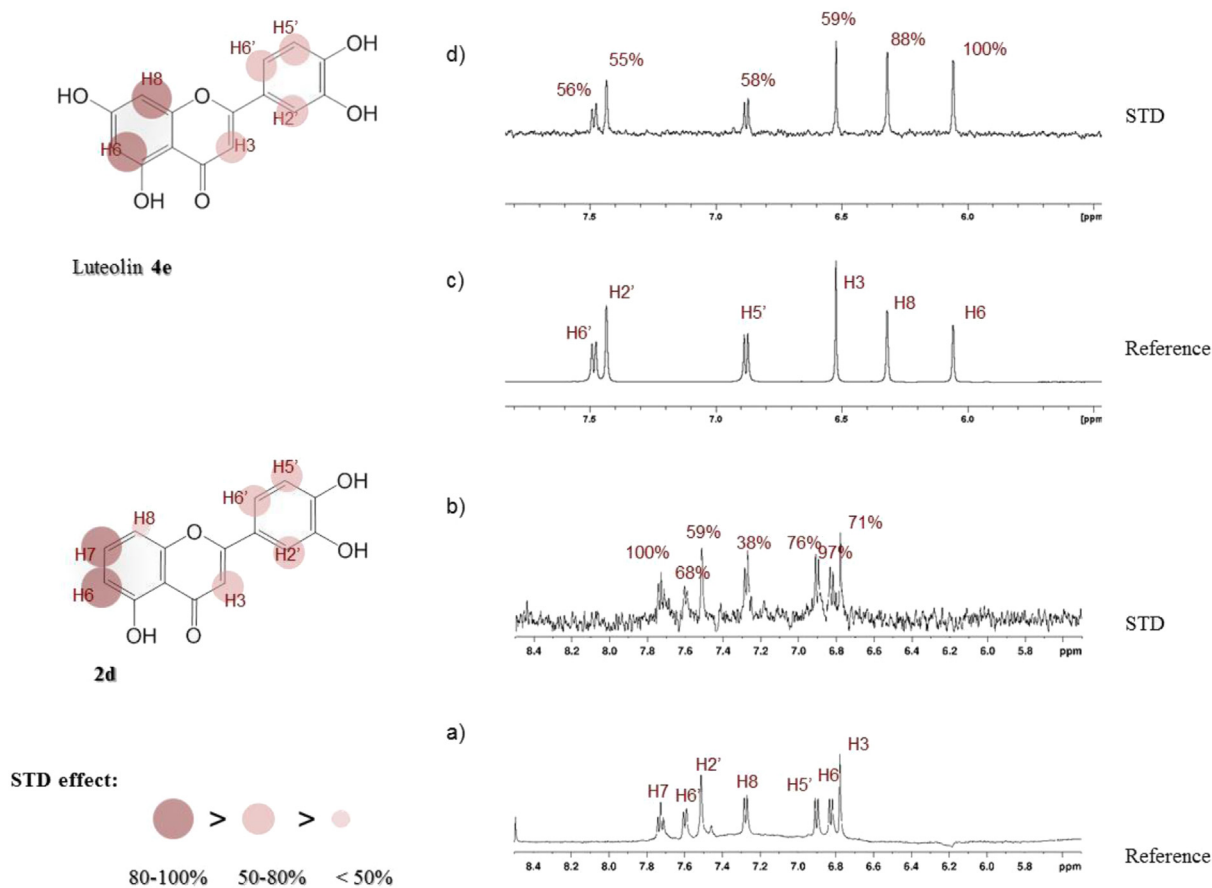


Fig. 2. Expansion of the aromatic region on the STD-<sup>1</sup>H NMR spectra of compound **2d**: a) and b); and of luteolin **4e**: c) and d); in the presence of LOX-1 at 600 MHz and 37 °C.

signals obtained for the flavonoids permitted its binding epitope to be determined.

#### 4. Discussion

This paper reinforces flavonoids as effective inhibitors of LTB<sub>4</sub> production in human neutrophils. It was also possible to establish a structure/activity relationship for the inhibitory activity.

Concerning the inhibition of LTB<sub>4</sub> production in human neutrophils by flavonoids, luteolin **4e** (IC<sub>50</sub> = 1.6 ± 0.3), **1d**, **3d**, **2d** and quercetin **4i** (IC<sub>50</sub> = 4.0 ± 1.2) proved to be the most actives, in descending order of potencies. All of them share the same substitution pattern in the B-ring, a catechol group. The number of hydroxyl groups in the A-ring did not seem to be a determinant feature for the inhibitory activity. However the presence of a 3-OH on the C-ring clearly diminished the studied activity, as quercetin **4i** was 2.5 times less active than luteolin **4e**. Considering the least active studied flavonoids, derivatives from group **1** (Fig. 1) presented low activities. The most active of them, 4'-hydroxyflavone **1c**, inhibited the production of LTB<sub>4</sub> just 38.4 ± 9.7% for the concentration of 40 μM. In group **2** (Fig. 1) it is possible to recognize precisely the same pattern of activity, in which 5,4'-dihydroxyflavone **2c** had an effect of 29.8 ± 6.5% for 40 μM. Flavonoids in group **3** (Fig. 1) seemed to be much better inhibitors than the previous, so the 7-OH in the A-ring may favor the activity. It should also be noted that, in group **5** (Fig. 1), it was once more clearly shown that the 3-OH group contributes to lowering the flavonoid activity, because comparing (±)-eriodictyol **5b** with taxifolin **5c**, the last one is considerable less potent than the first one, 53.4 ± 4.8% and 19.9 ± 2.2% of inhibition for 40 μM, respectively. Sadik et al.

[37] studied the inhibition of 15-LOX by a group of flavonoids, namely flavone, chrysin, apigenin, quercetin, luteolin, taxifolin, and naringenin, and concluded that the vital features for the inhibition of the pro-oxidants enzymes, like LOX, are the presence of orthodihydroxyl groups (a catechol moiety) in the A-ring and in the B-ring and the presence of the 2,3-double bond and a 4-oxo group in the C-ring, although the presence of the 3-OH group diminishes the inhibitory effect. Here, these assumptions are, in part, corroborated and further extended with new data. The flavanones from group **5** are clearly less active than the correspondent flavones and the flavonol quercetin, displaying the importance of the 2,3-double bond. In addition, in the same study it was concluded that luteolin **4e** was more effective than quercetin **4i**, and one of the reasons may be because the latter has an excessive number of OH groups, which lowers the hydrophobicity, restraining the flavonoid intercalation in the hydrophobic cavity that constitutes the access channel to the active site of the enzyme. In other studies, it was suggested that the planarity of the molecules may influence their capacity to interact with LOX [38,39]. Actually it was settled, as in the present study, that quercetin **4i** and luteolin **4e** were more potent than taxifolin **5c**, which reflects the marked non-planar character of this molecule. Also it was previously proposed that flavonoids act as electron acceptors from chemical groups near the LOX active center, the interaction being between the electron acceptor, flavonoid, and a nucleophilic group present in the LOX molecule. The antioxidant activity of flavonoids is based on two possible reducing pathways: electron-transfer from the molecule to the radical, leading to indirect H-abstraction and H-transfer from the molecule to the radical. It has been clearly proved that the B-ring is the most important site for the antioxidant capacity,

particularly when it has a catechol group, as in quercetin **4i** [16,40,41]. This may explain the good performance of quercetin **4i** in the inhibition of LOX and of the other flavonoids with higher activity, they all share this feature. Notwithstanding, there have been some studies arguing that the hydrogen donation, or the antioxidant activity, is not the only explanation for the inhibitory capacity of the production of LTs [42,43]. Loke et al. [44] tested the flavonoids quercetin **4i**, luteolin **4e** and taxifolin **5c**, and stated that the essential feature for a good inhibitory activity of LTB<sub>4</sub> production was the 2,3-double bond and that the 3-OH group had a minimal effect in this activity, as attested in our study. Moreover, they observed a dissociation between this inhibitory effect and the antioxidant properties of flavonoids as the 2,3-double bond and the absence of the 3-OH group have little effect in the antioxidant activity also determined in their study, by the formation of lipid hydroperoxides and F<sub>2</sub>-isoprostanes in AAPH-induced LDL oxidation. In reality there are various studies, as well as reviews, that ascertain the 2,3-double bond in conjugation with a 4-oxo function in the C-ring, providing electron delocalization from the B ring, and hydroxyl groups at positions 3 and 5, providing hydrogen bonding to the oxo group, as two of the main structural features for the antioxidant activity [15]. In another study Mahesha et al. [45] considered the isoflavones inhibition of LOX and reported that an electron donated by isoflavones was accepted by the ferric form (Fe<sup>3+</sup>) of LOX, which is reduced to resting ferrous form (Fe<sup>2+</sup>), thus inhibiting LOX, which may be an additional mechanism of action applicable to 5-LOX.

Once defined the effectiveness of the tested flavonoids, it was our aim to evaluate their type of LOX inhibition and simultaneously determine the corresponding structure–activity relationship. It is almost certain that there is a common mechanism of reaction between the different LOXs, because their catalyzed oxygenation involves a similar pair of double bonds on the fatty acid substrate and its coupling with O<sub>2</sub> [2]. In this sense, and because of the lack of sufficiently purified human enzymes (human 5-LOX), most of the structural research has been done on soybean LOX, as it was in this study [46]. In 1989, Belman et al. [47] had already done this kind of study, with soybean LOX, and at the time they reported that the use of AA as substrate rapidly inactivated the enzyme. The kinetics were linear for a very short period of time for accurate rates to be obtained. So the alternative for this kind of studies was the use of linoleic acid as substrate. The kinetic with linoleic acid were linear for a sufficiently long time before inactivation and rates could easily be obtained [2]. To completely characterize the LOX activity, qualitative kinetics profile and quantitative parameters should be considered. Quantitative parameters include estimation of V<sub>max</sub>, K<sub>m</sub> as well as K<sub>i</sub> [48]. Compounds **2d**, **4b**, luteolin **4e** and quercetin **4i** were found to be uncompetitive LOX inhibitors, since V<sub>max</sub> in the presence of increasing concentrations of drug was invariably lower than the V<sub>max</sub> observed in their absence, and the value of K<sub>m</sub> also decreased, thus parallel lines were obtained in their Lineweaver – Burk plot. They only bind to the enzyme–substrate complex [49]. Additionally comparing the K<sub>i</sub> of these three flavonoids, **2d** presents a K<sub>i</sub> of 18.0 μM, much lower than the ones of **4b**, luteolin **4e** and quercetin **4i**, 95.4 μM, 121.7 μM and 106.7 μM, respectively, which denote flavone **2d** as having a stronger affinity for the enzyme, than the other three flavonoids. From these flavonoids quercetin **4i** and luteolin **4e** have in common a catechol group in B-ring and a 5,7-dihydroxyl in the A-ring, so these features may determine this type of inhibition. On the contrary, the other studied flavonoids owned a mixed noncompetitive type of inhibition. This means that they are able to bind to both the free enzyme and the enzyme–substrate complex [49]. Flavonoids **1d**, **2d**, and **3d**, luteolin **4e** and quercetin **4i** have a catechol group in B-ring, however **1d** and **3d** have a different type of inhibition from the

others, possessing none OH group in A-ring or a 7-OH, respectively. On the other hand, when flavonoids present a 5-OH and a 7-OH in the A-ring the number and location of the OH groups in B-ring seem to define the type of inhibition. Flavone **4b** has a 3'-OH at the B-ring and an uncompetitive type of inhibition, but chrysin **4a** and apigenin **4c** which have none or a 4'-OH, respectively, present a mixed noncompetitive type. Flavonoid **2c** possess just two OH groups and have a mixed noncompetitive type of inhibition, interestingly flavones **1d** and chrysin **4a** have the same number of OH and the same type of inhibition. In this sense, the type of inhibition is not just defined by the number of OH groups present but more by the relative position occupied by these groups. Noteworthy, when studying the inhibition of 5-LOX the structure/activity relationship clearly denotes the catechol group in B-ring as the vital feature for a good activity. In the type of inhibition, this relationship seems to be dependent not just on the OH of the B-ring but also on those in the A-ring.

STD-NMR studies have also been performed in order to obtain an epitope mapping of the studied flavonoids that would indicate a distinct contact with the enzyme and thus understand which regions of the structure are in closer contact with LOX-1. NMR is a very useful tool to study the molecular interactions in solution and an essential technique to characterize events of molecular recognition and to obtain information about the interactions of small ligands with biologically relevant macromolecules (proteins or nucleic acids). The STD-NMR technic has been used for some years to characterize the ligand–receptor complexes [28]. The intensity of the saturation transfer is quantified and is an indicator of the affinity. When a molecule binds to the receptor, only the regions that are in close contact to the protein will receive magnetization transfer and appear in the difference spectrum owing to a more efficient saturation transfer.

Fig. 3 summarizes the results obtained for the inhibition constant and STD- NMR data for the studied compounds. For all compounds the higher percentages of STD response were obtained for the A-ring as can be seen in Fig. 3, in particular H6 and H8. This clearly indicates a higher magnetization transfer and thus a closer contact of this ring with LOX-1 and clearly evidences the pivotal role of this ring in the interaction with the enzyme. However the substitution pattern of B-ring seems to affect the interaction with LOX-1, since when there is no substituent, such as in chrysin **4a**, this ring receives less saturation indicating a weaker interaction (percentages of relative STD from H2' to H6' ≈ 50%). This interaction with LOX-1 does not seem to depend on the hydrophobicity of the molecule, since for compounds **1d** and chrysin **4a**, the A-ring is the one with a higher STD response.

From the epitope mapping analysis it is clear that the A-ring is in more intimate contact with LOX-1, whereas B-ring region is more solvent-exposed. However, no conclusion can be drawn regarding the site of binding.

Moreover, the features that made the flavonoids more active in the inhibition of LTB<sub>4</sub> production were not precisely the same that interacted more with LOX-1. This finding indicates that we should be cautious when comparing the two LOXs.

In conclusion, this complete systematic study of a series of flavonoids on the inhibition of LTB<sub>4</sub> production results in the achievement of solid structure–activity relationships. In addition, it was possible to define the features that contribute to the different types of LOX inhibition, with a clear cooperation between A and B-rings, indicating the existence of a substitution pattern in both of them which favor the activity. Finally the obtained results in the STD-NMR studies confirmed the importance of the substitution pattern of both rings for a higher interaction with LOX-1. The data collected allowed the establishment of the epitope mapping of these flavonoids with this enzyme. These results open the

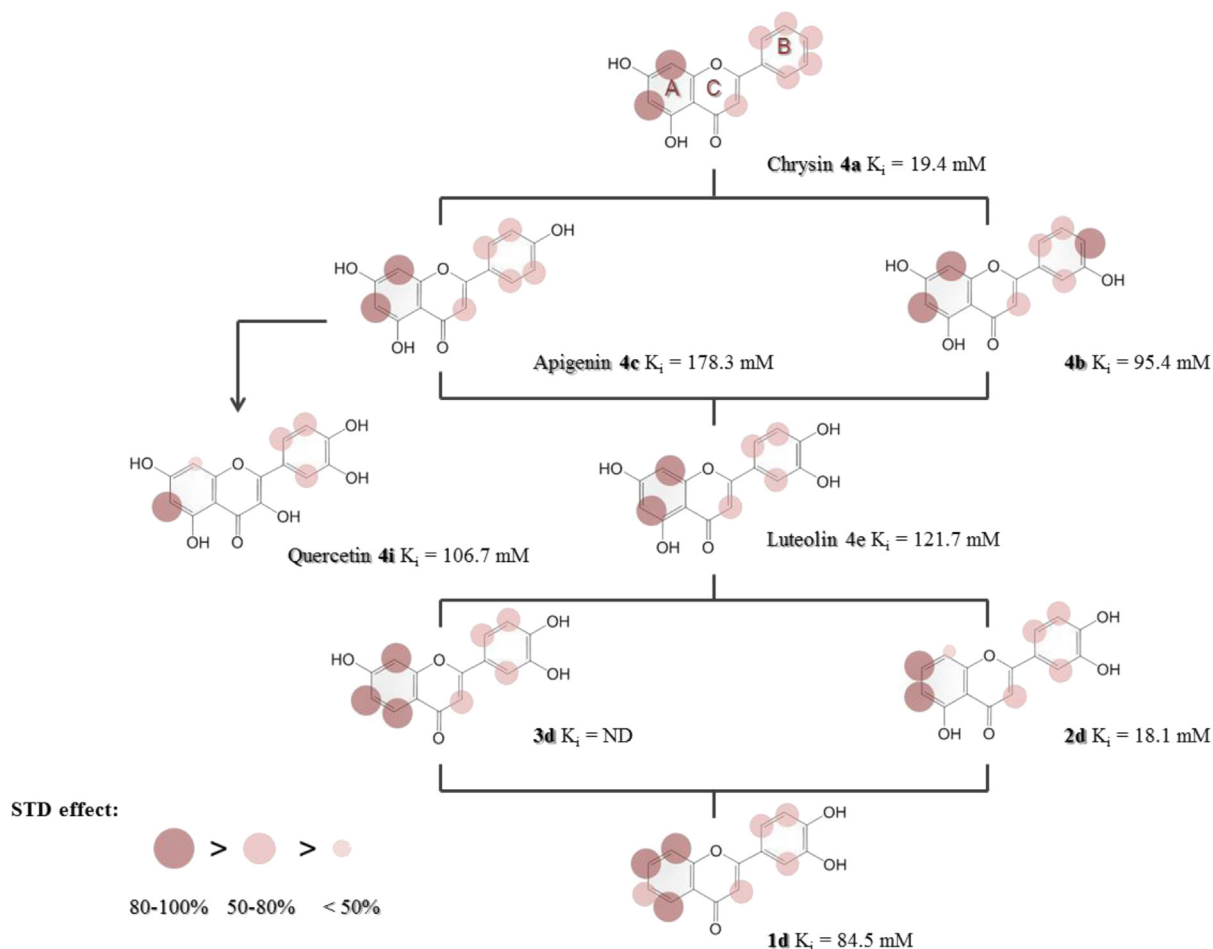


**Fig. 3.** Epitope mapping obtained for all the tested flavonoids with LOX-1.

opportunity to further explore the potential of these molecular entities, especially by increasing their anti-inflammatory effects, while maintaining of decreasing putative side effects, for a successful prospective pharmaceutical application.

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## ***CHAPTER III. Original Research***

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### **III.3. Flavonoids inhibit COX-1 and COX-2 enzymes and cytokine/chemokine production in human whole blood**

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My contribution for this work comprised all the performed studies.

# Flavonoids Inhibit COX-1 and COX-2 Enzymes and Cytokine/Chemokine Production in Human Whole Blood

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**Abstract**—Cyclooxygenase 2 (COX-2) and the production of cytokines/chemokines are important targets for the modulation of the inflammatory response. Although a large variety of inhibitors of these pathways have been commercialized, some of those inhibitors present severe side effects, governing the search for new molecules, as alternative anti-inflammatory agents. This study was undertaken to study an hitherto not evaluated group of flavonoids, concerning its capacity to inhibit COX-1 and COX-2 enzymes, as well as to inhibit the production of the cytokines and a chemokine, in a complex matrix involved in the systemic inflammatory process, the blood, aiming the establishment of a structure–activity relationship. The results obtained reveal promising flavonoids for the modulation of the inflammatory process, namely the ones presenting a catechol group in B ring, as some flavonoids were able to simultaneously inhibit the production of inflammatory prostaglandin E<sub>2</sub> and pro-inflammatory cytokines.

**KEY WORDS:** flavonoids; COX; human whole blood assay; cytokines; chemokine.

## INTRODUCTION

The inflammatory response involves a broad series of processes, where a variety of soluble mediators are involved in the activation of resident cells (such as fibroblasts, endothelial cells, tissue macrophages, and mast cells) and in the recruitment and activation of inflammatory cells (such as monocytes, lymphocytes, neutrophils, and eosinophils). Those soluble mediators may be organized into four main groups: (a) inflammatory lipid metabolites such as platelet-activating factor (PAF) and the derivatives of arachidonic acid (prostaglandins (PGs), leukotrienes

(LTs), lipoxins); (b) three cascades of soluble proteases/substrates (clotting, complement, and kinins), which generate numerous pro-inflammatory peptides; (c) nitric oxide, a potent endogenous vasodilator; and (d) cytokines, which are major determinants for the cellular infiltrate, the state of cellular activation, and the systemic responses to inflammation [1].

Cyclooxygenases (COXs) are determinant enzymes in the production of PGs with a varied chemistry, with different receptors and diverse mechanisms of modulation of their function. All these features determine a variety of physiological functions of PGs, namely, blood clotting, ovulation, initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone, and immune responses. PGs are synthesized in a broad range of tissue types and serve as autocrine or paracrine mediators to signal changes within the immediate environment [2].

There are three types of COX isoenzymes: 1, 2, and 3. Under physiological conditions, COX-1 is constitutively expressed at low levels, in virtually all tissues. COX-2 expression is more restricted, to the kidney, brain, testicles, and tracheal epithelial cells. COX-3 is expressed in specific sites, including the brain and the spinal cord, but its exact

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functions still needs to be disclosed. COX-1 is responsible for the constant production of PGs critical to the autocrine/paracrine responses to circulating hormones and maintenance of gastric mucosal integrity and platelet function, i.e., for the regulation of the homeostatic functions. On the other hand, COX-2 is one of the key enzymes implicated in the modulation of inflammation, and can be upregulated 20-fold in macrophages, monocytes, fibroblasts and endothelial cells by various stimuli during the inflammatory process. In contrast, COX-1 activity is unaffected or increased only marginally (two- to fourfold). If so, COX-2 expression is high and transient, leading to a burst of inflammatory PGs production in a tightly regulated time-limited manner [3, 4]. This hyperexpression of COX-2 results from the integrated function of several inflammatory mediators, including interleukins (ILs) and cytokines [i.e., IL-1, IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )], via transcriptional activation [5].

During the inflammatory process, various cytokines are produced. They are pleiotropic molecules that exert their effects locally or systemically in an autocrine or paracrine manner. Cytokines are part of a large and complex signaling network and their effects depend on the context in which they are working. These networks involve synergistic as well as antagonistic interactions and exhibit both negative and positive regulatory effects on various target cells [1, 6]. The cytokines may be divided in the ones participating in acute or in chronic inflammation; however, there are some that significantly contribute to both. Some of the cytokines playing a part in acute inflammation are IL-1, TNF- $\alpha$ , IL-6, and IL-11. The cytokines that participate in chronic inflammation can be subdivided into the ones mediating humoral responses, such as IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, and IL-13 and transforming growth factor- $\beta$  (TGF- $\beta$ ), and those mediating cellular responses, such as IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons (IFNs), interferon- $\gamma$  inducing factor (IGIF), TGF- $\beta$ , and TNF- $\alpha$  and - $\beta$ . Some cytokines, such as IL-1, significantly contribute to both acute and chronic inflammation [1].

There are also chemotactic cytokines, designated chemokines. They are responsible for the migration of circulating leukocytes to the sites of inflammation or injury [7]. The secretion of these chemokines is usually induced by pro-inflammatory cytokines such as IL-1, IL-2, TNF- $\alpha$ , interferon- $\gamma$  (IFN- $\gamma$ ), and the bacterial product, lipopolysaccharide. Likewise, the regulation of their production is mediated by other cytokines such as TGF- $\beta$ , IL-4, and IL-10, although the effects vary according with the chemokine and cell type [8]. The following chemokines IL-8, granulocyte colony-stimulating factor (G-CSF) and

granulocyte-macrophage colony-stimulating factor (GM-CSF) also participate in the acute inflammation [1].

The primary aim of these phenomena is the resolution of the inflammatory process. However, it becomes a problem when they surpass normal physiological control conducting to an exaggerated response, contributing to the pathogenesis of a variety of diseases. It is also why the overexpression of COX-2 and the overproduction of cytokines/chemokines are usually related to autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, or some forms of diabetes [3–5, 7].

In this sense, COX-2 and the cytokines/chemokines' production are ideal targets for the modulation of the inflammatory response. A large variety of non-selective and selective inhibitors of COX-2 have been commercialized. Notwithstanding some of those inhibitors revealed that inhibition of COX-2 enzymatic activity is not amenable to long-term treatment procedures, presenting severe side effects as gastrointestinal bleeding, thrombosis, and stroke [9–11]. Likewise, the anti-inflammatory therapies based on the inhibition of the production of cytokines and chemokines have also been pursued, glucocorticoids being one of the options; however, they also are potent immunosuppressive agents, which limit their effectiveness in therapy [12]. These limitations have been governing the search for other therapies, namely, the ones based on gene and antibodies therapies, as well as new molecules which may constitute an alternative to the clinically available inhibitors [13, 14]. One of these group of molecules may be polyphenols, more precisely, flavonoids. Flavonoids represent a wide variety of molecules that share a basic skeleton, an arylbenzopyran ring system, and the different rearrangements of this ring system originate different classes of flavonoids. All these classes are present ubiquitously in nature; which makes flavonoids an even interesting group of study molecules [15]. It has been proven that flavonoids are able to behave as developmental regulators/signaling molecules as well, by interacting with specific proteins central to intracellular signaling cascades [16]. Flavonoids have been already implicated as promising molecules in the resolution of the inflammatory process, by means of their antioxidant activity and by interfering with a variety range of enzymes and signaling cascades activated during this process [17–20].

Therefore, this study was undertaken to study the flavonoids' inhibition capacity of COX-1 and COX-2 enzymes, as well as to inhibit the production of the cytokines, IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ , and the chemokine, IL-8, in a complex matrix involved in the systemic inflammatory process, the blood, aiming the establishment of a structure-

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activity relationship. The chemical structures of the studied flavonoids are represented in Fig. 1.

## MATERIALS AND METHODS

## Reagents

The following reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, USA): dimethylsulfoxide (DMSO), acetylsalicylic acid, gentamicin sulfate, cremophor® EL, lipopolysaccharides from *Escherichia coli* 026:B6 (LPS), calcium ionophore (A23187), arachidonic acid (AA), RPMI 1640 medium, fetal bovine serum, L-glutamine, penicillin, streptomycin, 5Z-7-oxozeaenol, apigenin **4c**, chrysin **4a**, luteolin **4e**, (±)-naringenin **5a**, (±)-eriodictyol **5b**, and quercetin **4i**. Ethanol absolute was purchased from Fisher Chemical (Loughborough, Leic, UK). Acacetin **4d** and taxifolin **5c** were purchased from Fluka Chemie GmbH (Steinheim, Germany). The

flavonoids **4b**, chrysoeriol **4f**, diosmetin **4g** and **4h** were synthesized by co-authors of this manuscript, from the Departamento de Química and QOPNA, Universidade de Aveiro [19]. The other tested flavonoids were obtained from INDOFINE Chemical Company, Inc. (Stryker Lane Hillsborough, USA). The thromboxane synthase inhibitor (TXBSI) was synthesized by co-authors of this manuscript, from the Eberhard Karls University of Tübingen. The “PGE<sub>2</sub> Enzyme Immunoassay (EIA) Kit” was obtained from Enzo Life Sciences (Lausen, Switzerland). The “Multi-Analyte ELISArray Human Kit – Human Mix-N-Match”, for the cytokines/chemokine determination, was obtained from QIAGEN® (Boston, USA).

## Human Whole Blood Assay

The blood was collected from healthy human volunteers, following informed consent. Venous blood was collected by antecubital venipuncture, into heparin-Li<sup>+</sup> vacuum tubes.

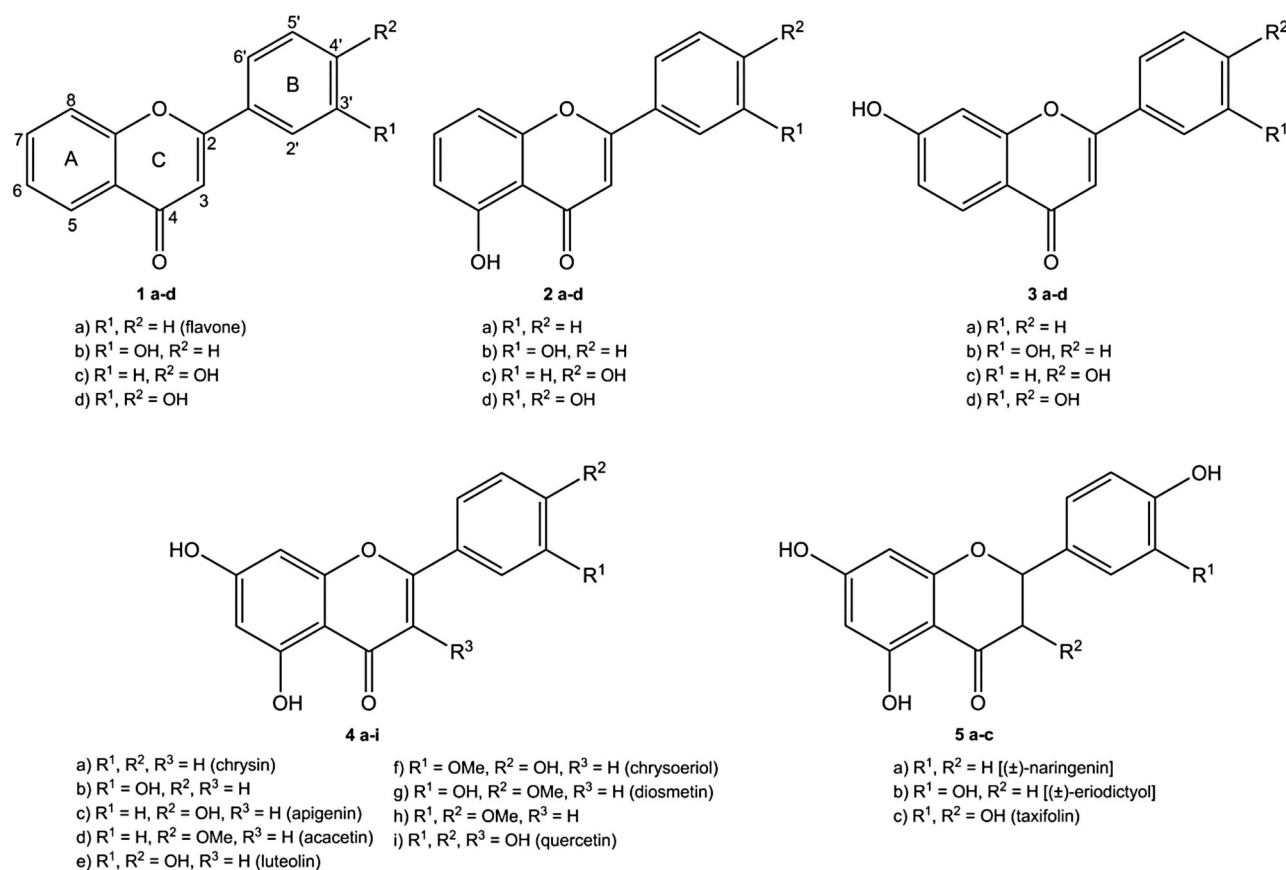


Fig. 1. Chemical structures of the studied flavonoids.

### COX-1 Assay

The human whole blood assay to assess the COX-1 inhibition was performed as previously reported, with modifications [21]. Collected blood (500  $\mu$ L) was placed in microtubes and incubated in a water bath at 37 °C with TXBSI (1  $\mu$ M) and the tested flavonoids (1.25–100  $\mu$ M) in DMSO for 15 min. The use of thromboxane synthase inhibitor, TXBSI, reduces the amount of A23187 needed and results in higher prostaglandin production. During this incubation period, the TXBSI and the tested flavonoids interact with all the cells and the other components of the blood. Then, the A23187 (12.2  $\mu$ g/mL) was added and the mixture was incubated for 15 min, allowing the triggering of COX-1 activity. The reaction was stopped by placing the samples on ice for 5 min. The samples were subsequently centrifuged at 1,000 g for 20 min, at 4 °C, to separate the blood cells from the supernatant, maintaining the low temperature. The supernatant was then collected and stored at –20 °C until use. The quantity of solvents used did not have inhibitory effects and neither affected the cellular viability (data not shown).

### COX-2 Assay

The human whole blood assay to assess the COX-2 inhibition was performed as previously reported, with modifications [21, 22]. Collected blood (800  $\mu$ L) was placed in a six-wells plate and incubated in a humidified incubator at 37 °C with TXBSI (1  $\mu$ M), the acetylsalicylic acid (10  $\mu$ g/mL), and the tested flavonoids (25–100  $\mu$ M) in DMSO/chromophor/ethanol 1 % (1:10) for 15 min. The use of TXBSI reduces the amount of LPS needed and its incubation period. Then, LPS (10  $\mu$ g/mL) was added and the mixture was incubated for 5 h, allowing the activation of COX-2. The reaction was stopped by adding DPBS–gentamicin buffer (1 mL) to the samples and placing them on ice for 10 min. The samples were subsequently centrifuged at 1,000g for 15 min, at 4 °C, to separate the blood cells from the supernatant, maintaining the low temperature. The supernatant was then collected and stored at –20 °C until use. The quantity of solvents used did not have inhibitory effects and neither affected the cellular viability (data not shown).

### Determination of PGE<sub>2</sub> Production

The amount of PGE<sub>2</sub> in the samples (thawed plasma supernatants) was measured using the above mentioned commercial EIA kit, according to the manufacturer's instructions, as an indicator of COX-1 and –2 activities. COX inhibitors, indomethacin (0.125–1  $\mu$ M) and celecoxib (0.5–50  $\mu$ M),

were used as positive controls. Results are expressed as the percent inhibition of control PGE<sub>2</sub> production. Each study corresponds to at least four experiments.

### Cytokines/Chemokine Assay

The human whole blood assay to assess the inhibition of production of the cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IFN- $\gamma$ ) and chemokine (IL-8) was performed as previously reported, with modifications [23]. The collected blood (50  $\mu$ L) was placed in a 24-wells plate with 950  $\mu$ L of RPMI 1640 medium [(pH = 7.4) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin] and incubated in a humidified incubator at 37 °C with the tested flavonoids (10 and 25  $\mu$ M) in DMSO/RPMI 1640 (1:1) for 30 min. Then, LPS (0.5 ng/mL) was added and the mixture was incubated for 16 h. For the determination of the produced IFN- $\gamma$ , 100  $\mu$ L of human whole blood with 900  $\mu$ L of RPMI 1640 medium and 10  $\mu$ g/mL of LPS were used. After this incubation period, the samples were subsequently centrifuged at 600 g for 5 min, at room temperature. The supernatant was then collected and stored at –20 °C until use. The quantity of solvents used did not have inhibitory effects and neither affected the cellular viability (data not shown).

### Determination of Cytokines/Chemokines Production

The amount of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IFN- $\gamma$ , and IL-8 in the samples (thawed plasma supernatants) was measured using the above mentioned commercial ELISA kit, according to the manufacturer's instructions. The 5Z-7-oxozeaenol (a transforming growth factor- $\beta$ -activated kinase 1 (TAK-1) inhibitor) [24], 1  $\mu$ M, was used as positive control, leading to a complete inhibition of the production of all the studied cytokines and chemokine. Due to the naturally occurring differences in human cytokine release, the value obtained for each volunteer was normalized to its LPS control. The results are expressed as the percent inhibition of control cytokines/chemokine production. Each study corresponds to at least three experiments.

## RESULTS

### Human Whole Blood Assay

#### COX-1 Assay

Among all the tested flavonoids, it was clear that half of them were good inhibitors of COX-1–PGE<sub>2</sub> production and the other half did not exhibit any activity up to the

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highest tested concentration, 100  $\mu$ M. The most active flavonoids were flavone **1a**, **2a**, **1d**, **1c**, and **3a**, with inhibitions of  $89.3 \pm 2.5$ ,  $80.4 \pm 3.9$ ,  $80.4 \pm 4.2$ ,  $78.3 \pm 6.3$ , and  $72.6 \pm 4.2$  %, respectively, for 100  $\mu$ M (Table 1). In Fig. 2, it is possible to identify flavone **2d** and chrysin **4a** as having good activities, but with inhibition's percentages lower than 70 %. Moreover, among the less active flavonoids **4h**, **3c**, **1b**, **3b**, and luteolin **4e** presented activities lower than 50 %, up to 100  $\mu$ M. The other tested flavonoids did not inhibit the COX-1-PGE<sub>2</sub> production. Taking in account the different substitution patterns of the studied flavonoids from group 1, the most active was flavone **1a**. From group 2, the most active were **2a** and **2d** and the other two flavonoids were not active. Flavone **3a** was the best of its group, followed by **3c** and **3b**, whereas **3d** was not active. Flavonoids of group 4 were less active, chrysin **4a** being the most active. Flavanones from group 5 were not active. Indomethacin was used as positive control and presented an activity of  $91.8 \pm 3.2$  %, for 1  $\mu$ M.

*COX-2 Assay*

The best activity of COX-2-PGE<sub>2</sub> production inhibition was obtained for flavonoids **1d** and **2d** with the following percentages of inhibition  $74.6 \pm 2.4$  and  $67.2 \pm 2.9$  %, respectively, for 100  $\mu$ M. Flavonoids **3d**, diosmetin **4g**, **1c** and luteolin **4e** were also quite active with percentages of inhibition of  $59.2 \pm 2.9$ ,  $56.9 \pm 6.1$ ,  $56.4 \pm 4.3$ , and  $56.4 \pm 6.9$  %, respectively, for 100  $\mu$ M (Table 1). The other flavonoids in Fig. 3 showed activities lower than 50 % up to the highest tested concentration, 100  $\mu$ M, chrysin **4a** and ( $\pm$ )-eriodictyol **5b** being the less active ones. The other tested flavonoids did not inhibit the COX-2-PGE<sub>2</sub> production, up to 100  $\mu$ M. Overall, from group 1, flavonoid **1d** was the most active,  $74.6 \pm 2.4$  %, followed by **1c** and flavone **1a**,  $38.6 \pm 1.8$  %, whereas **1b** was not active. In group 2, flavone **2d** was the only one which proved to be capable of inhibiting COX-2-PGE<sub>2</sub> production. Flavone **3d** was the most active of group 3,  $59.2 \pm 2.9$  %, followed by **3c**,  $41.7 \pm 2.8$  %. Flavones **3a** and **3b** were not active. In group 4, diosmetin **4g** ( $56.9 \pm 6.1$  %), luteolin **4e**, apigenin **4c**, chrysoeriol **4f** and chrysin **4a** ( $34.0 \pm 4.0$  %), were the only active flavonoids, in descending order of potencies. Flavanone ( $\pm$ )-eriodictyol **5b** was the only one that presented any activity in group 5. Celecoxib was used as positive control and presented an activity of  $72.8 \pm 2.9$  %, for 5  $\mu$ M.

*Cytokines/Chemokines Assay*

The flavonoids demonstrating the best inhibition patterns on COX-2's inhibition, **1d**, **2d**, **3d**, and luteolin **4e**, and

quercetin **4i**, shared a catechol group in B ring, and were selected to assess their ability to modulate the production of cytokines/chemokine. Flavonoid **3d** appeared as the most active in what concerns the inhibition of IL-1 $\beta$ 's production, with an inhibition activity of  $85.0 \pm 4.1$  %, for 25  $\mu$ M, the highest tested concentration. Flavonoids **1d** and luteolin **4e** were also good inhibitors of IL-1 $\beta$ 's production with activities of  $72.8 \pm 7.5$  and  $61.8 \pm 13.8$  %, respectively, for 25  $\mu$ M. The other tested flavonoids presented activities lower than 50 %. All the tested flavonoids appeared as great inhibitors of the production of IL-6, with percentages of inhibition always higher than 50 %, for 25  $\mu$ M, in the following order of potencies, luteolin **4e** ( $95.6 \pm 1.2$  %) > **1d** > **3d** > **2d** > quercetin **4i** ( $57.9 \pm 9.9$  %). The production of IL-8 was the less affected by the tested flavonoids. Nevertheless, flavonoids **3d** and **2d** had inhibition activities greater than 50 %, for 25  $\mu$ M,  $61.9 \pm 12.5$  and  $60.3 \pm 3.3$  %, respectively. In what concerns the inhibition of IFN- $\gamma$ 's production, the flavonoids under study displayed a great performance, with high percentages of activity, in the following order of potencies, luteolin **4e** ( $95.3 \pm 2.4$  %) > **3d** > **2d** > quercetin **4i** > **1d** ( $70.4 \pm 0.9$  %). Once more, the flavonoids under study proved to be good modulators of cytokines' production, inhibiting in a great extent the production of TNF- $\alpha$ . The best activity belonged to luteolin **4e**, followed by **1d**, **2d**, **3d**, and quercetin **4i**, with inhibitions of  $76.2 \pm 9.0$ ,  $72.8 \pm 3.5$ ,  $71.2 \pm 1.6$ ,  $67.5 \pm 1.5$  and  $59.1 \pm 3.3$  %, respectively. All the tested flavonoids demonstrated a concentration-dependent activity, data shown in Fig. 4.

## DISCUSSION

Flavonoids are promising molecules for the resolution of the chronic inflammatory processes. In this sense, this work aimed to study the structure-activity relationship between a hitherto not studied series of flavonoids and COXs-1 and -2 enzymes, using a human whole blood technique. Moreover, it was studied, in the same matrix, their ability to modulate the production of a series of cytokines and chemokine, which are important mediators of the inflammatory process.

The assays for the evaluation of the inhibition of the isolated enzymes are important as they give the exact information about the actual and sole impact of the drug on the enzyme, avoiding other endogenous influences. This kind of studies could also be performed in freshly isolated cells, with minimized protein content. However, these two kinds of assays present some pitfalls: they neglect the plasma binding of the drug, the other blood components, and the other interactions besides the drug-

**Table 1.** Inhibition and ratio of COX-1 and COX-2

| Compounds                       | R1  | R2  | R3 | Inhibition (%) $\pm$ SEM |                | COX-1 / COX-2 Ratio |
|---------------------------------|-----|-----|----|--------------------------|----------------|---------------------|
|                                 |     |     |    | COX-1                    | COX-2          |                     |
| <b>1a</b> Flavone               | H   | H   | -  | 89.3 $\pm$ 2.5           | 38.6 $\pm$ 1.8 | 2.3                 |
| <b>1b</b>                       | OH  | H   | -  | 49.5 $\pm$ 7.1           | NA             | -                   |
| <b>1c</b>                       | H   | OH  | -  | 78.3 $\pm$ 6.3           | 56.4 $\pm$ 4.3 | 1.4                 |
| <b>1d</b>                       | OH  | OH  | -  | 80.4 $\pm$ 4.2           | 74.6 $\pm$ 2.4 | 1.1                 |
| <b>2a</b>                       | H   | H   | -  | 80.4 $\pm$ 3.9           | NA             | -                   |
| <b>2d</b>                       | OH  | OH  | -  | 67.8 $\pm$ 3.1           | 67.2 $\pm$ 2.9 | 1.0                 |
| <b>3a</b>                       | H   | H   | -  | 72.6 $\pm$ 4.2           | NA             | -                   |
| <b>3b</b>                       | OH  | H   | -  | 37.1 $\pm$ 4.5           | NA             | -                   |
| <b>3c</b>                       | H   | OH  | -  | 43.1 $\pm$ 5.2           | 41.7 $\pm$ 2.8 | 1.0                 |
| <b>3d</b>                       | OH  | OH  | -  | NA                       | 59.2 $\pm$ 2.9 | -                   |
| <b>4a</b> Chrysin               | H   | H   | H  | 67.4 $\pm$ 5.5           | 34.0 $\pm$ 4.0 | 2.0                 |
| <b>4c</b> Apigenin              | H   | OH  | H  | NA                       | 45.2 $\pm$ 4.8 | -                   |
| <b>4e</b> Luteolin              | OH  | OH  | H  | 33.5 $\pm$ 7.9           | 56.4 $\pm$ 6.9 | 0.6                 |
| <b>4f</b> Chrysoeriol           | OMe | OH  | H  | NA                       | 35.6 $\pm$ 2.5 | -                   |
| <b>4g</b> Diosmetin             | OH  | OMe | H  | NA                       | 56.9 $\pm$ 6.1 | -                   |
| <b>4h</b>                       | OMe | OMe | H  | 44.6 $\pm$ 7.2           | NA             | -                   |
| <b>5b</b> ( $\pm$ )-Eriodictyol | OH  | H   | -  | NA                       | 25.5 $\pm$ 5.0 | -                   |

Production of PGE<sub>2</sub> in human whole blood by the indicated studied flavonoids determined by EIA (% , mean  $\pm$  SEM), for the highest tested concentration, 100  $\mu$ M. Each study corresponds to at least three experiments  
 NA no activity was found up to 100  $\mu$ M

cell interaction. The whole blood assay minimizes the introduction of artifacts that would occur during cell separation steps, all cell–cell interactions persist and it assures

the maintenance of the plasma protein levels, which resembles closely the *in vivo* pathophysiological conditions [21].

## Flavonoids Inhibit COX-1 and COX-2 Enzymes and Cytokine/Chemokine Production in Human Whole Blood

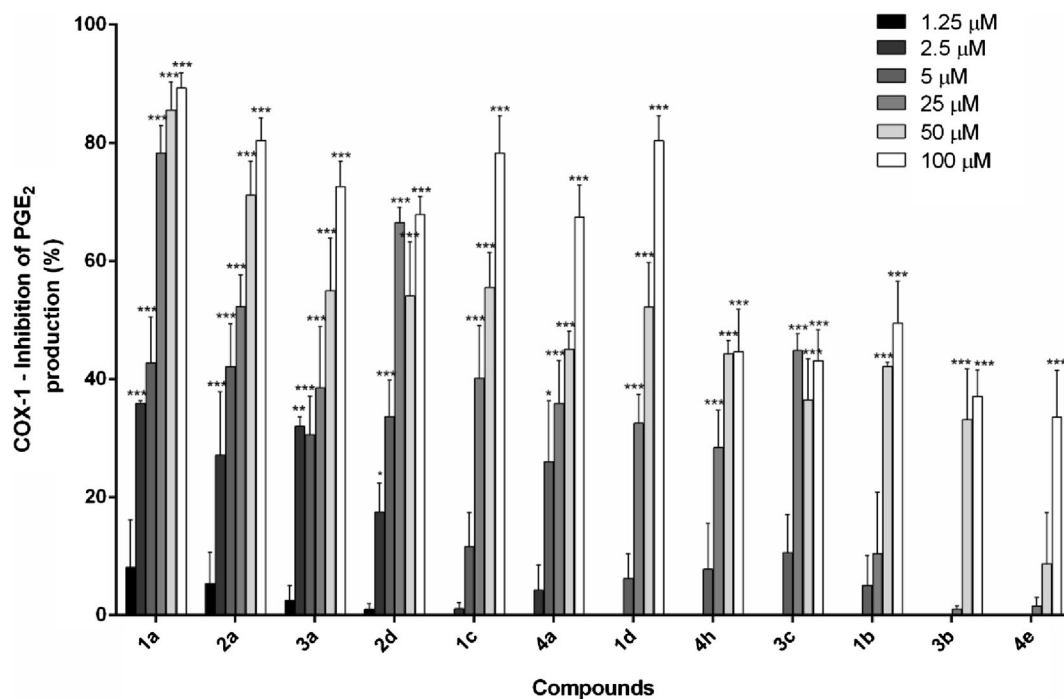


**Fig. 2.** Inhibition of COX-1—production of PGE<sub>2</sub> in human whole blood by the indicated studied flavonoids determined by EIA. Each value represents mean ± SEM of at least three experiments. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.1$  compared to the stimulated control (TXBSI/A23187).

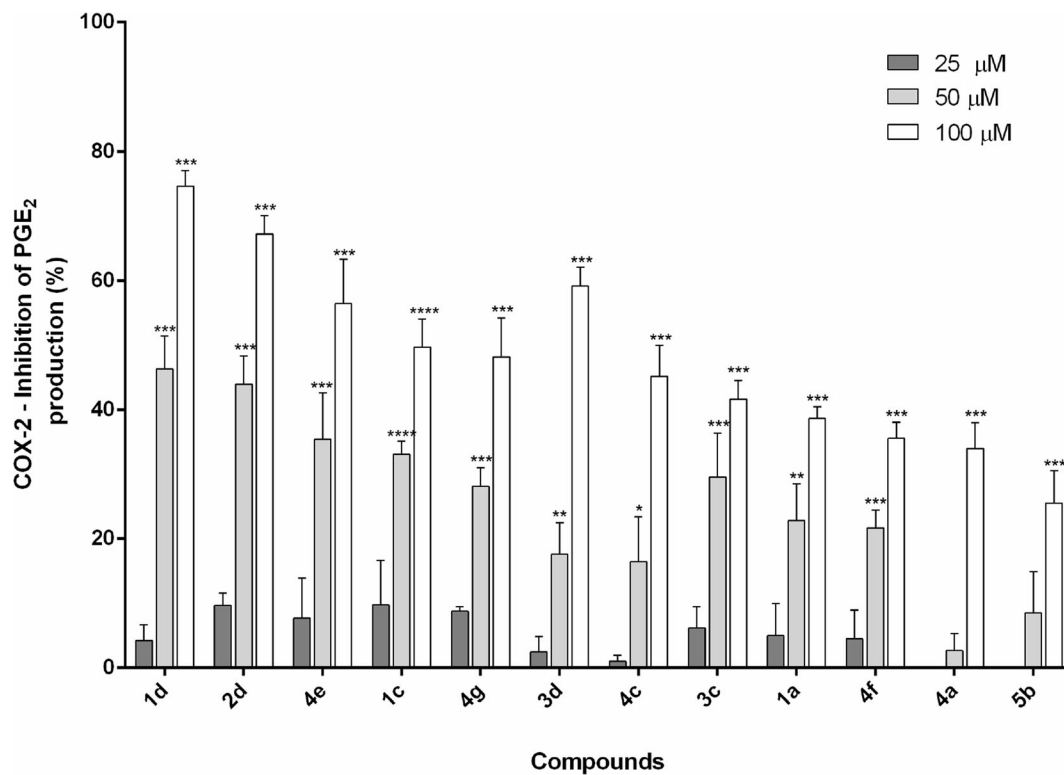


**Fig. 3.** Inhibition of COX-2—production of PGE<sub>2</sub> in human whole blood by the indicated studied flavonoids determined by EIA. Each value represents mean ± SEM of at least three experiments. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.1$  compared to the stimulated control (TXBSI/LPS).

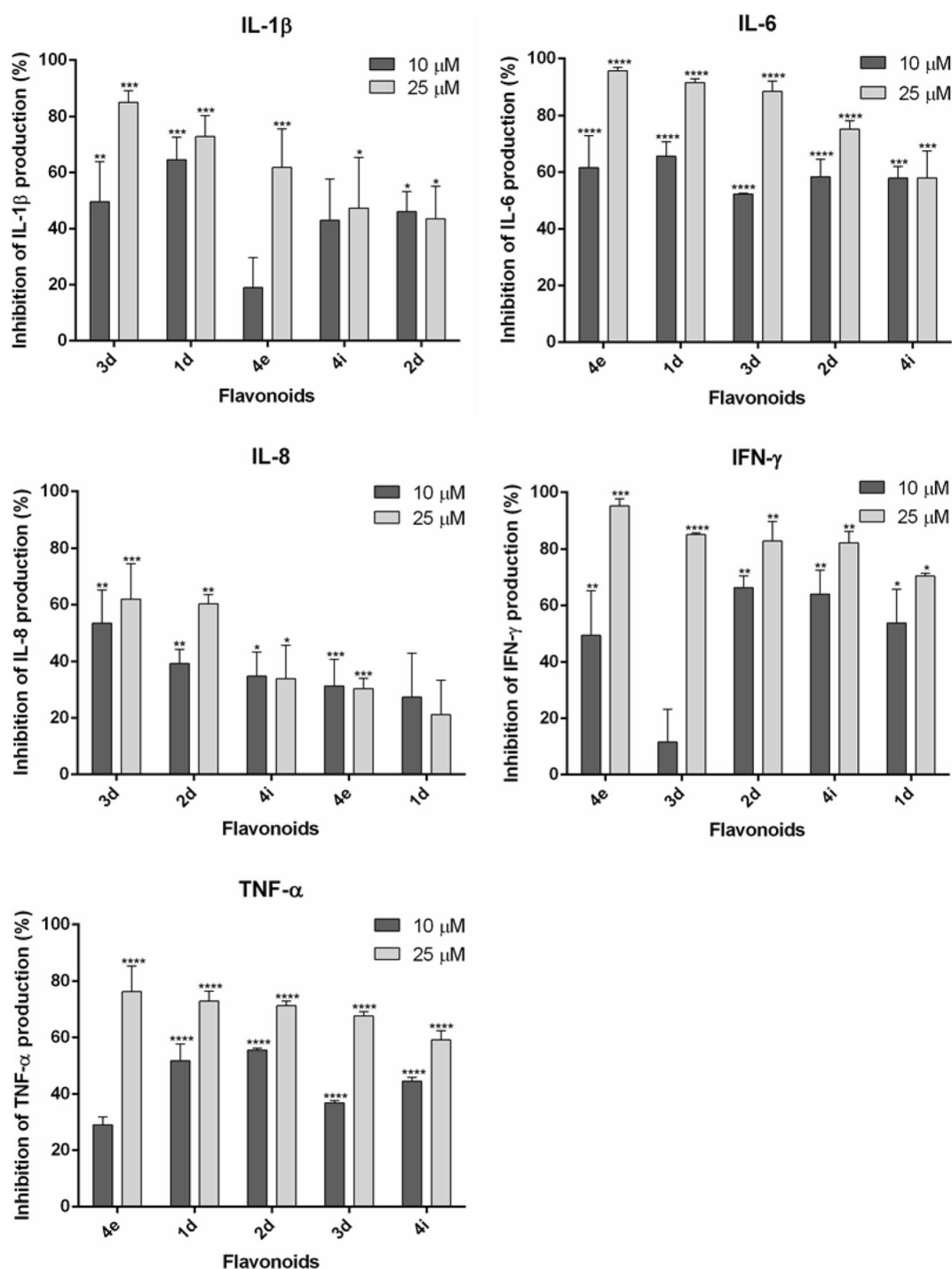


**Fig. 4.** Inhibition of cytokines/chemokine—production in human whole blood by the indicated studied flavonoids determined by EIA. Each value represents mean $\pm$ SEM of at least three experiments. \*\*\* $P$ <0.001, \*\* $P$ <0.01, \* $P$ <0.1 compared to the stimulated control (LPS).

The studied flavonoids revealed clear patterns of activity and some of them happened to be good inhibitors of

COX-1–PGE<sub>2</sub> production. Flavonoids **1a**, **2a**, and **3a** were three of the most active (Table 1). All of them hold none or

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just one hydroxyl (OH) group, being the less substituted flavonoids among them all. Flavonoids **4h**, **3c**, **1b**, **3b** and luteolin **4e** were active, but in a minor rate, with activities of inhibition lower than 50 %, up to 100  $\mu$ M. Overall, the less substituted flavonoid of each group, from **1** to **4**, were the ones that showed the best activity. Adding to this, flavones from group **1**, flavone **1a**, **1c**, and **1d** (Table 1) were the most active between the studied flavonoids. Their substitutions only occur in B ring, with none or two OH groups, which highlight once more that less substituted flavonoids may present a higher inhibition of COX-1. This may happen because the inhibitor-binding site within the active site in COX-1 is smaller and the interaction of less substituted molecules may be easier than a more substituted and big molecule. Besides, it was the reason why it was possible to selectively inhibit COX-2 and at the same time, avoid interfering with the homeostatic functions related to COX-1 [25, 26].

Moreover, the flavonoids of group **5** did not show any activity, which might mean that the lack of a double bound C2=C3 affects dramatically the studied activity. Wang and co-workers, who studied the effect of the flavonoids luteolin **4e**, quercetin **4i**, and ( $\pm$ )-naringenin **5a** in the inhibition of COX-1, have already reported that the C2=C3 double bound in C ring, which determines the co-planarity of the molecule, was essential for a higher COX-1 inhibitory activity. They also concluded that the presence of a 3-OH group in the C ring diminished slightly the COX-1 inhibitory activity. These findings are, in part, in agreement with the ones of the present study. However, in this study, quercetin **4i**, which has a 3-OH, did not present activity up to the highest tested concentration, 100  $\mu$ M. This discrepancy may be due to the different work material, the referred study used the microsomal isolated enzyme and in the present study it was used human whole blood, which is a most reliable matrix, more approximate to the real physiological conditions [27].

The studied flavonoids also demonstrated a promising inhibitory activity of COX-2-PGE<sub>2</sub> production. The most active were **1d**, **2d**, and **3d**, which have in common a catechol group in B ring. Luteolin **4e** was also a good inhibitor, sharing the same substitution pattern, but it was as good as flavonoids **4g** and **1c**. In group **5**, ( $\pm$ )-eriodictyol **5b** was the only one that showed activity. Once more, the importance of the catechol group in B ring was exposed. So far, it was clear that the structure that defines a good inhibitor of COX-1 is not the same for COX-2. The importance of the presence of a catechol group to the inhibition of COX-2, as already been reported in non-cellular *in vitro* studies and in isolated cells and it is now corroborated in a whole blood assay. The active site of COX-2 is

divided into three important regions: a hydrophobic pocket defined by Tyr<sup>385</sup>, Trp<sup>387</sup>, Phe<sup>518</sup>, Ala<sup>201</sup>, Tyr<sup>248</sup>, and Leu<sup>352</sup>; the entrance of the active site lined with the hydrophilic residues Arg<sup>120</sup>, Glu<sup>524</sup>, Tyr<sup>355</sup> and a side pocket lined by His<sup>90</sup>, Arg<sup>513</sup>, Val<sup>523</sup>, and Ser<sup>530</sup> [28]. Priscilla D'Mello and co-workers undertook a docking study where they found that the catechol group of B ring of luteolin was orientated towards the hydrophobic pocket, with 3',4'-dihydroxy groups forming H-bonds with Tyr<sup>385</sup> and Ser<sup>530</sup>. They also studied the interaction between chrysin and apigenin with COX-2, concluding that the 5-OH interacted with Tyr<sup>355</sup> forming an H-bond and the 4'-OH on the B ring of apigenin formed an additional H-bond with Tyr<sup>385</sup>; but, Ser<sup>530</sup> remained inaccessible, thereby underlining the importance of the catechol moiety. Moreover, they tested quercetin and found that although quercetin contains a catechol-like function on the B ring, it was not orientated towards the hydrophobic pocket. Instead, the A ring was orientated towards the pocket, forming H-bond with Ser<sup>530</sup> and the orientation enabled the 3-OH group to interact with Tyr<sup>355</sup>. Thus, they concluded that quercetin may not be a classical COX inhibitor but may be involved in the inhibition of transcription of COX-2 [29]. In a study with LPS-activated RAW264 macrophage cells, anthocyanidins with an ortho-dihydroxyphenyl structure on the B ring suppressed the COX-2 expression. The number of OH groups on the B ring appeared to be related to a molecular conformation that influences the interactions between flavonoids and enzymes such as tyrosine kinase and protein kinase C, which are involved in the transcriptional activity of COX-2 [30].

Feng and co-workers found that COX-2 is an oxidant stress-inducible gene, explaining the enhanced expression of COX-2 during inflammation. So, the radicals species produced by inflammatory cells could lead not only to the deleterious over-expression of COX-2, but also to the amplification of PGs during inflammation. The radical scavengers may inhibit the formation of PGs by the suppression of COX-2 expression [31]. Another study in RAW264.7 cells stated that COX was activated by peroxynitrite which may mean that this activation is particularly significant in cells with great amounts of nitric oxide and superoxide anion radical [32]. This may also explain why the flavonoids with a catechol group in B ring were good inhibitors of the production of PGE<sub>2</sub> via COX-2, because they have already proven to be great modulators of the *oxidative burst* during in the circumstances of an inflammation process, namely by the scavenge of reactive oxygen species [19].

The COX's inhibitory potential of flavonoids was found, for the first time, in 1980 [33]. Further ahead, several studies were developed and several flavones were found to be COX inhibitors, while some flavonols were LOX lipooxygenase (LOX) inhibitors. Moreover, it was found that reduction of the C2=C3 double bond and glycosylation reduced the inhibitory activity of COXs [34, 35]. Noteworthy is the interest in COX's inhibitory activity which has been growing since the discovery of the three isoforms of the enzyme, especially COX-2 because of its role both in the development and in the recovery of the inflammatory process. Thus, COX-2 is a very interesting drug target. Although the effect *in vivo* is difficult to predict, Lopez-Posadas and co-workers have already stated that some flavonoids probably downregulate the COX-2 expression and PG generation in conditions of intense oxidative stress, as in profound inflammatory reactions [36]. Furthermore, the series of flavonoids in this study have already proven to be very good modulators of the human neutrophil's oxidative burst, the first cells implicated in the inflammatory response. As so, these flavonoids appear by now as promising anti-inflammatory agents [19].

Table 1 exposes the selective inhibition of COX-2 by flavonoids **3d**, apigenin **4c**, chrysoeriol **4f**, diosmetin **4g** and (±)-eriodictyol **5b**; the first one is the most potent, followed by diosmetin **4g**, and (±)-eriodictyol **5b** was the less active of them. All of these flavonoids are substituted in B ring, presenting a catechol group or a methoxyl and an OH group and, in general, they are substituted in A ring with a OH group in positions 5 and 7. The exception is **3d**, which has only a 7-OH in A ring. Apigenin **4c** has already been described as a potent transcriptional inhibitor of COX-2 in mouse macrophages RAW 264.7, stimulated with LPS or IFN- $\gamma$ , *via* perturbation of NF- $\kappa$ B pathway [37]. Later on, Raso and co-workers studied the inhibitory effect of flavonoids in the PGE<sub>2</sub> production in a macrophage cell line J774A.1 stimulated with LPS and showed that the strongest active flavonoids examined possess a C2=C3 double bond and 5,7-dihydroxyl groups in the A ring, apigenin; highlighting once more the effect of flavonoids on macrophages [38]. As the ratio COX-1/COX-2 indicate the affinity for each study enzymes, if the ratio is lower than 1, it means that the flavonoid under study has more affinity for COX-2, which is the case of luteolin **4e**, the only one that showed to be more selective for COX-2, but the ratio value is not very low, 0.6.

Flavonoids **1b**, **2a**, **3a**, **3b**, and **4h** are COX-1 selective, but the most selective ones were **2a** and **3a**. The less potent was **3b** (Table 1). Yet again, these findings prove that the less substituted flavonoids are better inhibitors of

COX-1 and even selective for this enzyme. Analyzing the COX-1/COX-2 ratios, the studied flavonoids that revealed ratios higher than 1, i.e., have more affinity for COX-1, were flavone **1a**, **1c**, **1d** and chrysin **4a**. However, flavone **1a** was the one with a higher ratio value, 2.3, followed by chrysin **4a**, 2.0, so they have more affinity for COX-1. The activities of the flavonoids selective for COX-1 and with higher affinities for this enzyme may be explored in other directions, namely, in platelet studies, maybe revealing other interesting and promising biological effects. Inasmuch as the ideal inhibitor of COX-1, with antiplatelet effect, is the one that act by affecting platelet COX-1 activity, but only cause a marginal and transitory inhibitory effect on COX-2 [39].

Concerning the inflammatory diseases and the resolution of chronic inflammatory processes, the major problem of the existent anti-inflammatory drugs is the adverse reactions associated. The development of drugs that inhibit both COX-2 and 5-LOX may lead to compounds with enhanced efficacy, fewer side effects and with a wider spectrum of activity, when compared with selective COX inhibitors [40, 41]. In fact, flavonoids with a catechol group in B ring, namely, **3d** and luteolin **4e**, which in this study revealed to have a higher affinity for COX-2, have already proven to be very good inhibitors of human 5-LOX, so they are dual inhibitors, demonstrating a very auspicious pattern of activity for the resolution of the inflammatory processes [20].

Following this sense, the best inhibitors of COX-2, sharing a catechol group in B ring, **1d**, **2d**, **3d**, and luteolin **4e**, and quercetin **4i** were selected to assess their ability to modulate the production of IL-1 $\beta$ , IL-6, IL-8, IFN- $\gamma$ , and TNF- $\alpha$  in the same matrix used to study the COX activity, the human whole blood. Overall, the selected flavonoids proved to be exceptional inhibitors of cytokines' production for the highest tested concentration, 25  $\mu$ M. The lower activities were observed for the inhibition of IL-8.

It is well documented that cytokines and chemokines are essential keys of the inflammatory process. Actually, neutrophils, which are the first cells reaching the site of inflammation, may be primed by agents that include bacterial products and cytokines or chemokines, e.g., TNF- $\alpha$ , GM-CSF, IL-8 and IFN- $\gamma$ . Those neutrophils are then mobilized to the site of infection or inflammation, where they encounter activating signals to trigger bacterial killing and synthesize other factors, which can subsequently activate both neutrophils and other cells of the immune system. In particular, neutrophils have been shown to synthesize and secrete IL-8 in response to a number of stimuli, including TNF- $\alpha$  and GM-CSF [42]. Thus, a "balance" between the

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effects of pro-inflammatory (i.e., IL-1 $\beta$ , TNF $\alpha$ , IL-6, IL-8, and IFN- $\gamma$ ) and anti-inflammatory cytokines (i.e., IL-10, IL-4, TGF $\beta$ ) is believed to determine the outcome of diseases [43].

The studied flavonoids were able to effectively inhibit the production of IL-1 $\beta$ , in both tested concentrations of 10 and 25  $\mu$ M. Flavonoid **3d**, which has a 7-OH in the A ring presented the best activity (Fig. 4). However, the substitution did not seem determinant for the activity as flavonoid **1d**, which has none OH groups in A ring, presented almost the same activity. On the other hand, flavonoid **2d**, which has a 5-OH in the A ring was the less active, indicating that this feature may not favor the activity under study. Comparing the results obtained for luteolin **4e** and quercetin **4i**, it is possible to conclude that the 3-OH in the C ring did not influence in a positive way the activity, more on the contrary. In healthy subjects, IL-1 $\beta$  is barely detectable in the blood stream and during disease are in the femtomolar range, and this is explained by the tremendous potency of IL-1 $\beta$  to induce inflammatory responses, and it is why this cytokine is essential to be restrained in an inflammation process [44]. Also, it is already proven that IL-1 $\beta$  play a critical role in rheumatoid arthritis, as TNF- $\alpha$  which up-regulate each other in a positive feedback loop to cause most of the pathologic symptoms [45].

IL-6 can be induced by other pro-inflammatory cytokines, like IL-1 $\beta$  and TNF- $\alpha$ , and depending on the context, IL-6 is itself commonly regarded as pro-inflammatory, or as an anti-inflammatory cytokine [6]. Luteolin **4e** was the best inhibitor of IL-6 production (Fig. 4). Luteolin has a catechol group in B ring, as all the selected flavonoids, and two OH groups in the C5 and C7 of A ring. Quercetin **4i** was the less active, and its substitution pattern is equal to luteolin, but with an additional 3-OH in the C ring, so this OH did not favor the inhibition of IL-6 production, as what happened for IL-1 $\beta$ . The existence or not of an OH group in the A ring did not seem to be determinant for the activity as the other studied flavonoids presented almost the same activities.

IL-8 was the only chemokine studied, and it is the most potent chemokine in terms of neutrophils chemotaxis and enzyme release [46]. IL-8 can be induced on virtually all cells (leukocytes, fibroblasts, endothelial and epithelial cells and tumor cells) upon stimulation with a variety of products, including cytokines (IL-1 $\beta$ , TNF- $\alpha$ ), other chemoattractants (leukotriene B4) and bacterial material [LPS, N-Formyl-Met-Leu-Phe (fMLP)]. In an

experimental collagen-induced arthritis, administration of anti-IL-8 antibodies to the inflammatory joint reduced the inflammation, which may indicate IL-8 as a potential target for the resolution of this kind of inflammatory diseases [47]. In this study, the tested flavonoids were able to inhibit IL-8 production but in a less extent than the studied cytokines. Nevertheless, the presence of just one OH group in the A ring, independently of its position may be important for a higher activity as flavonoids **3d** and **2d** were the most active (Fig. 4). The presence of a 3-OH in C ring did not seem important for the activity as quercetin **4i** and luteolin **4e** presented almost the same activity. Finally, the number of OH seems important for the activity, as flavonoid **1d**, the less substituted, was the less active. It is important to note that IL-8 may be induced by cytokines such as IL-1 $\beta$  and TNF- $\alpha$ , so flavonoids which modulate the production of these cytokines, indirectly modulate the production of IL-8 [48].

IFN- is an important cytokine that has been implicated in the pathogenesis of a variety of autoimmune and chronic inflammatory conditions systemic lupus erythematosus, type I diabetes mellitus, adjuvant-induced arthritis [1]. All the selected flavonoids were effective inhibitors of the IFN- production, percentages higher than 60 %, for 25  $\mu$ M. Luteolin **4e** was the best one inhibiting the production almost completely (Fig. 4). Once more, the presence of OH groups in A ring appeared as an important feature to the activity as flavonoid **1d** was once more the less active.

The production of TNF- $\alpha$  was surely inhibited by the selected flavonoids, in a concentration-dependent manner. Analyzing the results, it may be concluded that the presence and the position of the OH groups in A ring is not a determinant for the activity, as luteolin **4e**, **1d**, **2d**, and **3d** were equally potent. On the other hand, the 3-OH in C ring did not contribute for the activity improvement, because quercetin **4i** was the less active. There are other reports of studies undertaken in isolated macrophages that did not support the relevance of the 3-OH. Although in different matrixes, these findings are in agreement with the ones found in the present study [43, 49]. TNF- $\alpha$  share several pro-inflammatory properties with IL-1 $\beta$ , like fever induction and stimulation of the production of collagenase and PGE<sub>2</sub> by synovial cells, contributing to the damage in inflammatory conditions such as rheumatoid arthritis. They further exert secondary inflammatory effects by stimulating IL-6 synthesis in several cell types. IL-6, along with TNF- $\alpha$  and IL-1, induce fever and the acute phase response, maintaining the inflammatory

response through a cascade of cytokines with overlapping properties [1]. Luteolin and quercetin have already proven to be effective inhibitors of the production of TNF- $\alpha$  and IL-6, although the majority of the studies were carried out in different matrices, which did not allow concrete conclusions about the effect in the human body [50, 51]. Xagorari and co-workers have reported that luteolin and quercetin inhibited LPS-stimulated TNF- $\alpha$  and IL-6 release in RAW 264.7 macrophages [52]. Luteolin also inhibited the production of TNF- $\alpha$  *in vivo*, in male ICR mice [53]. Another study was undertaken in mice, to which luteolin was administrated intraperitoneally and it attenuated the increase in serum TNF- $\alpha$  levels elicited by LPS [54]. In accordance with the literature, in this work, luteolin **4e** was the most active flavonoid in the inhibition of almost all cytokines, IL-6, IFN- $\gamma$ , and TNF- $\alpha$ , which makes this flavonoid a promise in the modulation of the inflammatory process.

All in all, the results of this work reveal promising flavonoids for the modulation of the inflammatory process, namely the ones presenting a catechol group in B ring. Better still, these flavonoids are able to simultaneously inhibit the production of inflammatory PGE<sub>2</sub> and pro-inflammatory cytokines.

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## ***CHAPTER III. Original Research***

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### **III.4. Modulation of apoptosis pathways in human neutrophils by flavonoids**

Manuscript submitted for publication

My contribution for this work comprised all the performed studies.

## **Modulation of apoptosis pathways in human neutrophils by flavonoids**

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### **Abstract**

Human neutrophils are key players in the inflammatory process, which is no less than a natural physiological response of the organism to tissue damage by injury or infection. Following its physiological activity at inflammatory sites, neutrophils are eliminated by apoptosis, as a natural and crucial phenomenon that should happen during the resolution of inflammation. During an uncontrolled inflammatory response neutrophils start the over-production of reactive oxygen species and cytokines, and the gene expression of transcription factors, which modulate the apoptotic process and may delay it, causing an imbalance in the neutrophils' homeostasis. Thus, the regulation of neutrophils' apoptosis may constitute a new therapeutic target and another key to understand and to contain the uncontrolled deleterious effects of chronic inflammation. The aim of this work was to clarify the effects of a group of selected flavones and a flavonol in human neutrophil's apoptosis process, and study the mechanisms by which this effect was produced, providing an insight on the structure/activity relationship. The studied flavonoids were effective pro-apoptotic molecules; this effect is potentially undertaken via caspases 3, 8 and 9, mainly through the extrinsic apoptotic pathway. All the flavonoids sharing a catechol group in B ring showed good pro-apoptotic activities. However, the 3-OH in C ring or a 5-OH in A ring do not accredit for apoptosis, more on the contrary. Moreover, the presence of just one OH group in B ring clearly lowered the pro-apoptotic effect. These flavonoids revealed a great potential to be used in chronic inflammation where the normal apoptotic function of neutrophils is altered

**Key words:** Flavonoids, Apoptosis, Caspase activation, Extrinsic pathway, Mitochondrial potential, p53

## **1. Introduction**

Inflammation is a natural physiological response of the organism to tissue damage by injury or infection. Neutrophils are the first cells arriving at the site, where their lifetime can increase several fold. [1] They constitute the first line of host defence against invading pathogens (bacteria, fungi, etc.). Under physiologic conditions, the turnover of neutrophils, with a lifetime of around 2-3 days, is a natural homeostatic event. During inflammatory response, the turnover of neutrophils is referred to increase to around 5.4 days, but also in this case, death is an essential cellular event to maintain neutrophil number, avoiding the evolution to chronic inflammation. [2-4] Moreover, apoptosis is crucial for the efficient disposal of the engulfed pathogens, for the generation and release of neutrophil extracellular traps (NETs), and for the production of cytokines through the clearance of apoptotic neutrophils by macrophages. Constitutive neutrophil death is indeed an essential mechanism for the modulation of neutrophil's homeostasis. [5] The delayed apoptosis of neutrophils has been related with numerous acute and chronic inflammatory diseases, namely in Kawasaki disease, [6] inflammatory bowel disease, [7], and rheumatoid arthritis [8] among others. In fact, in the event of inflammatory processes, neutrophils produce a myriad of inflammatory mediators, namely, reactive species, and pro-inflammatory cytokines; however, in a chronic state these mediators are over-produced and lead to the damage of the adjacent healthy tissues, propagating the inflammation. As so, this delayed apoptosis and clearance of neutrophils may contribute to unwanted and exaggerated inflammation. The precise regulation of neutrophils' apoptosis prevents the release of toxic intracellular components that can damage healthy tissue. [2, 9]

Various molecules are involved in the survival of neutrophils; they are designated as pro-survival stimuli, and comprise cytokines, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and interferon gamma (IFN- $\gamma$ ), adenosine triphosphate (ATP), and leukotriene B<sub>4</sub> (LTB<sub>4</sub>), among others. [5] It is also well established that the apoptotic process is regulated by a cascade of signalling pathways, involving anti-apoptotic and pro-apoptotic transcription factors. For example, the up-regulation of p53 triggers the extrinsic apoptotic cascade, [10] while some transcription factors may display anti-apoptotic effects, like nuclear factor kappa B (NF- $\kappa$ B) and signal transducer and activator of transcription 3 (STAT-3), or pro-apoptotic effects, like c-Jun N-terminal kinase (JNK), p38 (mitogen-activated protein kinases) and apoptosis inducing factor (AIF). [5]

It is therefore of utmost importance to discover and study molecules capable of modulating and control neutrophils' apoptosis to provide a fine balance between their

immune functions and their safe clearance, and consequently modulate the inflammatory process. Flavonoids are natural polyphenols that can be widely found in nature. They are well-known as having a myriad of biologic activities, including positive modulating effects in inflammation. [11] These compounds exhibit antioxidant activity; modulate eicosanoids, cytokines, chemokines' production and gene expression of transcription factors. [12, 13] Therefore, flavonoids, as molecules capable of modulating these inflammatory mediators may have also an important modulating activity of the apoptosis process of neutrophils, and this way to contribute to the resolution of inflammatory processes.

The aim of this work was to clarify the effects of a group of selected flavones and flavonol (figure 1) in human neutrophil's apoptosis process, and to study the mechanisms by which this effect was produced, and provide an insight on the structure/activity relationship.

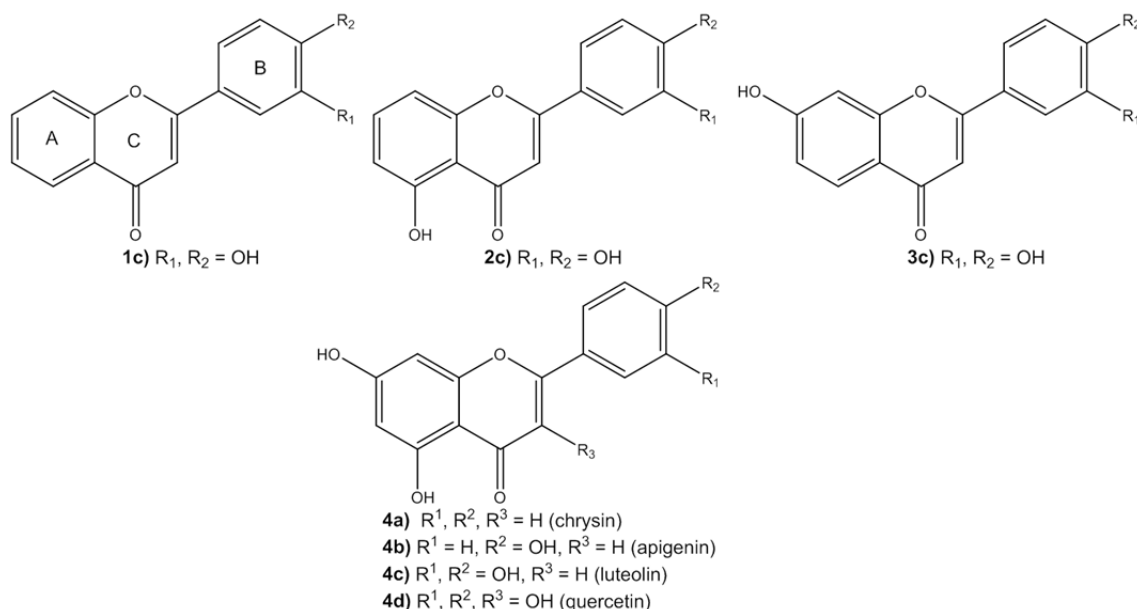


**Figure 1** - Chemical structures of the studied flavonoids.

## **2. Materials and Methods**

### **2.1. Materials**

The following reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, USA): dimethylsulfoxide (DMSO), RPMI 1640 medium, fetal bovine serum, L-glutamine, penicillin, streptomycin, nutlin-3, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), chrysin, apigenin, luteolin, and quercetin. The other tested flavonoids were obtained from Indofine Chemical Company, Inc. (Stryker Lane Hillsborough, USA). Annexin-V-FLUOS Staining Kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany).

Hemacolor® was obtained from Merck (Darmstadt, Germany). Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK, and BD™ MitoScreen Flow Cytometry Mitochondrial Membrane Potential Detection Kit were purchased from BD Biosciences (San Diego, USA). DC Protein Assay was purchased from Bio-Rad (California, USA). Nuclear Extract Kit and TransAM® p53 Transcription Factor Assay Kit were purchased from Active Motif (La Hulpe, Belgium).

## **2.2. Methods**

### **2.2.1. Isolation of Human Neutrophils**

Following informed consent, venous blood was collected by antecubital venepuncture from healthy human volunteer, into vacuum tubes with K<sub>3</sub>EDTA. The isolation of human neutrophils was performed by the density gradient centrifugation method as previously reported. [14] RPMI 1640 medium [(pH = 7.4) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin] was used as incubation media in all experiments, in which isolated neutrophils were exposed to the tested flavonoids.

### **2.2.2. Assessment of neutrophils' apoptosis**

#### **2.2.2.1. Cytomorphological alterations**

Following incubation of isolated neutrophils ( $1 \times 10^6$  / mL) with the tested flavonoids (50 µM), in DMSO:RPMI (1:1), for 4 h, at 37 °C, in a humidified incubator, samples were cytocentrifuged, stained with Hemacolor®, and counted under light microscopy (100 x) to determine the proportion of cells displaying characteristic apoptotic morphology. At least 400 cells were counted per slide. [15]

#### **2.2.2.2. Annexin-V binding assay**

Following incubation of the isolated neutrophils ( $1 \times 10^6$  / mL) with the tested flavonoids (0 - 50 µM), in DMSO:RPMI (1:1), for 4 h, at 37 °C, in a humidified incubator, neutrophils were analysed by flow cytometry, using the commercial Annexin-V-FLUOS Staining Kit according to the manufacturer's instructions, allowing the simultaneous staining with annexin-V labelled with fluorescein and propidium iodide. The fluorescence signals were collected using a BD Accuri™ C6 flow cytometer. A polygon gate was set according to the neutrophils' light scattering properties (in a forward vs. side scatter plot) excluding cell debris and other blood cells, restricting the analysis to neutrophils only. At least 10,000 cells were collected and their fluorescence signal detected, in logarithmic mode and the

data were analysed using the BD Accuri™ C6 software. The green fluorescence due to Annexin-V conjugated with FITC was followed in channel 1 (FL1) and plotted as a histogram of FL1 staining. Fluorescence due to the propidium iodide incorporation was followed in channel 3 (FL3). [15]

#### **2.2.2.3. Caspases-3, -8 and -9 activities**

The involvement of caspases -3, -8 and -9 in flavonoid – induced apoptosis were studied using three irreversible inhibitors of these caspases: Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK, respectively, which are able to block apoptosis. The isolated neutrophils ( $1 \times 10^6$  / mL) were treated with the caspase inhibitor under study, Z-DEVD-FMK (10  $\mu$ M), Z-IETD-FMK (5  $\mu$ M), or Z-LEHD-FMK (10  $\mu$ M), in RPMI medium, for 30 min, at 37 °C, in a humidified incubator. Flavonoids (50  $\mu$ M), in DMSO:RPMI (1:1), were then added and incubated for 4 h, in the same conditions. Apoptotic neutrophils were analysed by flow cytometry, using the commercial Annexin-V-FLUOS Staining Kit, as described above in 2.2.2.2. [15]

#### **2.2.3. Assessment of neutrophils' mitochondrial potential**

To evaluate the flavonoids' effect on mitochondrial membrane's potential ( $\Delta\Psi_m$ ), isolated neutrophils ( $1 \times 10^6$  / mL) were incubated with the tested flavonoids (50  $\mu$ M), in DMSO : RPMI (1:1), for 4 h, at 37 °C, in a humidified incubator. Afterwards, alterations of the mitochondrial potential were analysed by flow cytometry, using the commercial BD™ MitoScreen Flow Cytometry Mitochondrial Membrane Potential Detection Kit, according to manufacturers' instructions. Briefly, samples were collected and centrifuged at 400 g, 5 min, at room temperature. The supernatants were discarded and 500  $\mu$ L of JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) work solution were added and the pellet resuspended. This mixture was incubated for 15min, in a humidified incubator. After the end of the incubation time, 2 mL of Assay Buffer 1x were added, followed by a centrifugation, 400 g, 5 min, at room temperature. The supernatant was discarded and the pellet resuspended in 500  $\mu$ L of Assay Buffer 1x. The fluorescence signals were collected using a BD Accuri™ C6 flow cytometer, as described above. JC-1 is a lipophilic fluorochrome that is used to evaluate the status of the  $\Delta\Psi_m$ . In live cells JC-1 penetrates the plasma membrane of cells as monomers. Healthy mitochondrial membrane is polarized and JC-1 is rapidly taken up. This uptake increases the concentration gradient of JC-1 leading to the formation of JC-1 aggregates (known as J-aggregates) within the mitochondria. JC-1 aggregates show a red spectral shift resulting in higher levels of red fluorescence emission which is measured in the FL-2 channel. On the other hand, in apoptotic cells with depolarized  $\Delta\Psi_m$ , JC-1 persist as monomers, and

do not aggregate; this leads to reduced fluorescence in the FL-2 channel and to the appearance of a green spectral shift resulting in higher levels of green fluorescence emission which is measured in the FL-1 channel. [16]

#### **2.2.4. Assessment of p53 activation in neutrophils**

Isolated neutrophils ( $8 \times 10^6$  / mL) were exposed to the tested flavonoids (50  $\mu$ M), in DMSO : RPMI (1:1), for 4 h, 6h, 8h and 24h, at 37 °C, in a humidified incubator. After each incubation time, the nuclear extract was isolated, using the Nuclear Extract Kit, according to the manufacturers' instructions, with the suggested sonication (2 cycles of 12 s pulses) before the last centrifugation. The protein was quantified by the Lowry method (DC Protein Assay), and the cells' lysates were diluted in complete lysis buffer to 3.5  $\mu$ g/mL. The p53 activation was evaluated by TransAM® p53 Transcription Factor Assay Kit, according to the manufacturers' instructions.

#### **2.2.5. Statistical analysis**

Statistics were calculated using GraphPad Prism™ (version 6.0; GraphPad Software). Results are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical comparison between groups was estimated using the one-way analysis of variance (ANOVA), followed by the Bonferroni's post-hoc test. In all cases, p-values lower than 0.05 were considered as statistically significant. Inhibitory patterns were calculated with GraphPad Software.

### **3. Results**

#### **3.1. Effect of flavonoids on neutrophils' apoptosis**

All the tested flavonoids were able to induce neutrophil's apoptosis in the present experimental conditions. Despite the fact that these flavonoids have only slight structural differences, they present different activities. The flavones luteolin **4c**, **3c**, chrysin **4a** and **1c** were clearly the most active, increasing apoptosis in  $82 \pm 6$  %,  $74 \pm 11$  %,  $74 \pm 10$  % and  $66 \pm 7$  %, respectively , for 50  $\mu$ M (figure 2). The other tested flavonoids, quercetin **4d**, **2c** and apigenin **4b** resulted in a lower pro-apoptotic effect (less than 50 %):  $36 \pm 6$ %,  $41 \pm 7$  % and  $10 \pm 4$ %, respectively (figure 2). Figure 3 displays the representative images obtained for luteolin **4c**, **3c**, chrysin **4a** and **1c** through the cellular morphological study. It is possible to observe the cytomorphological alterations of apoptotic neutrophils, which appear with nuclear condensation and cellular shrinkage, when compared to healthy

ones. In this figure is also displayed the representative flow cytometry plots of annexin-V [(annexin-V; x axis)/PI (y axis)] binding.

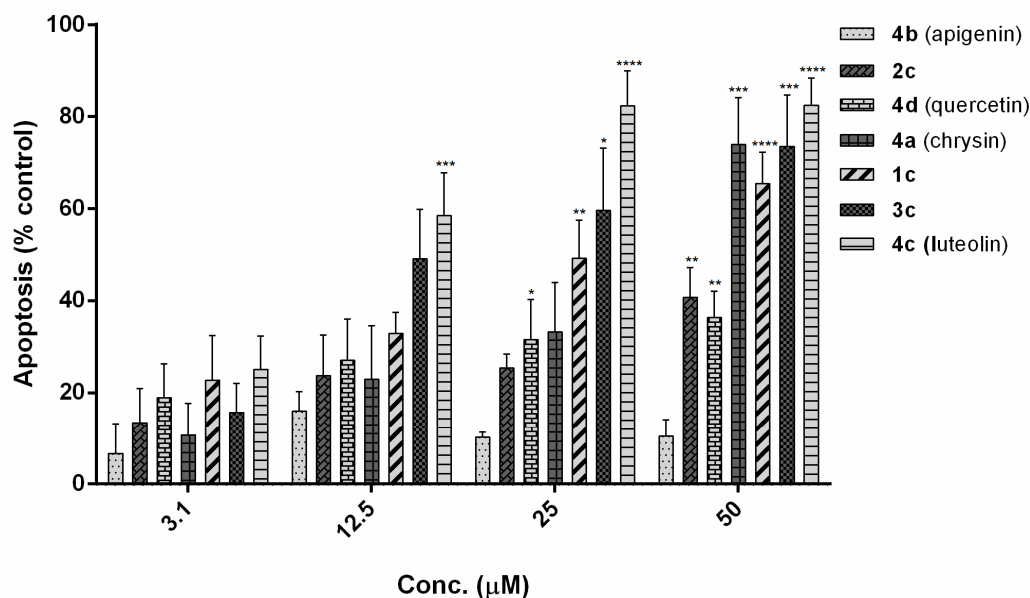


**Figure 2** - Effect of flavonoids (3.1 - 50 µM) on human neutrophils' apoptosis, assessed by flow cytometric analysis of annexin-V binding assay. \*\*\*\* $p \leq 0.001$ , \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$ , and \* $p \leq 0.05$ , compared with the control assay (without flavonoid). Values are given as the mean  $\pm$  SEM ( $n \geq 3$ ).

### **3.2. Effect of caspases-3, -8 and -9 inhibitors on flavonoid-induced apoptosis**

The flavonoids under study were treated with specific and irreversible caspases inhibitors to verify its influence on the flavonoids-induced apoptosis. Figure 4 reveals that in the presence of Z-DEVD-FMK, a caspase-3 inhibitor, the induction of apoptosis decreased for all the flavonoids, to percentages lower than 20 %; the only exception was for flavone **1c**, for which the induction of apoptosis decreased, but just to  $39 \pm 1$  %. This significant decrease of apoptosis percentage indicates that flavonoids induce apoptosis via caspase-3. In what concerns caspase-8, the activity of all flavonoids was affected once more. Figure 4 clearly shows a diminished percentage of apoptosis for all the flavonoids in the presence of this inhibitor, almost all to values below 20 %, for 50 µM. This means that when caspase-8 is inhibited the flavonoids are not capable of inducing apoptosis in a large extent, which indicates that caspase-8 is also involved in the observed pro-apoptotic

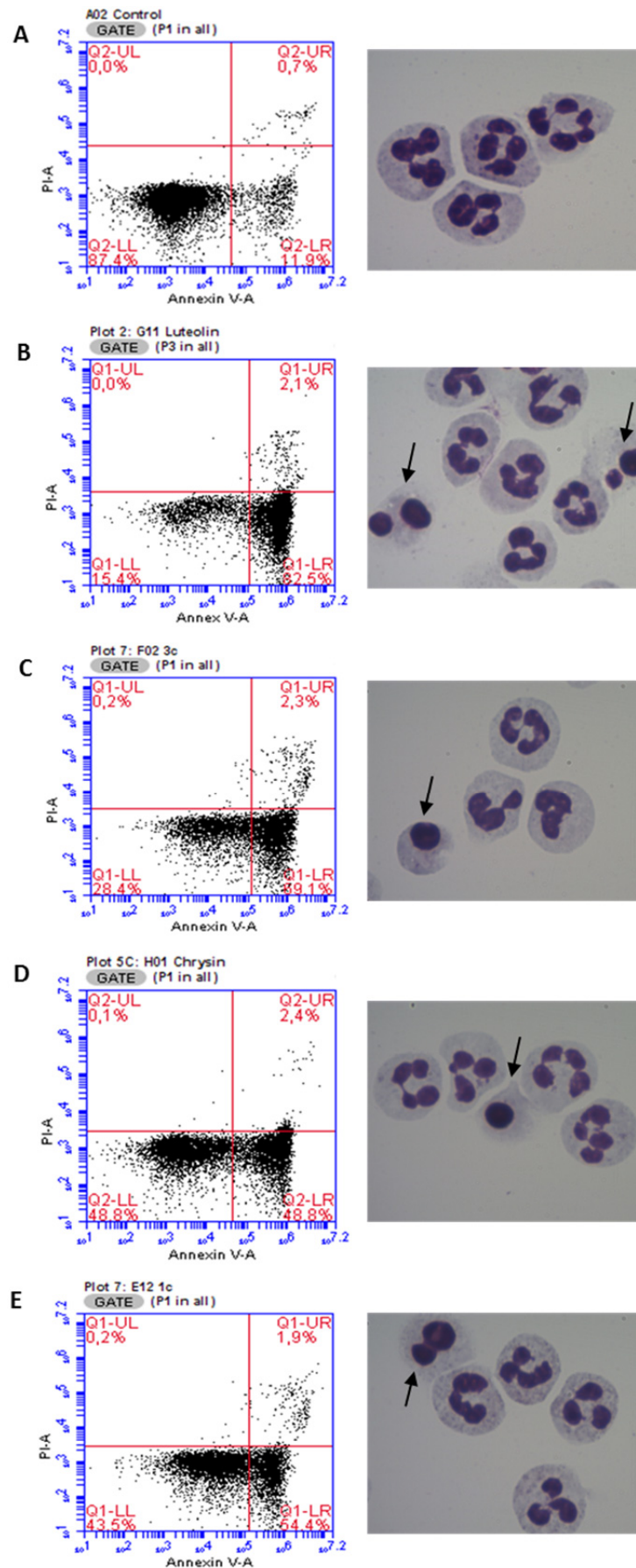


**Figure 3** - Representative flow cytometry plots of annexin-V [(annexin-V; x axis)/PI (y axis)] binding and representative images of cytocentrifuge preparations after staining with

Hemacolor® for control (without flavonoid) (**A**), 50  $\mu$ M of luteolin **4c** (**B**), 50  $\mu$ M of flavone **3c** (**C**), 50  $\mu$ M of chrysin **4a** (**D**), 50  $\mu$ M of flavone **1c** (**E**), after 4h of incubation. Arrowheads indicate an example of apoptotic neutrophil.

effect. For apigenin **4b**, this effect was not so pronounced since its capacity to induce apoptosis was already low, when compared to the other tested flavonoids. In the presence of , Z-LEHD-FMK, caspase-9 inhibitor, the pronounced decrease of apoptosis percentage is also plain; however, it is possible to distinguish two groups: one formed by luteolin **4c**, flavone **1c**, and chrysin **4a**, flavone **3c**, quercetin **4d**, in which apoptosis percentages were near 20 %, for 50  $\mu$ M; and the other integrating flavone **2c**, and apigenin **4b**, in which apoptosis percentages almost equalled the control (without flavonoid), for 50  $\mu$ M (figure 4).

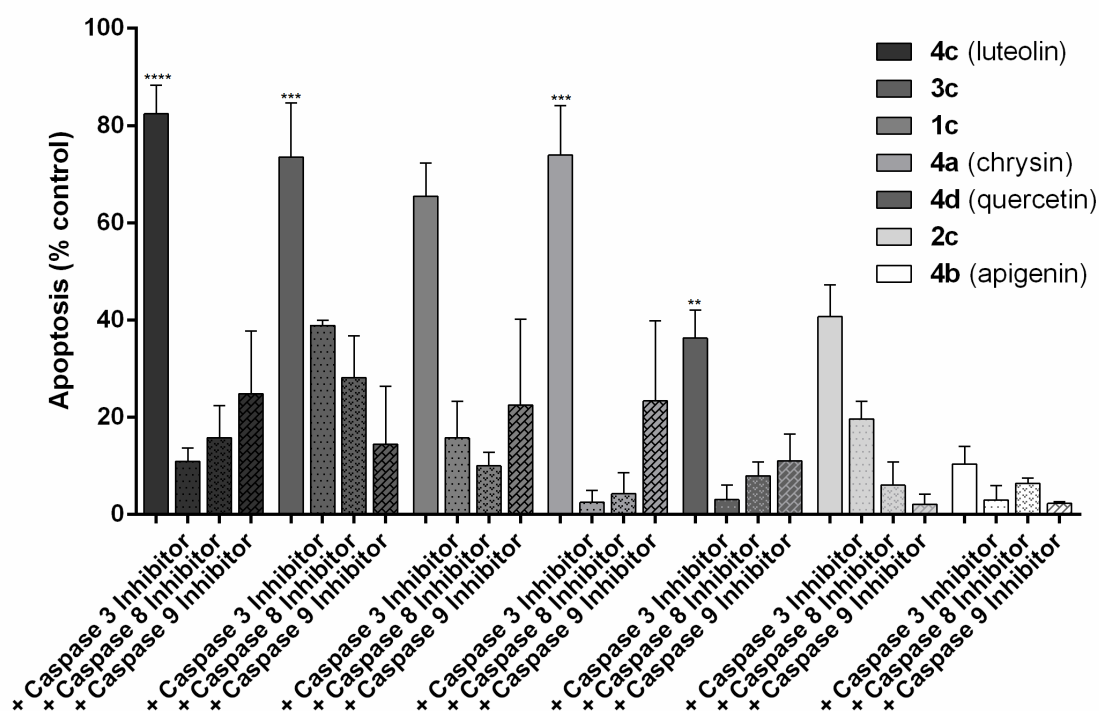


**Figure 4** - The effect of caspases inhibitors in flavonoids' pro-apoptotic activity. Neutrophils were cultured with or without the caspase 3, caspase 8 or caspase 9 inhibitors, in the presence of the studied flavonoids (50  $\mu$ M), before assessment of neutrophil apoptosis by flow cytometry analysis. \*\*\*\* $p \leq 0.001$ , \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$ , and \* $p \leq 0.05$ , compared with the control assay (without flavonoid). The values are given as the mean  $\pm$  SEM ( $n \geq 3$ ).

### **3.3. Influence of flavonoids on neutrophils' mitochondrial potential**

Figure 5 represents the percentage of cells expressing  $\Delta\Psi_m$  after 4h of incubation with the tested flavonoids. The values obtained under influence of the tested flavonoids were close to the control (without flavonoid). As so, none of the tested flavonoids affected the mitochondrial potential, at the present experimental conditions. CCCP was used as positive control (50 - 150  $\mu\text{M}$ ).

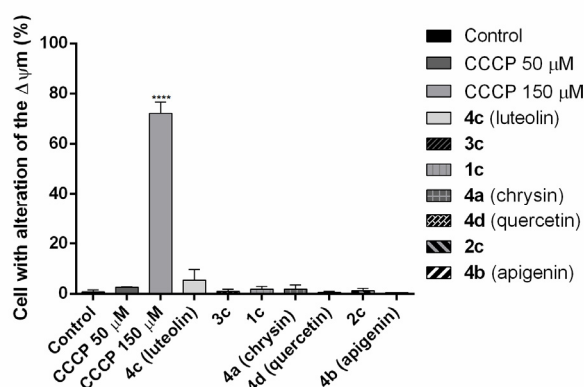


**Figure 5** - Effect of flavonoids in the  $\Delta\Psi_m$ , assessed by flow cytometric analysis, and expressed as the percentage of cells expressing  $\Delta\Psi_m$ , after the incubation period. \*\*\*\* $p \leq 0.001$ , compared with the control assay (without flavonoid). The values are given as the mean  $\pm$  SEM ( $n \geq 3$ ).

### **3.4. Activation of neutrophils' p53 by the studied flavonoids**

Under the studied experimental conditions, none of the studied flavonoids was capable of activating p53, for all the incubation periods tested, 4, 6, 8 and 24h (data not shown). Nutlin-3a (40  $\mu\text{M}$ ) was used as positive control for which an effective activation was observed (data not shown). [17]

## **4. Discussion**

Neutrophils are key players in the regulation of the inflammatory response; they are considered both inflammatory effectors and immunoregulatory cells. [18] It is now evident that neutrophils' demise is mediated by a complex network of intracellular death / survival signaling pathways and variety of extracellular stimuli, namely by pro-inflammatory cytokines. [2] The flavonoids selected for this study were previously shown to be effective inhibitor of these pro-inflammatory cytokines [19], which make them potential modulators of apoptosis. The regulation of neutrophils' apoptosis may constitute a new therapeutic target and another key to understand and to contain the uncontrolled deleterious effects of

chronic inflammation, a condition that affects millions of people, characterized by permanent affliction and disability. [20] In this work, several assays were undertaken pursuing the idea that these flavonoids would be able to modulate neutrophil's apoptosis. Moreover, it was bidden to understand in which apoptotic pathway this flavonoids were acting and finally their putative activating effect on p53.

Five of the flavonoids selected for these study share a functional substitution pattern, a catechol group in B-ring, that has already demonstrated to be determinant for various of the anti-inflammatory effects of flavonoids [19, 21, 22]; those are flavones **1c**, **2c**, **3c**, luteolin **4c** and quercetin **4d**, although they differ in the number of OH groups in A and C rings. Even though, chrysin **4a** and apigenin **4b**, which has none or just one OH group in B ring were tested to ascertain if the catechol group is indeed essential for the activities under study.

All the tested flavonoids induced neutrophil's apoptosis. Apigenin **4b**, which has only one OH group in B-ring, was the less potent, with an apoptosis induction of only  $10.4 \pm 3.6$  % for the highest tested concentration, 50  $\mu$ M (figure 2). Luteolin **4c**, flavonoids **3c** and **2c**, and chrysin **4a** were the ones that led to a greater increase of apoptosis, compared to the control (without flavonoid) (figure 2). The first three flavonoids have in common the catechol group in B ring which may explain their proximity of potencies; however, chrysin **4a** has no OH group in B ring, and therefore this result does not corroborate the determinant role of the catechol group for the activity under study. On the other hand, it is possible to conclude that just one OH group in B-ring is not good enough to induce apoptosis, as confirmed in apigenin **4b**, which only displayed a weak effect,  $10 \pm 4$  %, for 50  $\mu$ M; quercetin **4d** and flavone **2c**, in their turn, had similar effects, by inducing apoptosis in  $36 \pm 6$  %,  $41 \pm 7$  %, respectively, for 50  $\mu$ M; revealing that a 3-OH in C ring, for quercetin, and a 5-OH in A ring, for **2c**, do not accredit for apoptosis, more on the contrary. Lucas and co-workers have already demonstrated that luteolin and apigenin induced neutrophils' apoptosis, as did other plant flavone, wogonin, in a time- and concentration- dependent manner; however, they did not establish a structure/activity relationship. [23] Liu and co-workers showed that quercetin was able to prevent delayed apoptosis in neutrophils exposed to lipopolysaccharide (LPS), probably by quercetin ability to induce apoptosis, reversing the LPS effect; [24] this result corroborates the observations made in the present study. More recently, a study of the pro-apoptotic effects of chlorinated flavonoid in human neutrophils attested once more their involvement on the caspase-3 mediated apoptosis. [15] All in all, these results prove that the studied flavonoids are promising molecules to solve the inflammatory process by means of neutrophils' apoptosis induction; avoiding the *neutrophilia* phenomenon, found in various

infectious and chronic diseases, and consequently, the overproduction and release of toxic metabolites that always lead to tissue injury and subsequent organ dysfunctions. [5] Generally, the apoptosis process occurs through two different pathways: intrinsic and extrinsic. The intrinsic pathway is usually characterized by the loss of mitochondrial membrane's integrity and release of cytochrome c, apoptotic protease activating factor 1 (Apaf-1) and other pro-apoptotic factors into the cytoplasm. The mitochondrial membrane's potential is highly dependent on the ratio between pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) factors. The cytochrome c, Apaf-1, dATP and procaspase-9 associate to form a complex designated as apoptosome that activates caspase-9 through autocatalysis. The extrinsic pathway is characterized by the triggering of the members of the death receptor super-family, such as tumor necrosis factor receptors (TNFR), Fas, etc. The ligands bind to the receptor, leading to the recruitment of specific adaptor proteins Fas-associated protein with Death Domain (FADD) / tumor necrosis factor receptor type 1-associated death domain (TRADD) and recruitment and processing of procaspase-8 molecules. The latter can in turn activate Bid, which represents a crosstalk between extrinsic and intrinsic death pathways, which may explain why this pathway may also activate caspase 9. Caspase-9 and caspase-8 activate caspases-3 through the cleavage of procaspase-3 that, in turn, activate other executor caspases and cleave cellular targets [2, 10] As so, it is essential to understand by which pathways these flavonoids exert their pro-apoptotic effects. For this purpose, three selective and irreversible caspase inhibitors were tested: Z-DEVD-FMK, for caspase-3, Z-IETD-FMK, for caspase-8, and Z-LEHD-FMK for caspase-9. In general, the obtained results indicate that flavonoids exert their effects via activation of caspases, as all the inhibitors used affected the apoptosis percentages of flavonoids. This is not new for apigenin and luteolin, as Lucas and co-workers, in the same work referred above, stated that their effect in apoptosis was achieved through direct effect in caspases, although they used a broad spectrum caspase inhibitor. [23] In this work we aimed to investigate the deeper effect in each caspase. The caspase-3 inhibitor significantly decreased the apoptosis percentage of all flavonoids, down to values almost equal to the control (without flavonoid), for flavone **1c** in a slighter extent, indicating that they exert their effect via caspase-3. Zielinska-Przyjemska and co-workers studied naringenin, naringin and hesperidin and concluded that they induce caspase-3 activity through their antioxidant effects. They observed that production of ROS inhibited the caspase-3 activation, and consequently the apoptosis process. The tested flavonoids reversed this process. [25] Interestingly, the flavonoids studied in the present work have also proved to modulate the production of several ROS [21]; so they may be able to modulate apoptosis, through direct or indirect inhibition of caspase-3 activity. However, as caspase-3 is common to the intrinsic and extrinsic apoptotic pathways, this result needed

to be further investigated. For caspase-8, as for caspase-3, the pro-apoptotic effect of all flavonoids was reduced in the presence of the selective inhibitor, nearly to the control levels. Flavone **1c** was the one that was less affected by the caspase inhibitor, similar to what happened for caspase-3. Generally, it is possible to conclude that caspase-8, associated with the extrinsic apoptotic pathway, is also involved in the pro-apoptotic effect of flavonoids. For caspase-9 two kinds of behaviour were observed, luteolin **4c**, flavone **1c**, and chrysin **4a**, flavone **3c**, quercetin **4d**, presented apoptosis percentages higher or near 20 %; and flavone **2c**, and apigenin **4b** lowered their activities to values below 20 %. The pro-apoptotic effects of the first group leave some doubts in what extent they are dependent on caspase-9, as the apoptotic induction is affected, but in a smaller extent. The ability of flavonoids to induce apoptosis has been described by several authors. However, this kind of activity is more explored for flavonoids as anti-cancer drugs, so the studies are mainly done in cancer cell lines. [26, 27] Luteolin, for example, is described as an inductor of caspases-3, -8 and -9 in human malignant cells. [28] Chrysin is also reported as able to induce apoptosis by the degradation of caspases-3 and-8, but once more in human cancer cell lines. [29] The studies of caspases inhibition in inflammatory cells, namely in neutrophils, are scarce; and, as it is well proved, the apoptosis modulation effect vary among cell types; this being one of the reasons why this kind of study is important for a better understanding of the mechanisms by which flavonoids act in each cell type. [30, 31] To our knowledge, it is the first time the effect of flavonoids **1c** to **4d** on caspases -8 and -9 activity is evaluated, in neutrophils.

A study of the alteration of the mitochondrial membrane potential was undertaken, as it is involved in the intrinsic pathway. It was observed that none of the flavonoids altered the mitochondrial potential (figure 5), which does not corroborate the intrinsic pathway. We can speculate that one of the hallmarks of neutrophil physiology is a reduced number of mitochondria, and for this reason no effect was detected, however we obtained positive results when we used the positive control CCCP, discarding this hypothesis. Moreover, through the quantification of cytochrome c we would be able to corroborate these results, but because of the low number of mitochondria that kind of study is not possible. [5] The lower effects on the apoptosis induction observed for some flavonoids in the presence of caspase-9 inhibitor may be related to the fact that caspase-9 is involved in the intrinsic pathway of apoptosis or that caspase 8 may trigger caspase 9 activation through the crosstalk between extrinsic and intrinsic death pathways, as mentioned above. This last hypothesis is supported by the fact that the studies undertaken do not corroborate a key role of the intrinsic pathway.

Finally, as the transcription signalling cascade is also implicated in apoptosis regulation, this work aim to understand the effect of the flavonoids on p53 activation. The p53

transcription pathway is intimately related with inflammation and can act at least in two stages: as a common inhibitor of NF- $\kappa$ B-dependent transcription and as a positive regulator of neutrophils clearance by macrophages. [32] The up-regulation of p53 triggers the extrinsic pathway of apoptosis, and as the studied flavonoids seem to act through this pathway, they may also be related to the p53 activation. [10] However, under the studied experimental conditions no effect of the studied flavonoids was observed in p53 activation. In this work the spontaneous apoptosis was studied, but it is known that under inflammation conditions neutrophils overproduce ROS, as already mentioned. These ROS cause apoptosis by DNA damage but also by p53 activation; it is possible that the studied flavonoids as inhibitors of ROS production and activity may indirectly interfere with the p53 activation. [2, 21] Several cytokines, tumor necrosis factor alpha (TNF- $\alpha$ ), interferon gamma (IFN- $\gamma$ ), and chemokine interleukin 8 (IL-8) have also been reported to delay neutrophils' apoptosis, contributing to extended inflammatory states. [1] The flavonoids studied in this work, particularly, luteolin **4c**, and flavones **1c** and **3c**, have already demonstrated to be effective inhibitors of these pro-inflammatory mediators in human whole blood [19], which may contribute to boost their efficiency in human neutrophils' apoptosis induction.

In conclusion, the studied flavonoids were effective pro-apoptotic molecules; this effect is potentially undertaken via caspases 3, 8 and 9, but no mitochondrial involvement could be observed, indicating a key role of the extrinsic pathway. Thus, the majority of the studied flavonoids revealed a great potential to be used in chronic inflammation where the normal apoptotic function of neutrophils is altered.

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## ***CHAPTER IV. Discussion and Conclusions***

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### **IV.1. Integrated Discussion**

As mentioned before, chronic inflammatory diseases are responsible for the permanent affliction, disability, and, many times, premature death of millions of patients. They constitute a heavy burden for modern society. Those diseases include rheumatoid arthritis Crohn's disease, Alzheimer's disease, diabetes, among others.(1, 2)

Although inflammation is defined as a natural response of the body to infection, irritation or other injuries, it may trigger or aggravate several pathologic processes when the anti-inflammatory defences of the organism are not enough to contain and antagonize the deleterious activities of the overproduced pro-inflammatory mediators. The mechanisms involved in inflammatory processes are well known; however, it is also clear that they are governed by a series of mediators that favour the inflammation and others that counteract those actions, resulting in complex networks that difficult the development of the ideal anti-inflammatory drugs.(3) Additionally, the available treatments, albeit successful in some cases, have numerous and severe side effects. These facts justify the constant pursuit for alternative therapeutic approaches for chronic inflammation diseases.

In recent years, flavonoids have been associated with various health benefits, namely antioxidant (4), antiviral (5), antibacterial (6), anti-inflammatory (7), vasodilatory (8), anticancer (9), and anti-ischaemic (10) properties. These properties, associated with their ubiquitous distribution in nature, and their presence in the great majority of foods, as part of our daily diet,(11) confer flavonoids great value-added molecules. Taking in account these potential anti-inflammatory properties, flavonoids started to be considered a valuable alternative to modulate and prevent the inflammatory process; and moreover, to be the base for the synthesis of more potent and efficient anti-inflammatory drugs. Thus, a large number of studies have been performed, and it has become clear that the activities of flavonoids may vary significantly, depending on slight variations in their chemical structure or other characteristics such as the spatial conformation or the lipophilicity of the molecules.(2) In general, the studies available in the literature lack a coherent and rational approach of the flavonoids activity, since, frequently, only a small number of flavonoids are studied using the same techniques; there are a variety of assays for studying the same properties, most of them in non-cellular systems, not allowing a reliable comparison among them; most of the time the studies are done with the same flavonoids, not

exploring new synthesized, and never tested flavonoids, or with plant extracts, which difficulties the conclusions taken for each one of the flavonoids included in the extract. The work developed in this thesis intended to extend and rationalize the current knowledge on the alleged anti-inflammatory properties of flavonoids by elucidating the mechanisms of action related with their structure (structure-activity relationships). For this purpose, a group of 24 flavonoids were selected. They belong to three flavonoid classes, flavones, flavanones and flavonols, which have different patterns of substitution in A-, B- and C-rings (figure 1).

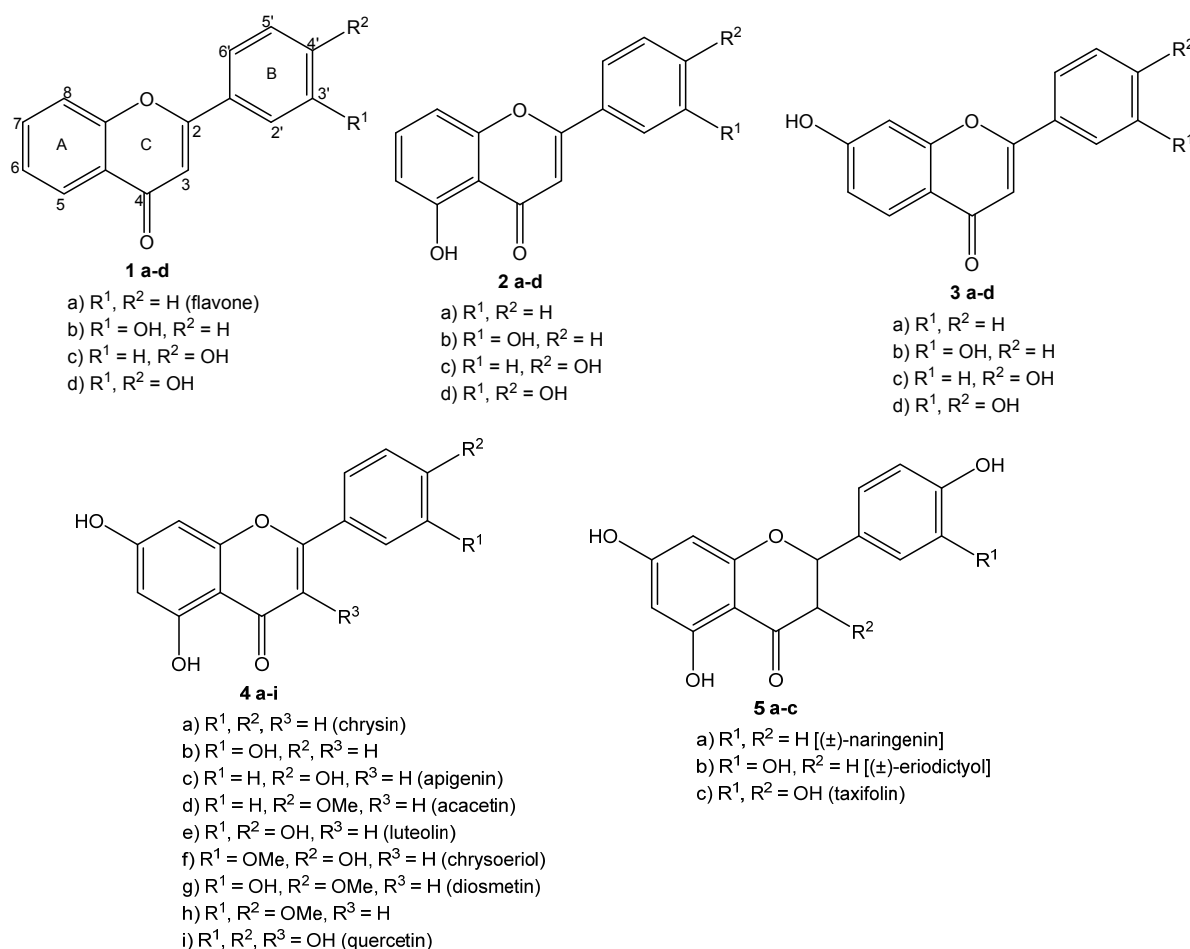


**Figure 1** - Chemical structures of the studied flavonoids.

Neutrophils are the first cells arriving at the inflammation loci and are part of the first line of defence of the innate immune system. During the inflammatory response, neutrophils greatly increase its number, life span, mobility, tissue influx ability, and phagocytic capacity.(12) Their role in inflammation is even more relevant because they are responsible for the production of an extraordinary array of oxygen-dependent and oxygen-independent microbicidal weapons to destroy and remove infectious agents. The oxygen-dependent mechanisms involve the production of reactive oxygen species (ROS) and

oxygen-independent mechanisms include most of the other neutrophil functions, such as chemotaxis, phagocytosis, degranulation, and the release of lytic enzymes and bactericidal peptides. These factors are essential for host defence and inflammation, but their overproduction, especially when high levels of ROS are overproduced in a sustained form, a process designated as oxidative burst, may cause or magnify damage in the inflammatory sites.(13, 14)

The first work of this thesis (section III.1) aimed the study of the effect of the selected flavonoids in the human neutrophil's oxidative burst, and to establish a relation between the activity found and their structure. Pursuing this idea, a series of studies were undertaken in phorbol-12-myristate-13-acetate (PMA)-activated human neutrophils and four luminescent and fluorescent probes were used to assess ROS production. The studied flavonoids were undoubtedly good modulators of the human neutrophils' oxidative burst. Concerning the structure-activity relationship, the overall obtained results stress out the determinant importance of the catechol group in B-ring. Another main feature that contributed for higher activity was the presence of a 3'-methoxyl group in the B-ring. Of note, the current study extended knowledge on the importance of the hydroxylations in the A-ring. It became clear that the substitution pattern in A-ring, i.e. the number and position of OH groups, only implicated slight differences in the activity. The presence of the 3-OH in the C-ring and the C2=C3 double bond just became important features when the H<sub>2</sub>O<sub>2</sub> and HOCl reactive species were studied. Noteworthy the accepted general idea is that a flavonoid is a good antioxidant when it gathers a combination of essential features: OH groups in B-ring that are able to form hydrogen bounds with 3-OH, aligning the three rings; the combination of the 3- and 5-OH groups with the 4-oxo group in C ring allows electron delocalisation from the 4-oxo group to both substituents.(2) Actually, in this work it was attested the importance of these features, but just for some specific reactive species, proving that flavonoids do not act just by scavenging ROS, but also probably by enzymes inactivation; and finally their overall substitution pattern also affects its lipophilicity, which may explain some of the different results obtained here in this *in vitro*, cellular assay. To the best of our knowledge, some of the flavonoids tested in this work were never studied for this kind of modulation activity, allowing us to do a better integration of the obtained results. All in all, the studied flavonoids that showed the most promising activities in this study were flavones **2d** and **1d**, chrysoeriol **4f** and luteolin **4e**.

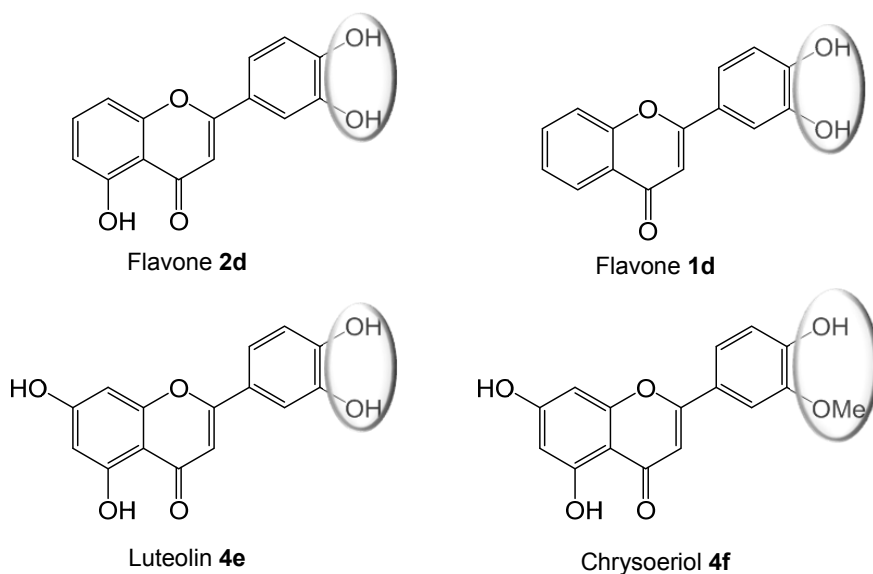


**Figure 2** - Studied flavonoids that presented the best modulation of human neutrophils' oxidative burst activity.

Three of the most active flavonoids (figure 2) share the catechol group in B-ring. As it was mentioned, it is well documented that this feature determines an excellent scavenging of ROS, because it donates hydrogen and an electron, stabilizing them and yielding relatively stable flavonoid radicals. However, this does not explain the reason why a methoxylated flavonoid is among the most active ones. In fact, the replacement of an OH by a methoxyl group increases the lipophilicity of the molecules,(15) enabling the access of the flavonoid to the cell. Landi-Librandi and co-workers undertook a study with liposomal flavonoids and concluded that the ability to inhibit the chemiluminescence of luminol was a function of the lipophilicity of the compounds.(16) Moreover, the methylation of the catechol arrangement in the B-ring is described as having the capacity to convert the flavonoid into an nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase inhibitor, which may be one more reason for the observed activity of chrysoeriol **4f**. (17, 18) Another important enzyme in the oxidative burst process is myeloperoxidase (MPO), which is implicated in the HOCl formation, and a docking study indicated that the B-ring's hydroxyl group(s) of flavonoids may be important for entering the pocket, reaching the active site of MPO.(19)

Once revealed the great capacity of the tested flavonoids to modulate oxidative burst of neutrophils, as an important deleterious process of inflammation, it became of great interest to evaluate if these molecules may comprise activities in other pathways related to inflammation. As so, the second work of this thesis (III.2) was focused in the modulation of the production of leukotriene (LT) $B_4$  and the type of inhibition and interaction between flavonoids and lipoxygenase (LOX), the enzyme involved in the production of this LT.

Leukotrienes (LTs) are identified as mediators of a series of inflammatory diseases, namely rheumatoid arthritis, inflammatory bowel disease, psoriasis, allergic rhinitis, bronchial asthma, atherosclerosis and certain types of cancer. (20) LTB<sub>4</sub>, in particular, participates in a range of pathophysiological events and exerts its activity by the recruitment of leukocytes to the inflammation sites. It has been demonstrated to be a potent neutrophil chemoattractant in humans *in vivo*. (21, 22) Studying the inhibition of LTB<sub>4</sub> production in human neutrophils, three groups of flavonoids were established, with different potencies. The most active ones, comprised luteolin **4e**, flavones **1d**, **2d** and **3d**, and quercetin **4i**, which share the same substitution pattern in the B-ring, a catechol group (figure 3).

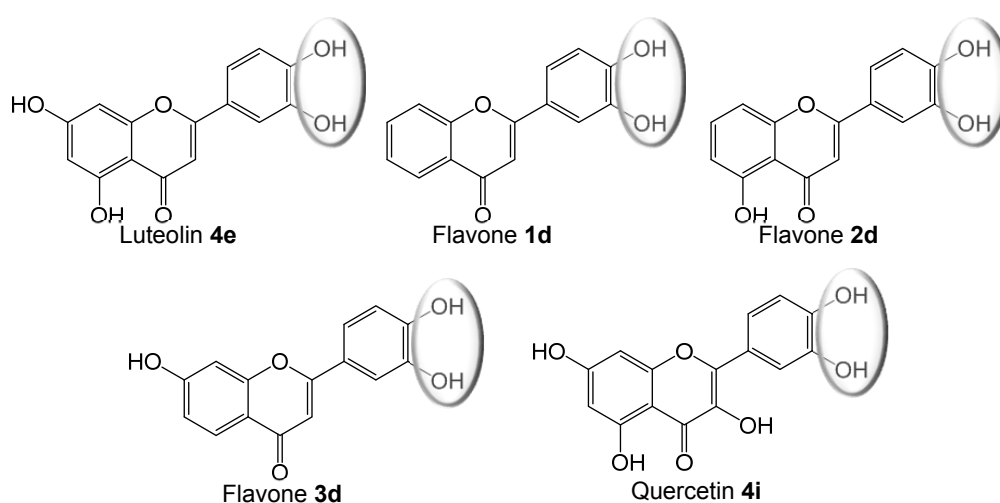


**Figure 3** - Studied flavonoids that presented the best inhibition of LTB<sub>4</sub> production.

As it can be seen in figure 3, the number of OH groups in the A-ring did not seem to be a determinant feature for the inhibitory activity. The 3-OH in C-ring, although being important was not essential for a better activity, as quercetin **4i** was 2.5 times less active than luteolin **4e**. This is in agreement with the study of Sadik and co-workers.(23) Interestingly, some of the flavonoids with more activity in this study were equally active in the modulation of the oxidative burst. This may be because these compounds may act as electron acceptors from chemical groups near the LOX active centre, the interaction being between the electron acceptor, flavonoid, and a nucleophilic group present in the LOX molecule.(24) This explanation is not widely accepted. Hernandez and co-workers studied a group of flavonoids that did not match these structural features and they inhibited the production of LTB<sub>4</sub> by LOX, proving that flavonoids do not have just this mode of action; they actually proposed that the studied flavonols indeed directly inhibit 5-LOX, but also interact with other proteins, such perhaps the cofactor 5-lipoxygenase activating protein (FLAP), or different protein kinases [such as protein kinase A, protein kinase C, protein

tyrosine kinases and by mitogen-activated protein kinase (MAPK) kinase] that can modulate the 5-LOX activity.(25)

The most active flavonoids on the inhibition of LTB<sub>4</sub> production assay and other four flavonoids with lower activity were selected for the study of their type of inhibition and to disclose a relationship between the flavonoid's structure and the type of LOX inhibition. Noteworthy, the type of inhibition seemed to be dependent not just on the OH of the B-ring but also on those in the A-ring. Flavonoids **2d**, **4b**, luteolin **4e** and quercetin **4i** were found to be uncompetitive LOX inhibitors complex. Luteolin **4e** and Quercetin **4i** have in common a catechol group in B-ring and a 5,7-dihydroxyl in the A-ring, so these features may be related to this type of inhibition. On the contrary, the other studied flavonoids owned a mixed non-competitive type of inhibition. In this sense, the type of inhibition is not just defined by the number of OH groups present but more by the relative position occupied by these groups. The interaction between these same flavonoids and LOX were after evaluated by saturation transfer difference nuclear magnetic resonance (STD-NMR) spectroscopy and the results obtained corroborated the ones obtained for the type of inhibition assays.

Revealed the activity of the selected solid group of flavonoids on the production of LTB<sub>4</sub>, other major groups of inflammatory mediators had to be studied: prostaglandins (PGs), cytokines and chemokines. The third work of this thesis (III.3) comprised the study of the flavonoids effects in prostaglandin (PG)E<sub>2</sub>, cytokines [interleukin (IL)-6, IL-1 $\beta$ , tumour necrosis factor-alpha (TNF- $\alpha$ ), and interferon-gamma (IFN- $\gamma$ )] and chemokine (IL-8) production, in human whole blood, and the establishment of the respective structure-activity relationship. PGE<sub>2</sub> is a PG formed via cyclooxygenase (COX)-1 and COX-2, in the arachidonic acid cascade. COX-1 is constitutively expressed and responsible for the regulation of the homeostatic functions. COX-2 is implicated in the modulation of inflammation, and can be up-regulated 20-fold the inflammatory process. This hyper-expression of COX-2 results from the integrated function of several inflammatory mediators, including ILs and cytokines (i.e. IL-1, IL-6, and TNF-  $\alpha$ ), via transcriptional activation.(26)

The studied flavonoids revealed clear patterns of activity and some of them happened to be good inhibitors of COX-1 - PGE<sub>2</sub> production. Flavonoids **1a**, **2a**, and **3a** were three of the most active; they have in common none or just one OH group, being the less substituted flavonoids among them all (figure 4). Less substituted flavonoids have easier access to the small inhibitor binding active site of COX-1, than the more substituted ones. It was the reason why it was possible to selectively inhibit COX-2 and, at the same time, avoid interfering with the homeostatic functions related to COX-1. (27, 28) It was also

clear that the lack of a C2=C3 double bond in C-ring, which determines the co-planarity of the molecule, affected dramatically the studied activity. This corroborates the results obtained by Wang and co-workers. (29)

In what respects COX-2 assay, the most active flavonoids were **1d**, **2d**, and **3d**, which have in common a catechol group in B ring (figure 4). Luteolin **4e** was also a good inhibitor, sharing the same substitution pattern. So far, it was clear that the structure that defines a good inhibitor of COX-1 is not the same for COX-2 (figure 4), allowing the idea of the ultimate selective activity.

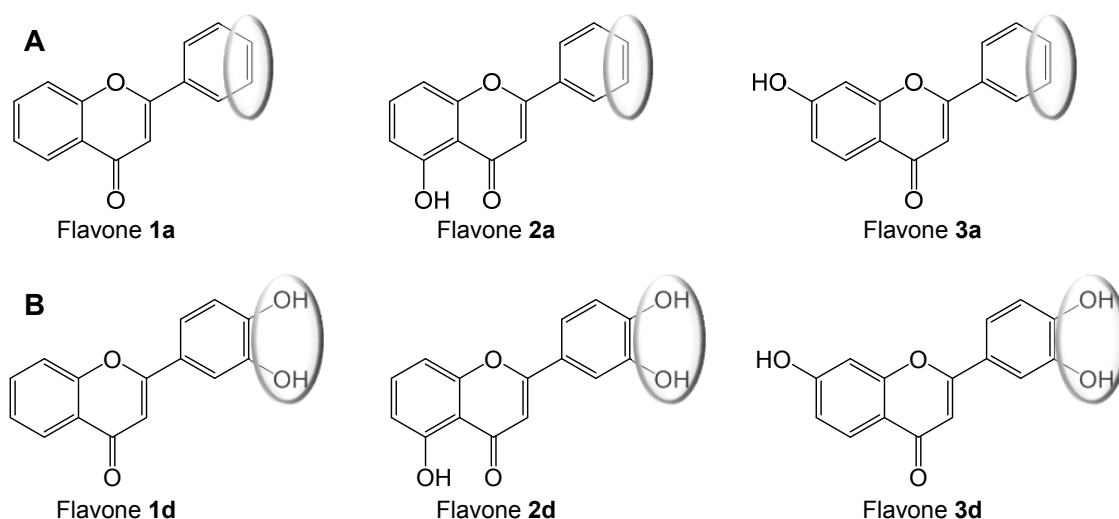


**Figure 4** - Studied flavonoids that presented the best inhibition of PGE<sub>2</sub> production - via COX-1 (**A**) and - via COX-2 (**B**).

The importance of the presence of a catechol group to the inhibition of COX-2, has already been reported in non-cellular in vitro studies and in isolated cells and it was now corroborated in a whole blood assay. (30) The OH groups on the B-ring appear to be related to a molecular conformation that influences the interactions between flavonoids and COX-2, being this group oriented to the hydrophobic pocket of the enzyme, (31) but also to the interactions between flavonoids and enzymes such as tyrosine kinase and protein kinase C, which are involved in the transcriptional activity of COX-2.(32) The ratio between COX-1 and COX-2 inhibitions revealed flavonoids **1b**, **2a**, **3a**, **3b**, and **4h** as COX-1 selective, and **3d**, apigenin **4c**, chrysoeriol **4f**, diosmetin **4g** and (±)-eriodictyol **5b** as COX-2 selective. The activities of the flavonoids selective for COX-1 and with higher affinities for this enzyme may be explored in other directions, namely, in platelet studies, maybe revealing other interesting and promising biological effects.(33) The COX-2 selective flavonoids, namely **3d** that was one of the most active, are promising candidates to modulate the COX-2 activity.

The best flavonoid inhibitors of COX-2, sharing a catechol group in B ring, **1d**, **2d**, **3d**, and luteolin **4e**, and quercetin **4i** were selected to assess their ability to modulate the production of IL-1 $\beta$ , IL-6, IL-8, IFN- $\gamma$ , and TNF- $\alpha$  in the same matrix used to study the COX activity, the human whole blood. Overall, the selected flavonoids proved to be exceptional inhibitors of cytokines' production. The lower activities were observed for the inhibition of IL-8. Luteolin **4e** was the most active flavonoid in the inhibition of almost all cytokines, IL-6, IFN- $\gamma$ , and TNF- $\alpha$ , which makes this flavonoid a promise in the modulation of the inflammatory process. Various studies can be found in the literature, mainly for luteolin and quercetin, which corroborate some of the results obtained in this study; however, they were performed in different models, invalidating a reasonable comparison.(34, 35)

The final work of this thesis (III.4) was devoted to an essential process to the healthy organism in the case of a chronic disease. It is the apoptosis process. In this study, we aimed to evaluate the effect of the most promising anti-inflammatory flavonoids until now, and two, less active, in the human neutrophils apoptosis and to study the mechanisms by which this effect was produced, and provide an insight on the structure/activity relationship. The delayed apoptosis of neutrophils has been related with numerous acute and chronic inflammatory diseases.(36-38) In fact, in the event of inflammatory processes, neutrophils over-produce a myriad of inflammatory mediators, as already described, which lead to the damage of the adjacent healthy tissues, propagating the inflammation. As so, this delayed apoptosis and clearance of neutrophils may contribute to solve the unwanted and exaggerated inflammation.(39)

All the tested flavonoids induced neutrophil's apoptosis. Luteolin **4c**, flavonoids **3c** and **2c**, and chrysin **4a** were the ones that led to a greater increase of apoptosis. Some studies have already defined the flavonoid's ability to modulate neutrophils apoptosis, but did not establish a relationship to their structure.(40, 41) Based on the results obtained in present work, we may conclude that catechol group is important for the activity, but not determinant, as chrysin **4a** has no substitution in B-ring and was equally active (figure 5).

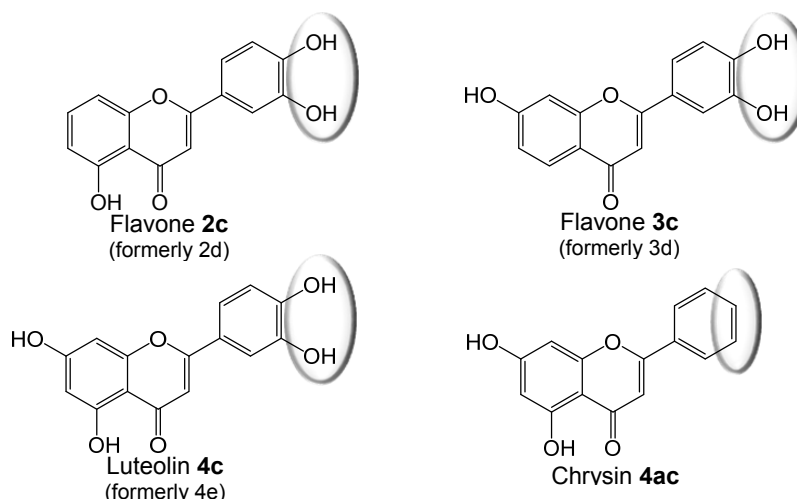


**Figure 5** - Studied flavonoids that presented the best pro-apoptotic effect.

Furthermore it was studied which caspases pathways were involved in the pro-apoptotic effect. The studied flavonoids were effective pro-apoptotic molecules; this effect is potentially undertaken via caspases 3, 8 and 9, but no mitochondrial involvement could be observed, indicating a key role of the extrinsic pathway. The studies of caspases inhibition in inflammatory cells, namely in neutrophils, are scarce; and, as it is well described, the apoptosis modulation effect vary among cell types; this being one of the reasons why this kind of study is important for a better understanding of the mechanisms by which flavonoids act in each cell type.(42) p53 is a transcription factor directly related to apoptosis induction and its up-regulation triggers the extrinsic pathway of apoptosis.(43) However, under the studied experimental conditions no effect of the studied flavonoids was observed in p53 activation.

Combining and integrating all the works developed under this thesis, it is clear that a pattern of action of these flavonoids was found. Overall, flavonoids 3',4'- dihydroxyflavone, 5,3',4'-trihydroxyflavone, 7,3',4'-trihydroxyflavone, and luteolin (figure 6) were undoubtedly good modulators of the pro-inflammatory mediators, which may represent excellent new alternatives for the resolution of inflammatory processes.

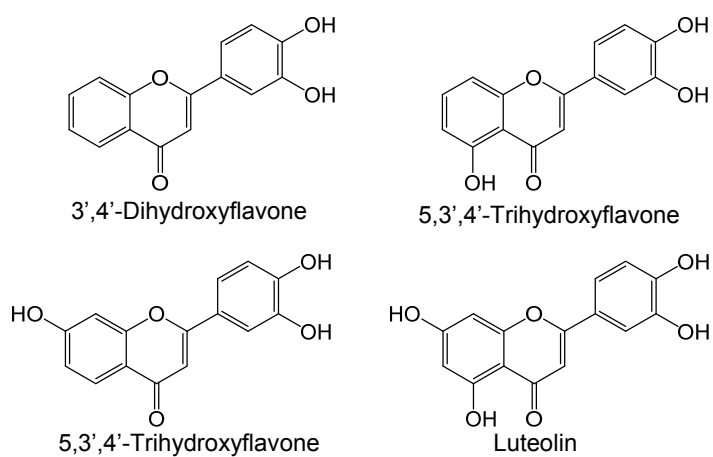


**Figure 6** - Chemical structures of the most promising flavonoids found as modulators of the pro-inflammatory mediators.

## IV.2. Conclusions

From the results obtained in the course of the performed studies it may be concluded that:

- The structural features of flavonoids clear influence the modulation of all the pro-inflammatory mediators;
- All the tested flavonoids were able to modulate the human neutrophils' oxidative burst;
- The catechol moiety in B-ring was essential for a greater inhibition of ROS production;
- The catechol moiety in B-ring was essential for a greater inhibition of LTB<sub>4</sub> production, independently of the substitution pattern in A-ring;
- The type of inhibition of soybean LOX, as the flavonoids interaction with it, are not only dependent on the OH groups in B-ring, but also in A-ring;
- The less substituted flavonoids were better inhibitors of PGE<sub>2</sub> production, via COX-1;
- The catechol moiety in B-ring was essential for a greater inhibition of PGE<sub>2</sub> production, via COX-2;
- The flavonoids that inhibited the PGE<sub>2</sub> production, via COX-2, simultaneously inhibited the production of pro-inflammatory cytokines;
- The selected flavonoids are pro-apoptotic molecules, and exert their effects via caspases, but no mitochondrial involvement could be observed, indicating a key role of the extrinsic pathway;
- Flavonoids 3',4'-dihydroxyflavone, 5,3',4'-trihydroxyflavone, 7,3',4'-trihydroxyflavone, and luteolin, comprising the essential structural features referred, are undoubtedly promising new alternatives for the resolution of inflammatory processes.

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