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FACULDADE DE FARMÁCIA
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EVALUATION OF THE ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF SYNTHETIC 2-STYRYLCHROMONES

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Evaluation of the antioxidant and anti-inflammatory activities of synthetic 2-styrylchromones

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"Só sei que nada sei"

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Ao Filipe

Aos meus pais, Elisabete e José

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I. **Gomes A**, Fernandes E, Silva AMS, Santos CMM, Pinto DCGA, Cavaleiro JAS, Lima JLFC. 2-Styrylchromones: Novel strong scavengers of reactive oxygen and nitrogen species. Bioorg Med Chem 2007; 15 (18): 6027-36.

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II. **Gomes A**, Fernandes E, Silva AMS, Santos CMM, Pinto DCGA, Cavaleiro JAS, Lima JLFC. Interactions of 2-styrylchromones with pro-inflammatory enzymes and oxidative species. Inflamm Res 2007; 56 (Suppl. 3): S459.

III. **Gomes A**, Fernandes E, Silva AMS, Santos CMM, Pinto DCGA, Cavaleiro JAS, Lima JLFC. Inhibition of leukotriene B_4 production in human neutrophils by 2-styrylchromones. Free Radic Res 2007; 41 (Suppl. 1): S30.

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I. **Gomes A**, Fernandes E, Lima JLFC. Evaluation of antioxidant and anti-inflammatory activities of synthetic chromones. 4º Encontro do *REQUIMTE*, Fátima, 31 de Março e 1 de Abril 2006 (comunicação em painel).

II. **Gomes A**, Fernandes E, Silva AMS, Santos CMM, Pinto DCGA, Cavaleiro JAS, Lima JLFC. Singlet oxygen scavenging activity by 2-styrylchromones. 13th Biennial Congress of the International Society for Free Radical Research, Davos, Suíça, 15-19 de Agosto de 2006 (comunicação em painel).

III. **Gomes A**, Fernandes E, Lima JLFC. Scavenging activity of flavonoids against singlet oxygen. XX Encontro Nacional da Sociedade Portuguesa de Química, Campus da Caparica – FCT/UNL, 14-16 de Dezembro de 2006 (comunicação em painel).

IV. **Gomes A**, Fernandes E, Lima JLFC. Metodologia de micro-análise para avaliação da actividade captadora de ácido hipocloroso. SPQ-ANALITICA'07, Lisboa, 29-30 de Março de 2007 (comunicação em painel).

V. **Gomes A**, Fernandes E, Silva AMS, Santos CMM, Pinto DCGA, Cavaleiro JAS, Lima JLFC. Interactions of 2-styrylchromones with pro-inflammatory enzymes and oxidative species. 8th World Congress on Inflammation, 16-20 de Junho de 2007, Copenhaga, Dinamarca (comunicação em painel).

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VII. **Gomes A**, Fernandes E, Garcia MBQ, Silva AMS, Santos CMM, Pinto DCGA, Cavaleiro JAS, Lima JLFC. The ROS and RNS scavenging activity of 2-styrylchromones are effectively reproduced by cyclic voltammetry analysis. AOAC Europe Section International Workshop – Enforcement of European Legislation on Food and Water: Analytical and Toxicological Aspects - e II

Encontro Nacional de Bromatologia, Hidrologia e Toxicologia, Lisboa, 17-18 de Abril de 2008 (comunicação em painel).

VIII. **Gomes A**, Fernandes E, Garcia MBQ, Silva AMS, Pinto DCGA, Santos CMM, Cavaleiro JAS, Lima JLFC. Cyclic voltammetric analysis of 2-styrylchromones. XXI Encontro Nacional da Sociedade Portuguesa de Química, Porto 11-13 de Junho de 2008 (comunicação em painel).

IX. **Gomes A**, Fernandes E, Silva AMS, Santos CMM, Pinto DCGA, Cavaleiro JAS, Lima JLFC. Anti-inflammatory potential of 2-styrylchromones regarding their interference with arachidonic acid metabolic pathways. 14th Biennial Meeting of the Society for Free Radical Research International, Pequim 18-22 de Outubro de 2008 (comunicação em painel).

X. **Gomes A**, Fernandes E, Silva AMS, Santos CMM, Pinto DCGA, Cavaleiro JAS, Lima JLFC. Novel 2-styrylchromones with high anti-inflammatory potential through prevention of LTB_4 production by human leukocytes, inhibition of COX-1 activity and scavenging of ROS and RNS. 1º Encontro Nacional de Química Terapêutica, Porto 13-15 de Novembro de 2008 (comunicação em painel).

ABSTRACT

Inflammation is the first response of the body to infection, irritation or other injuries. It is an essential process to neutralize aggressor agents and to repair damaged tissues, assuring, this way, the survival of the host. However, it is often uncontrolled in chronic inflammatory and autoimmune diseases, namely rheumatoid arthritis and Crohn's disease, and when it is linked to an allergic response like asthma and anaphylactic shock. Due to the permanent affliction, disability, and, many times, premature death of the millions of patients suffering from these diseases, chronic inflammation is associated with severe socio-economic problems. Unfortunately, the available anti-inflammatory treatments aren't always sufficiently effective and frequently present numerous and severe side effects especially in long-term use. Thus, there is an increasing interest in the search for new molecules with improved activities and better safety profiles.

2-Styrylchromones (2-SC) are chromone derivatives characterized by the attachment of a styryl group to the C2-position of the chromone structure. Although scarce in nature, with only three natural derivatives known, 2-SC have been demonstrated to bear important biological activities, most of them revealed in studies with synthesized derivatives. Indeed, compounds from this family exhibited antiallergic, antitumor, affinity and selectivity for A_3 adenosine receptors, antiviral, and antioxidant properties. Due to their structural similarity with flavones (2-phenylchromones), it is expected that 2-SC are also endowed with anti-inflammatory activity since a large number of studies show the effectiveness of flavones, as well as other flavonoids, in reducing inflammatory markers by interfering, through diverse mechanisms, in inflammatory processes. Thus, the general aim of this dissertation was to assess the anti-inflammatory potential of a series of 2-SC.

The first experimental approach in the scope of this dissertation was to evaluate the scavenging activity against reactive oxygen species (ROS) and reactive nitrogen species (RNS) of 2-SC. There is a considerable possibility that anti-inflammatory agents act, in part, as antioxidants since there are many evidences that an overgeneration of ROS and RNS occurs during inflammatory processes, contributing to the tissue damage. Some of the studied compounds proved to be extremely efficient scavengers of the different ROS and RNS, showing, in some cases, IC_{50}s below 1 μM. The hydroxylation pattern of 2-SC, especially in the B-ring but also in the A-ring, modulates the activity of these compounds, the 3',4'-dihydroxy derivatives being the most effective scavengers. By comparing the potency of 2-SC with structural-similar flavonoids it became clear that the styryl pattern also contributes to the observed antioxidant activity. A second experimental approach was performed in order to understand the mechanism of the scavenging activity of 2-SC through

the study of their electrochemical behaviour by cyclic voltammetry. The obtained results could be correlated with those of the scavenging assays, that is, higher scavenging effects corresponded to lower values of oxidation potentials, with significant correlation coefficients. Thus, in this family of compounds, oxidation potentials obtained by cyclic voltammetry seem to be applicable as a general indicator of radical scavenging activity.

One of the most studied mechanisms in the anti-inflammatory therapeutics is the inhibition of arachidonic acid metabolism by the enzymes cyclooxygenase (COX)-1 and COX-2. In addition, the 5-lipoxigenase (LOX) pathway of arachidonic acid metabolism is generating an increasing interest. Thus, the third experimental work included in this dissertation consisted in evaluating the COX-1 and COX-2 inhibitory capacity of 2-SC as well as the effect of these compounds on the leukotriene (LT)B_4 production by stimulated human polymorphonuclear leukocytes (PMNL). Some of the tested 2-SC were able to inhibit both COX-1 activity and LTB_4 production, which makes them dual inhibitors of the COX and 5-LOX pathways. The most effective compounds in this study were those having structural moieties with proved antioxidant activity, such as 3',4'-dihydroxy and 4'-hydroxy substituents. The mechanism by which 2-SC inhibit the production of LTB_4 probably involves the inhibition of the enzyme 5-LOX, whereas the inhibition of COX-1 by these compounds is likely to consist in the scavenging of the radical intermediates involved in COX enzyme catalysis.

Nuclear factor kappa B (NF-κB) is one of the most important inducible transcription factors whose modulation triggers a cascade of signaling events, some of which are potential key targets for intervention in the treatment of inflammatory conditions. Thus, the research of the anti-inflammatory potential of 2-SC was continued by the study of the inhibition of NF-κB activation stimulated by lipopolysaccharide (LPS) in a monocytic cell line (THP-1). Three of the tested 2-SC were able to significantly inhibit NF-κB activation. The effective compounds were then elected to be further evaluated on their inhibitory effects in the LPS-induced production of pro-inflammatory cytokines [tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8] in THP-1 cells. All the three compounds were able to reduce the production of TNF-α and IL-6, two of them reduced the production of IL-1β, and the most effective of all also reduced the production of IL-8.

In conclusion, the research developed in the scope of the present dissertation demonstrated the anti-inflammatory capacity of some of the tested 2-SC and allowed to establish important structure-activity relationships. The compound 3',4',5-trihydroxy-2-styrylchromone (**1C**) stood up as the most promising of the tested 2-SC due to its constant effectiveness in the different performed studies. We believe, therefore, that this compound is worthy be used in further pre-clinical experiments and clinical trials as a potential anti-inflammatory drug.

RESUMO

A inflamação é a primeira resposta do organismo à infecção, irritação e outras lesões. É um processo essencial para neutralizar os agentes agressores e para reparar os tecidos danificados, assegurando assim a sobrevivência do hospedeiro. No entanto, este processo torna-se, por vezes, descontrolado, como acontece em doenças inflamatórias e auto-imunes crónicas, nomeadamente a artrite reumatóide e a doença de Crohn, ou quando está associado a respostas alérgicas como na asma ou no choque anafilático. Devido ao sofrimento permanente, incapacidade e, muitas vezes, morte prematura de milhões de pacientes que sofrem destas doenças, a inflamação crónica está associada a graves problemas sócio-económicos. Infelizmente, os tratamentos anti-inflamatórios existentes nem sempre são suficientemente eficazes e muitas vezes apresentam efeitos secundários numerosos e graves especialmente quando utilizados a longo prazo. Assim, tem havido um crescente interesse na procura de novas moléculas com maior actividade e melhores perfis de segurança.

As 2-estirilcromonas (2-SC) são derivados de cromonas caracterizados pela existência de um grupo estiril ligado à posição C2 da estrutura cromona base. Embora raras na natureza, com apenas três derivados naturais conhecidos, as 2-SC têm demonstrado possuir importantes actividades biológicas, a maioria delas revelada em estudos efectuados com derivados de síntese. Na verdade, os compostos desta família já exibiram propriedades anti-alérgicas, anti-tumorais, de afinidade e selectividade para os receptores de adenosina A_3, antivirais e anti-oxidantes. Devido à sua semelhança estrutural com as flavonas (2-fenilcromonas), é esperado que as 2-SC sejam também capazes de exercer efeito anti-inflamatório dado que um vasto número de estudos demonstra a eficácia das flavonas, bem como de outros flavonóides, para reduzir marcadores da inflamação pela interferência, por diversos mecanismos, no processo inflamatório. Assim, o objectivo geral desta tese foi o de estudar o potencial anti-inflamatório de uma série de 2-SC.

A primeira abordagem experimental efectuada no âmbito desta tese foi avaliar a actividade captadora de espécies reactivas de oxigénio (ROS) e espécies reactivas de azoto (RNS) por 2-SC. Existe uma possibilidade considerável de que os agentes anti-inflamatórios actuem, em parte, como antioxidantes, já que há várias evidências de que durante o processo inflamatório ocorre uma geração acentuada de ROS e RNS, contribuindo para os danos tecidulares. Alguns dos compostos estudados provaram ser captadores extremamente eficientes de diferentes ROS e RNS, demonstrando, nalguns casos, IC_{50}s abaixo de 1 μM. O padrão de hidroxilação das 2-SC, especialmente no anel B mas também no anel A, define a actividade destes compostos, sendo que os derivados

3',4'-di-hidroxi se apresentam como os captadores mais eficazes. Comparando a potência das 2-SC com flavonóides estruturalmente semelhantes tornou-se claro que o grupo estiril também contribui para a actividade antioxidante observada. Uma segunda abordagem experimental foi realizada de forma a perceber o mecanismo inerente à actividade captadora das 2-SC através do estudo das suas propriedades electroquímicas por voltametria cíclica. Os resultados obtidos neste estudo puderam ser correlacionados com os obtidos nos estudos de actividade captadora, isto é, elevados efeitos captadores corresponderam a baixos valores de potencial de oxidação, com coeficientes de correlação significativos. Assim, nesta família de compostos, os potenciais de oxidação obtidos por voltametria cíclica parecem ser aplicáveis como indicadores gerais da actividade captadora de radicais.

Um dos mais estudados mecanismos na terapêutica anti-inflamatória é o da inibição do metabolismo do ácido araquidónico pelas enzimas ciclo-oxigenase (COX)-1 e COX-2. Para além disso, o percurso metabólico do ácido araquidónico por acção da 5-lipoxigenase (5-LOX) tem gerado um interesse crescente. Assim, o terceiro trabalho experimental incluído nesta tese consistiu em avaliar a capacidade de inibição das enzimas COX-1 e COX-2 por 2-SC bem como o efeito deste compostos na produção de leucotrieno (LT)-B_4 por leucócitos polimorfonucleares humanos (PMNL). Alguns dos compostos testados foram capazes de inibir quer a actividade da COX-1, quer a produção de LTB_4, o que faz deles duplos inibidores dos percursos da COX e 5-LOX. Os compostos mais eficazes foram aqueles que possuem fracções estruturais com comprovada actividade anti-oxidante, tais como os substituintes 3',4'-di-hidroxi e 4'-hidroxi. O mecanismo pelo qual as 2-SC inibem a produção de LTB_4 envolve, provavelmente, a inibição da enzima 5-LOX, enquanto que a inibição da actividade da COX-1 por estes compostos consiste, presumivelmente, na captação de intermediários radicalares envolvidos na catálise enzimática da COX.

O factor nuclear kapa B (NF-κB) é um dos mais importantes factores de transcrição indutíveis cuja modulação desencadeia trajectos de sinalização, sendo alguns deles potenciais elementos chave para intervenção no tratamento de doenças inflamatórias. Assim, a pesquisa do potencial anti-inflamatório das 2-SC continuou pelo estudo de inibição da activação do NF-κB estimulada por lipopolissacarídeo (LPS) numa linha celular monocítica (THP-1). Três das 2-SC testadas foram capazes de inibir significativamente a activação do NF-κB. Os compostos eficazes foram seguidamente eleitos para uma posterior avaliação dos efeitos inibidores da produção de citocinas pró-inflamatórias [factor de necrose tumoral (TNF)-α, interleucina (IL)-1β, IL-6 e IL-8] induzida por LPS em células THP-1. Os três compostos foram capazes de reduzir a produção de TNF-α e IL-6, dois deles reduziram a produção de IL-1β, e o mais eficaz reduziu também a produção de IL-8.

Em conclusão, a pesquisa desenvolvida no âmbito desta tese demonstrou a capacidade anti-inflamatória de algumas das 2-SC testadas e permitiu estabelecer relações estrutura-actividade importantes. O composto 3',4',5-tri-hidroxi-2-estirilcromona (1C) revelou-se como o mais promissor dos compostos testados devido à constante eficácia demonstrada nos diferentes estudos realizados. Acreditamos, assim, que este composto deverá ser testado em futuros ensaios pré-clínicos e clínicos como potencial fármaco anti-inflamatório.

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ABBREVIATIONS LIST

AAPH, α,α'-azodiisobutyramidine dihydrochloride

ANOVA, one-way analysis of variance

AP-1, activator protein-1

AUC, area under the curve

BDE, bond dissociation energy

$CO_3^{\bullet-}$, carbonate radical

COX, cyclooxygenase

COXIB, COX-2 selective inhibitor

DAF-2, 4,5-diaminofluorescein

DAF-2T, triazolofluorescein

DHR, dihydrorhodamine 123

DMSO, dimethyl sulfoxide

DTPA, diethylenetriamine pentaacetic acid

EDTA, ethylenediamine tetraacetic acid

EGTA, ethylene glycol tetraacetic acid

EIA, enzyme immunoassay

ELISA, enzyme-linked immunosorbent assay

Ep_{ox}, oxidation potential

FBS, fetal bovine serum

FLAP, 5-lipoxygenase activating protein

GSH, reduced glutathione

GSSG, oxidized glutathione

H_2O_2, hydrogen peroxide

HBSS, Hanks' balanced salt solution

5-HETE, 5(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid

$HO^{\bullet}$, hydroxyl radical

HOCl, hypochlorous acid

5-HPETE, 5(*S*)-hydroperoxy-6,8,11,14-eicosa-tetraenoic acid

HRV, human rhinovirus

IC_{50}, half maximal inhibitory concentration

IκB, inhibitor of NF-κB

IKK, IκB kinase

IL, interleukin

Ip, peak current

IP, ionization potential

LHD, lactate dehydrogenase

LDL, low density lipoproteins

LOX, lipoxygenase

LPS, lipopolysaccharide

LT, leukotriene

MPO, myeloperoxidase

NADH, β-nicotinamide adenine dinucleotide (reduced form)

NADPH, nicotinamide adenine dinucleotide phosphate (reduced form)

NBT, nitroblue tetrazolium chloride

NDGA, nordihydroguaiaretic acid

$NDPO_2$, disodium 3,3'-(1,4-naphthalene)bispropionate

NF-κB, nuclear factor kappa B

$^{\bullet}NO$, nitric oxide

$^{\bullet}NO_2$, nitrogen dioxide radical

NOC-5, 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene

NOS, nitric oxide synthases

Nox, NADPH oxidase

NSAID, non-steroidal anti-inflammatory drug

$^1O_2$, singlet oxygen

$O_2^{\bullet -}$, superoxide radical

$ONOO^-$, peroxynitrite anion

$ONOOCO_2^-$, nitrosoperoxycarbonate anion

ORAC, oxygen radical absorbance capacity

5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid

PG, prostaglandin

PMNL, polymorphonuclear leukocytes

PMS, phenazine methosulfate

RNS, reactive nitrogen species

$ROO^{\cdot}$, peroxyl radical

ROS, reactive oxygen species

RPMI, Roswell Park Memorial Institute

2-SC, 2-styrylchromones

SOD, superoxide dismutase

t-BHP, *tert*-butylhydroperoxide

TLR, toll-like receptor

TNF, tumor necrosis factor

TXA_2, thromboxane A_2

XO, xanthine oxidase

OUTLINE OF THE DISSERTATION

The present dissertation is structured in three main chapters:

Chapter I – General introduction

This chapter is divided in two sections:

I.1. Theoretical background

This section is divided in two subsections. The first subsection (I.1.1) consists in a summary of the biological activities of 2-SC known before the original research performed in the ambit of the present dissertation, with reference to the most relevant chemical structures. The second subsection (I.1.2) is a review article about flavonoids and their anti-inflammatory properties, *in vitro* and *in vivo*. Although the original research performed in the scope of this dissertation was not directed to flavonoids, this group of compounds presents significant structural similarities with 2-SC. Thus, we believe that the compilation of the existent knowledge about the role of flavonoids in inflammation helped to choose the most appropriate approach to follow in the study of 2-SC's anti-inflammatory potential and to interpret the results obtained. In this article, an overview about the inflammatory process is also given.

I.2. General and specific objectives of the dissertation

The general and specific objectives of the dissertation are provided in this section.

Chapter II – Original research

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

Chapter III – Discussion and Conclusions

This chapter is divided in two sections, the first consisting in an integrated discussion of the performed studies and the second in the general conclusions of the dissertation.

CHAPTER I

GENERAL INTRODUCTION

I.1. Theoretical background

I.1.1. 2-STYRYLCHROMONES: CHEMISTRY AND BIOLOGICAL ACTIVITIES

2-Styrylchromones (2-SC) (Figure 1) are a group of chromone (1-benzopyran-4-one; 1,4-benzopyrone; 4-oxo-4*H*-1-benzopyran) derivatives characterized by the attachment of a styryl group to the C2-position of the chromone structure. Until very recently, only two natural compounds were known, hormothamnione (**1**) and 6-desmethoxyhormothamnione (**2**) (Figure 2). These compounds were extracted from the marine cryptophyte *Chrysophaeum taylori*, although this alga had been identified in the first place as cyanobacterium *Hormothamnion enteromorphoides* [1, 2]. Recently, Yoon *et al* [3] isolated 5-hydroxy-2-styrylchromone (**3**) (Figure 2) from *Imperata cylindrica*, although the synthesis of this compound had been previously described [4].

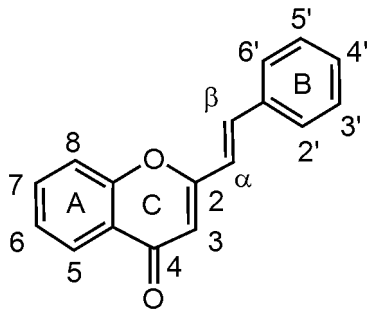
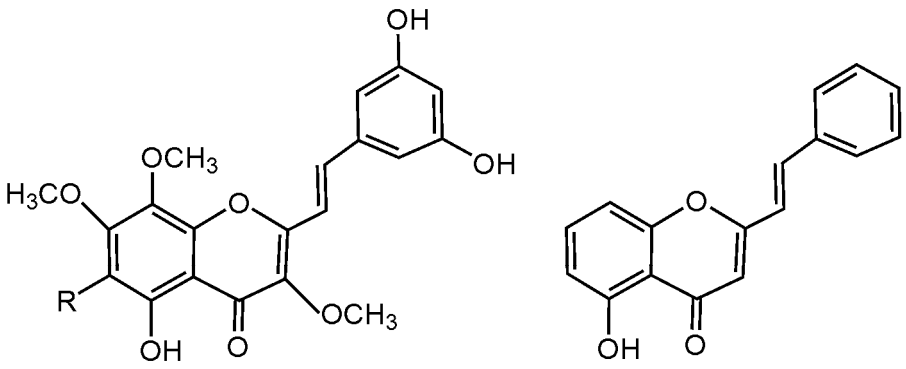


Figure 1 – Molecular scaffold of 2-styrylchromones.



1: R=OCH_3

2: R=H

3

Figure 2 - Chemical structures of the 2-styrychromone natural derivatives: hormothamnione (**1**), 6-desmethoxyhormothamnione (**2**), and 5-hydroxy-2-styrylchromone (**3**).

Although scarce in nature, a considerable number of 2-styrylchromone derivatives has been synthesized since before the isolation of first natural compounds. The most recent synthetic strategies found in literature deal with improvements of the most promising approaches, such as: aldol condensation/oxidative cyclization and Baker-Venkataraman rearrangement (see [5] for review).

Natural and synthetic 2-SC have been demonstrated to bear important biological activities which will be described here.

I.1.1.1. Antiallergic activity

The first biological activity shown by synthetic 2-SC was reported by Doria *et al* [6]. In that study, some of the tested 6-carboxilic acid-substituted 2-SC, when orally administrated, were able to display antiallergic activity in the passive cutaneous anaphylaxis test performed in rats (Figure 3). Moreover, the compounds **4**, **5**, **6**, and **8** (Figure 3) were significantly more potent than disodium cromoglycate, an antiallergic drug used in clinical practice, in the passive cutaneous anaphylaxis test, when administrated parenterally, and in the inhibition of histamine release from passively sensitized rat peritoneal cells. Since many different molecules were tested in this study, beyond those referred here, some structure-activity relationships were postulated. Thus, the substitution at the 3-position with lower alky groups, ethyl (**4**) and *n*-propyl (**5**), increased the oral activity. A further improvement of biological responses could be obtained in the 3-*n*-propyl series by introducing a single methyl into the aryl moiety (**6** and **8**). The vinyl moiety seems to be essential for significant antianaphylatic activity.

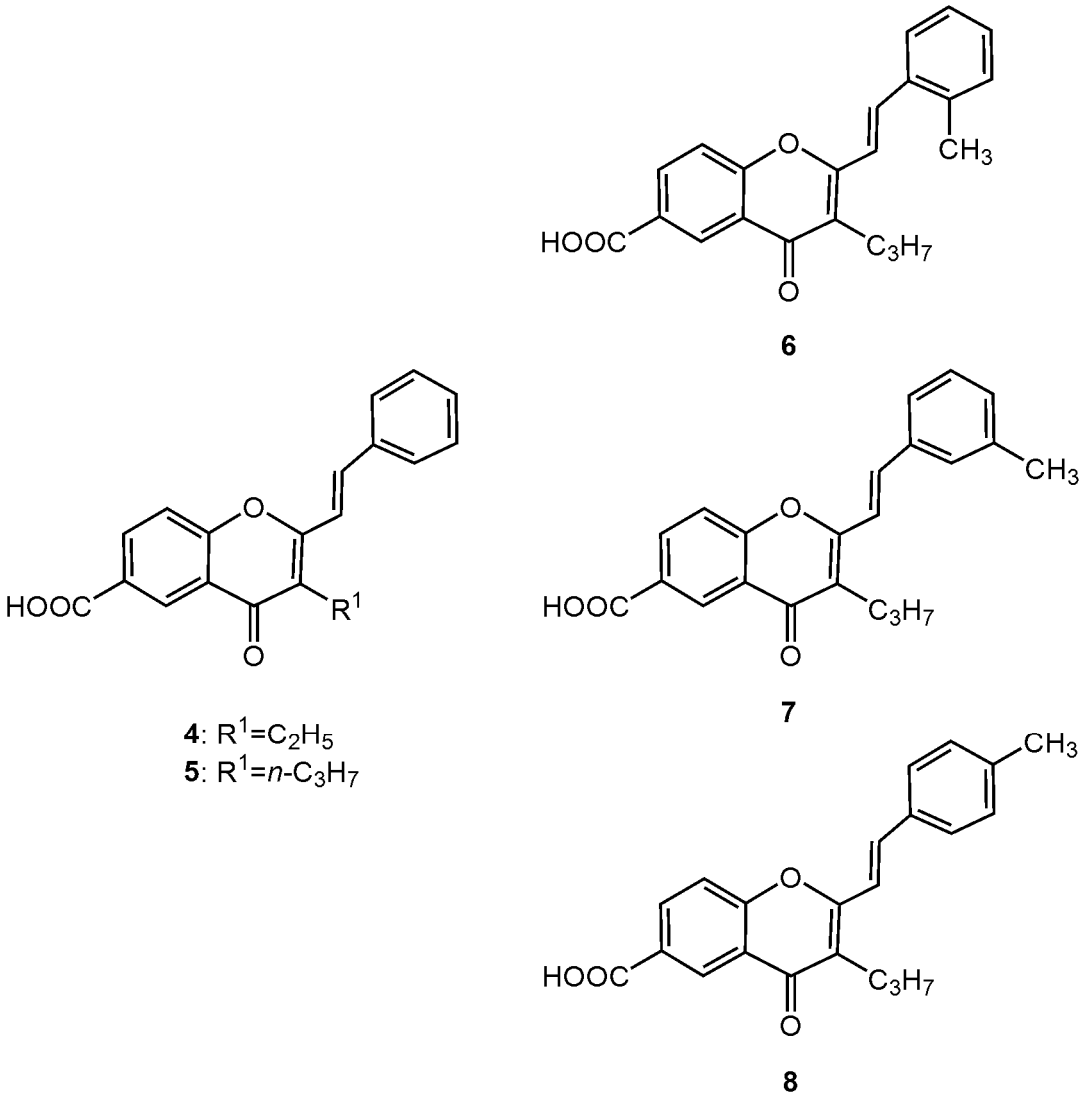


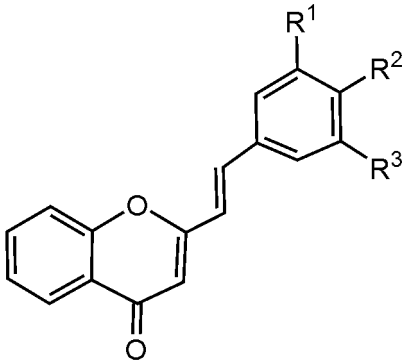
Figure 3 – Chemical structures of 2-styrylchromones studied for antiallergic activity.

I.1.1.2. Antitumor activity

The antitumor potential of 2-SC was first described with the natural derivatives hormothamnione (**1**) and 6-desmethoxyhormothamnione (**2**). Hormothamnione was shown to be a potent cytotoxic agent to P388 lymphocytic leukemia and HL-60 human promyelocytic leukemia cell lines, through the inhibition of RNA synthesis [1] and 6-desmethoxyhormothamnione showed good cytotoxicity to 9 KB cells, derived from a human epidermoid carcinoma of the nasopharynx [2].

Since the isolation of the natural derivatives, other 2-SC, obtained by synthesis, were shown to cause cytotoxicity in tumor cell lines. Six newly-synthesized 2-SC (Figure 4) showed tumor-specific cytotoxic activity when tested in normal oral human cells and tumor human cell lines [7]. Compounds **11** and **13** showed the highest tumor specificity. These

compounds induced apoptosis characterized by internucleosomal DNA fragmentation and caspase 3-activation. The methoxy groups were suggested to be involved in the induction of tumor-specific cytotoxicity by the formation of cytotoxic quinones or orthoquinones due to enzymatical demethylation of those groups. Recently, Marinho *et al* [8] verified that 4'-methoxy-2-styrylchromone (**11**), previously identified as a potent growth inhibitor of tumor cell lines, was less efficient in inhibiting the growth of normal cells, which confirmed the higher sensitivity of tumor cell lines to the toxicity of the tested compound. The mechanism underlying the tumor cell growth inhibitory effect involves a blockage at the G_2/M phase of the cell cycle. On the other hand, the G_2/M arrest was not observed in the non-tumor cell line. Additional results suggested that **11** can affect the *in vitro* assembly of tubulin into microtubules, acting as a microtubule-stabilizing agent.



9: R^1, R^2, R^3=H
10: R^1, R^3=H; R^2=CH_3
11: R^1, R^3=H; R^2=OCH_3
12: R^1=H; R^2, R^3=OCH_3
13: R^1, R^2, R^3=OCH_3
14: R^1=O-CH_2-O=R^2; R^3=H

Figure 4 - Chemical structures of 2-styrylchromones studied for antitumor activity.

More recently a series of 2-styrylchromone analogs was synthesized and evaluated for their antiproliferative effects in five human carcinoma cell lines: PC-3 (prostate carcinoma cell), A549 (non-small cell lung adenocarcinoma cell), BT483 (mammary gland adenocarcinoma cell), HeLa (cervical epithelioid carcinoma cell) and SKHep (hepatocellular carcinoma cell) [9]. The synthesis of the 2-stryrylchromone analogs was based on modifications of B-ring (Figure 5A) and A-ring (Figure 5B).

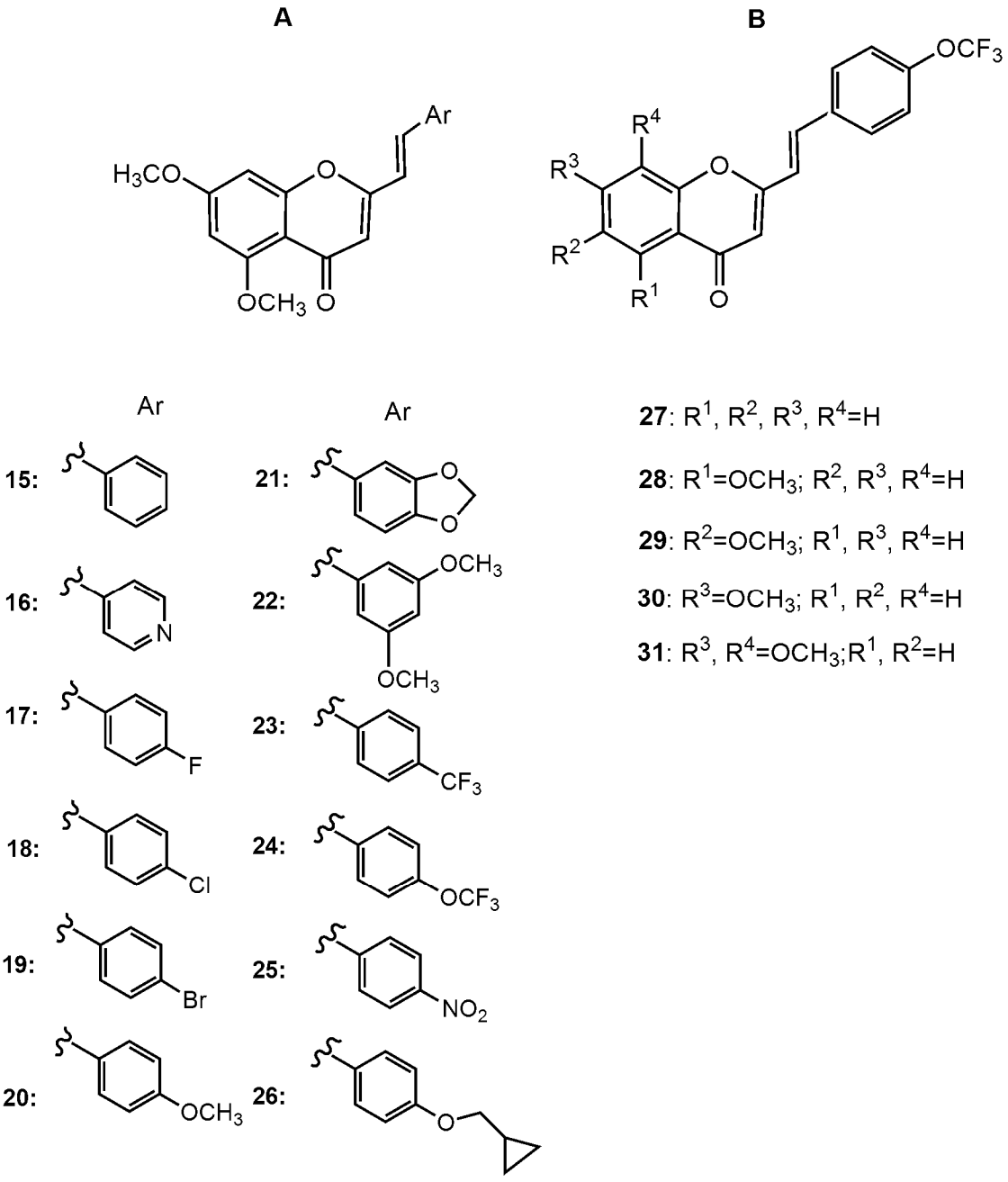


Figure 5 - Chemical structures of 2-styrylchromones studied for antitumor activity. (A) Analogues modified in the B-ring. (B) Analogues modified in the A-ring.

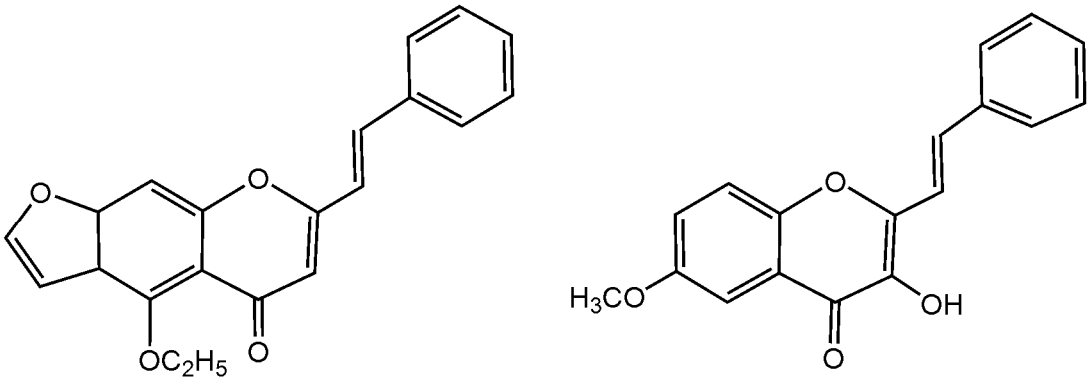
The results obtained with PC-3 cells showed that compound **27** exhibited moderate antiproliferative activity, indicating selective sensitivity of this cell line in response to 2-SC, which could be attributed to both steric and electronic effects of A-ring rather than B-ring moiety. From the B-ring modified analogs, **15**, **17**, **22**, **24**, **25**, and **26** exhibited moderate antiproliferative activity against A549 cells. Substitution of 4'-phenyl (**15**) for 4' pyridiyl (**16**) abolished the effect. The same happened by changing 4'-fluoro substituent (**17**) for bulkier substituents such as 4'-chloro (**18**), 4'-bromo (**19**), 4'-methoxy (**20**) and 4'-trifluoromethyl (**23**). Among the modified A-ring 2-styrylchromone analogs, only **27** demonstrated a

moderate activity. Results from BT483 cells showed that most compounds exhibited moderate activity. Nevertheless, compounds **15** and **19** were not active against this cell line. On the other hand, **22** and **26** exhibited higher activities than the other compounds. Among modified A-ring analogs, most of the tested compounds exhibited moderate activities except **29**, showing no activity, which was suggested to be due to steric hindrance caused by the 6-methoxy group. Compound **27**, bearing no substituent, showed the most potent antiproliferative activity. HeLa cells seemed to be the most sensitive to the tested compounds among the five cell lines. Compound **31** exhibited the most potent antiproliferative activity while **24** was not active among the B-ring modified analogs. SKHep cells were not as sensitive as HeLa cells. The most active compounds against this cell line were **24** and **31**. In order to examine the association between 2-styrylchromone-induced antiproliferation and cell cycle arrest the authors exposed HeLa cells to compounds **15**, **23**, and **31**. The results suggested that some of the compounds may induce the antiproliferative effect on HeLa cells through distinct mechanisms such as G_1 phase cell cycle arrest and DNA fragmentation [9].

I.1.1.3. Affinity and selectivity for A_3 adenosine receptors

Karton *et al* [10] investigated the structure-activity relationships of a series of flavonoid derivatives concerning their affinity to adenosine receptors in an effort to develop novel A_3 adenosine receptor antagonists. Selective antagonists of A_3 receptors have potential for the treatment of allergic, inflammatory and possibly ischemic disorders [11].

One of the compounds that showed a strong affinity to A_3 receptor was a 2-styryl derivative of the natural furanochromone visnagin (**32**) (Figure 6). In addition, this compound showed a considerable selectivity for A_3 vs. A_1 and A_{2A} receptors. The compound 3-hydroxy-6-methoxy-2-styrylchromone (**33**) also showed some selectivity for A_3 receptors. Although no functional antagonism was detected for these compounds, they can be an interesting scaffold for developing novel molecules with improved antagonist activity and specificity.



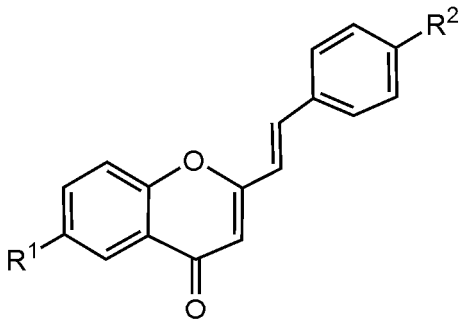
32 **33**

Figure 6 - Chemical structures of 2-styrylchromones studied for affinity and selectivity for A_3 adenosine receptors.

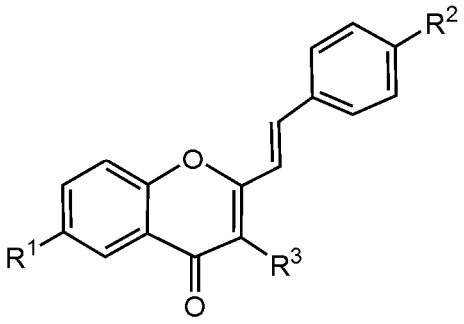
I.1.1.4. Antiviral activity

Several 2-SC (Figure 7: **34**-**41**) were tested for their antiviral activity against human rhinovirus (HRV), which is the most frequent cause of common cold [12]. The antiviral potency was evaluated in HeLa cell cultures infected with HRV 1B and 14, selected as representative serotypes for viral groups B and A of HRV, respectively. 2-SC **35**, **38**, **40**, and **41** displayed higher potency with serotype 14 than the others. The most active compound against both serotypes was **38**, followed by **40**, and **41**. Compound **35** showed moderate activity against both serotypes. The introduction of a second chlorine atom, in position 6 (**39**), increased the potency against HRV 1B and negated the antiviral effect on serotype 14. In order to improve the antiviral effect of the first series of tested 2-SC, the authors introduced hydroxy or methoxy groups in position 3 of the C-ring (Figure 7: **42-57**) [13]. The introduction of these groups generally enhanced the antiviral potency against both tested serotypes. Only the 3-substituted 4′-nitro-2-SC (**45** and **53**) were less potent than the corresponding analogue, without substituent in position 3 (**38**). In contrast to the previous results, the introduction of the 6-chlorine atom generally led to reduced activity in the series of 3-substituted 2-SC. Only when a strong electron-withdrawing nitro group was present at position 4′, 6-chloro substitution resulted in analogues with higher potency against both serotypes. Thus, compounds **49** and **57** emerged as the most potent 3-hydroxy- and 3-methoxy-2-SC, respectively, but at same time they exhibited significant cytotoxicity. The authors also tested the influence of the introduction of a fluorine atom in position 6 in 3-unsubstituted and 3-hydroxy- or methoxy-substituted 2-SC (Figure 7: **58**-**60**) [14]. As a result, the 3-unsubstituted compound (**58**) showed a weak potency against both serotypes, while the introduction of a 3-hydroxy or a 3-methoxy substituent enhanced the activity of 6-

fluoro-2-SC (**59** and **60**) against serotype 14 and led to the loss of efficacy against HRV 1B [14]. The specific activity against serotype 14 of the 3-substituted compounds can be ascribed to the presence of a fluorine atom in position 6.

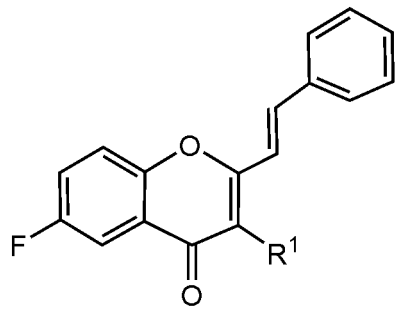


34: R^1=H; R^2=H
35: R^1=H; R^2=Cl
36: R^1=H; R^2=CH_3
37: R^1=H; R^2=OCH_3
38: R^1=H; R^2=NO_2
39: R^1=Cl; R^2=Cl
40: R^1=H; R^2=OH
41: R^1=H; R^2=NH_2



R^3=OH; OCH_3

42	**50**	R^1=H; R^2=H
43	**51**	R^1=H; R^2=Cl
44	**52**	R^1=H; R^2=OCH_3
45	**53**	R^1=H; R^2=NO_2
46	**54**	R^1=Cl; R^2=H
47	**55**	R^1=Cl; R^2=Cl
48	**56**	R^1=Cl; R^2=OCH_3
49	**57**	R^1=Cl; R^2=NO_2



58: R^1=H
59: R^1=OH
60: R^1=OCH_3

Figure 7 - Chemical structures of 2-styrylchromones studied for antiviral activity.

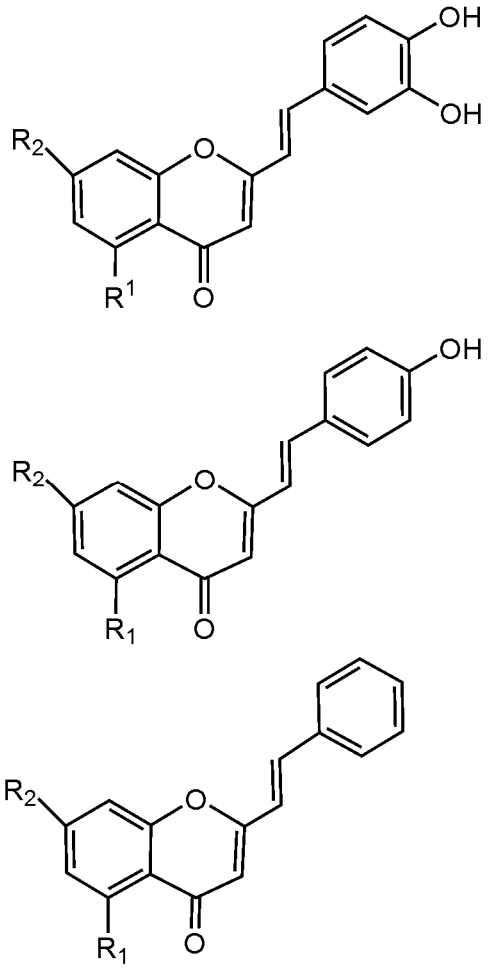
I.1.1.5. Antioxidant activity

The antioxidant properties of 2-SC have been shown in cellular [15] and non-cellular [16, 17] systems. In the first case, the authors evaluated the possible protective activity of six synthetic polyhydroxylated 2-SC (Figure 8: **61-63**; **65-67**) against the *tert*-

butylhydroperoxide (*t*-BHP)-induced pro-oxidant hepatotoxicity in freshly isolated rat hepatocytes. All the studied 2-SC exhibited hepatoprotective activity, which was reflected on the preservation of the integrity of the plasma membrane. It was evident that the 3',4'-dihydroxy (catechol) derivatives (**61-63**) were much more potent than the 4'-methoxy (phenol) (**65-67**) derivatives. The differences between the two groups of compounds were also observed in the qualitative and quantitative preservation of biochemical homeostasis. In fact, while the hepatoprotective effect for the catechol derivatives involved the prevention of lipid peroxidation and the inhibition of reduced glutathione (GSH) depletion and oxidized glutathione (GSSG) formation, the phenol derivatives only partially prevented lipid peroxidation, and had no effect on glutathione levels [15].

Several polyhydroxylated 2-SC (Figure 8: **61-63**; **65-67**; **68-71**) were tested [16] for their inhibitory effect on xanthine oxidase (XO). XO is a highly versatile enzyme, widely distributed among species and within the various tissues of mammals [18]. In XO-catalyzed reactions, oxygen is reduced by one or two electrons giving rise to superoxide radical ($O_2^{\bullet -}$) or hydrogen peroxide (H_2O_2) [19]. Consequently, XO is considered to be an important biological source of reactive oxygen species. Furthermore, it is known that an extensive metabolism of xanthine by XO will increase body uric acid levels. Due to the low solubility of uric acid, there is a tendency for urate crystals to be deposited in the urinary tract and in the synovial fluid of joints, a process associated with painful inflammation designated gout [20]. Fernandes *et al* [16] found out that the tested catechol derivatives (**61-63**) are considerably more potent inhibitors of XO than the phenol derivatives (**65-67**) and than the compounds with no hydroxy substituents in B-ring (**68-71**). The hydroxylation pattern in the benzopyrone moiety was important for the potency of XO inhibition. The presence of 5,7-dihydroxy substituents in the benzopyrone (**61**, **65**, and **68**) lead to an increase in activity, when compared with a single hydroxy substitution.

Filipe *et al* [17] studied the inhibitory effect of polyhydroxylated 2-SC (Figure 8: **61-64**) on Cu^{2+}-induced oxidation of isolated human serum low density lipoproteins (LDL), an *in vitro* model of lipid peroxidation. The most active compounds were **63** and **64**. The other tested compounds (**61** and **62**) were thought to be less active due to different partitioning of the hydrophobic 2-SC into LDL. The electron donating properties of the compounds, tested by pulse radiolysis in different micellar solutions helped to interpret the results. Hence, the compounds were shown to be equally capable of reacting with $O_2^{\bullet -}$ and tryptophan radical ($^{\bullet}$Trp) in cationic micelles, while the superiority of compound **64** over the other three 2-SC was made clear when electron transfer reactions were carried out in neutral micelles.



61: $R^1=R^2=OH$
62: $R^1=H$, $R^2=OH$
63: $R^1=OH$, $R^2=H$
64: $R^1=R^2=H$

65: $R^1=R^2=OH$
66: $R^1=H$, $R^2=OH$
67: $R^1=OH$, $R^2=H$
68: $R^1=R^2=H$

69: $R^1=R^2=OH$
70: $R^1=H$, $R^2=OH$
71: $R^1=OH$, $R^2=H$
72: $R^1=R^2=H$

Figure 8 - Chemical structures of 2-styrylchromones studied for antioxidant activity.

Table 1 – Summary of the most effective 2-styrylchromones in the studied biological activities. [1]When several compounds were active only the most potent are referred.

Biological activity	**Specific effect(s)**	**Active compound(s)[1]**	**References**
Antiallergic	Antiallergic activity in the passive cutaneous anaphylaxis test in the rat.	**4**-**8**	[6]
	Inhibition of histamine release from passively sensitized rat peritoneal cells.	**4**, **5**, **7**, **8**	
Antitumor	Cytotoxicity to P388 and HL-60 tumor cell lines	**1**	[1]
	Cytotoxicity to 9 KB tumor cells	**2**	[2]
	Tumor-specific cytotoxic effect	**11**, **13**	[7]
	Tumor-specific antiproliferative effect	**11**	[8]
	Antiproliferative effect against human carcinoma cell lines: PC-3, A549, BT483, HeLa, SKHep	PC-3: **27**; A549: **22**, **27**; BT483: **22**, **26**, **27**; HeLa: **31**; SKHep: **24**, **31**	[9]
Affinity for A_3 adenosine receptors	Binding to A_3 receptor; selectivity for A_3 vs. A_1 and A_{2A} receptors	**32**, **33**	[10]
Antiviral	Activity against HRV (serotypes 1B and 14)	Serotype 1B: **39** Serotype 14: **59**, **60** Both serotypes: **38**, **40**, **41**, **49**, **57**	[12-14]
Antioxidant	Protective activity against *t*-BHP-induced pro-oxidant hepatotoxicity in rat hepatocytes	**61-63**	[15]
	Inhibition of XO	**61-63**, **65**, **68**	[16]
	Inhibition of Cu^{2+}-induced oxidation of isolated human serum LDL	**63**, **64**	[17]

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I.1.2. MOLECULAR MECHANISMS OF ANTI-INFLAMMATORY ACTIVITY MEDIATED BY FLAVONOIDS

Reprinted from Current Medicinal Chemistry

Molecular Mechanisms of Anti-Inflammatory Activity Mediated by Flavonoids

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Abstract: Flavonoids (or bioflavonoids) are naturally occurring compounds, ubiquitous in all vascular plants. These compounds have been considered to possess anti-inflammatory properties, both *in vitro* and *in vivo*. Although not fully understood, these health-promoting effects have been mainly related to their interactions with several key enzymes, signaling cascades involving cytokines and regulatory transcription factors, and antioxidant systems. The biological effects of flavonoids will depend not only on these pharmacodynamic features but also on their pharmacokinetics, which are dependent on their chemical structure, administered dose schedule and route of administration. Thus, the therapeutic outcome mediated by flavonoids will result from a complex and interactive network of effects, whose prediction require a deep and integrated knowledge of those pharmacokinetic and pharmacodynamic factors.

The aim of the present review is thus to provide an integrated update on the bioavailability and biotransformation of flavonoids and the mechanisms of activity at the molecular, cellular, organ and organism levels that may contribute to their anti-inflammatory effects.

Keywords: Flavonoids, inflammation, cell signaling, antioxidant, ROS, RNS, arachidonic acid metabolism, cytokines, transcription factors.

1. INTRODUCTION

Flavonoids (or bioflavonoids) are naturally occurring compounds, ubiquitous in all vascular plants. These compounds have long been recognized to possess anti-hepatotoxic, anti-inflammatory, anti-atherogenic, anti-allergic, anti-osteoporotic and anti-cancer activities (reviewed in [1]). Additionally, these compounds have been considered to have beneficial effects in age-associated diseases such as cardiovascular and neurodegenerative diseases and some forms of cancer [2-6]. The potential health-promoting properties of flavonoids have been focused mainly on their antioxidant effects. However, it has become clear that the mechanism of action of flavonoids extends beyond the modulation of oxidative stress. More likely, the protective effects of flavonoids are linked to the modulation of intracellular signaling pathways, which are vital to cellular function.

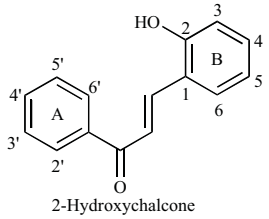
Inflammation is the first response of the body to infection, irritation or other injuries and is considered as a non-specific immune response aiming to neutralize the aggressor agents, and to repair damaged tissues, assuring this way the survival of the organism. The symptoms of inflammation are usually characterized by the following Latin expressions: *rubor* (redness), *calor* (warmth), *tumor* (swelling), *dolor* (pain), and *functio laesa* (loss of function). 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 [7]. Due to the permanent affliction, disability, and, many times, premature death of the millions of patients suffering from these diseases, chronic inflammation is associated with severe socio-economic problems. Unfortunately, the available treatments, although being successful in some cases, have numerous and severe side effects [8,9]. Therefore, there has been a constant pursuit for alternative therapeutic approaches, specially the use of naturally occurring molecules or their synthetic derivatives. Flavonoids, in particular, have been deeply studied in that focus in the last two decades, showing, many times, promising results. These compounds have been considered to possess anti-inflammatory properties, both *in vitro* and *in vivo*, resulting from their interactions with several key enzymes, signaling cascades involving cytokines and regulatory transcription factors, and antioxidant systems. Importantly, it is not always possible to extrapolate the effects observed *in vitro* to the *in vivo* situation. The *in vitro vs in vivo* differences are often attributed to biological changes in tissues and cells after their isolation and culture, but another important factor contributes for these differences, the pharmacokinetics of the studied compounds. Indeed, the biological effects of flavonoids will depend not only on pharmacodynamics but also on pharmacokinetics, which are dependent on their chemical structure, administered dose schedule and route of administration. Thus, the therapeutic outcome mediated by flavonoids will result from a complex and interactive network of effects, whose prediction requires a deep and integrated knowledge of those pharmacokinetic and pharmacodynamic factors.

The aim of the present review is thus to provide an integrated update on the bioavailability and biotransformation of flavonoids and the mechanisms of activity at the molecular, cellular, organ and organism levels that may contribute to their anti-inflammatory effects.

2. FLAVONOIDS

2.1. Structure and Nomenclature

Flavonoids are a group of about 4000 naturally occurring polyphenolic compounds that are nearly ubiquitous in plants and are recognized as the pigments responsible for the colours of leaves in autumn and the many shades of yellow, orange, and red in flowers and food [10].



2-Hydroxychalcone

Fig. (1). Chemical structure of 2-hydroxychalcone.

Natural flavonoids are all derived from a common biosynthetic pathway, which incorporates precursors from both shikimat and acetate-malonate pathways and the first flavonoid produced immediately following the confluence of the two pathways is a C-15 2-hydroxychalcone (Fig. (**1**)). This flavonoid forms the central core

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E-mail: egracas@ff.up.pt

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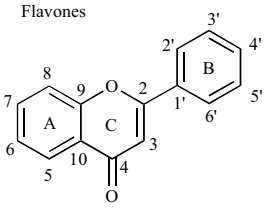
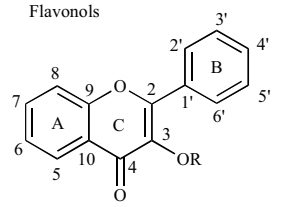
Flavones



	5	6	7	8	3	3'	4'
Chrysin	OH		OH				
Baicalein	OH	OH	OH				
Apigenin	OH		OH				OH
Acacetin							CH_3
Scutellarein	OH	OH	OH				OH
Cirsiliol	OH	OCH_3	OCH_3			OH	OH
Luteolin	OH		OH			OH	OH
Diosmetin	OH		OH			OH	CH_3
Wogonin	OH		OH	OCH_3			
Nobiletin	OCH_3	OCH_3	OCH_3	OCH_3		OCH_3	OCH_3
Guaphaliin	OH		OH	OCH_3	OCH_3		

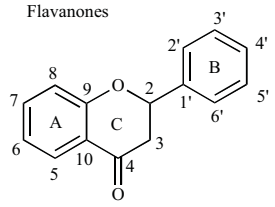
Flavonols



	R	5	6	7	3'	4'	5'
Kaempferol	H	OH		OH		OH	
Quercitrin	H	OH		OH	OH	OH	
Morin	H	OH		OH		OH	
Myricetin	H	OH		OH		OH	OH
Quercetagetin	H	OH	OH	OH	OH	OH	
Rutin	Rut	OH		OH	OH	OH	
Tiliroside	Glc (6''-*O*-*p*-coumaroyl)	OH		OH		OH	
Myricitrin	Rha	OH		OH		OH	OH
Quercitrin	Rha	OH		OH	OH	OH	

Rut – rutinose; Glc – Glucose; Rha – rhamnose

Flavanones



	3	5	7	3'	4'
Eridictyol		OH	OH	OH	OH
Naringenin		OH	OH		OH
Naringin		OH	ORut		OH
Hesperetin		OH	OH	OH	OCH_3
Hesperidin		OH	ORut	OH	OCH_3
Taxifolin	OH	OH	OH	OH	OH
Pinocembrin		OH	OH		

Rut-rutinose

Fig. (2). Flavones, flavonols and flavanones.

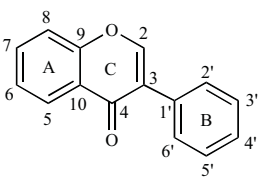
for all other flavonoids, which are obtained by means of the subsequent ring closure of the 2-hydroxychalcone. These flavonoids are characterized as containing a common structure consisting of two aromatic rings (A and B) that are bound together by 3 carbon atoms that combine with an oxygen atom and two carbons of the aromatic A-ring to form a third 6-member heterocycle ring (C-ring) [11]. The carbon atoms are identified with ordinary numerals for A and C-rings and "primed numerals" for the B-ring, although a modified number system is used for chalcones [12]. The flavonoids can be further divided into 7 classes, based on the oxidation state and functional groups of the C-ring, as well as the connection of the B-ring to the C-ring (Figs. (**2**), (**3**), and (**4**)). The structure of the C-ring may be an heterocyclic pyran, which yields flavan-3-ols (catechins) (without unsaturated bonds on C-ring) and anthocyanidins (with 2 double bonds) (Fig. (**3**)); or a pyrone, which yields flavonols (with one double bond C_2=C_3 and a 3-OH group), flavones (with one double bond C_2=C_3), flavanones or dihydroflavones (without the double bond C_2=C_3) (Fig. (**2**)), and isoflavones (with one double bond C_2=C_3 but where the connection of the B-ring is through the C_3 of C-ring instead of the C_2 as it occurs for the other flavonoids (Fig. (**3**)). The term *4-oxo-flavonoids* is often used to describe flavonoids, such as flavones, flavonols, and flavanones that carry a carbonyl group on C_4 of C-ring. Finally, there is the class of neoflavonoids with some differences from the other C-ring pyrone flavonoids *i.e.* the carbonyl group is on C_2 instead of on C_4, the double bond is in C_3=C_4, and the connection of the B-ring is through the C_4 of C-ring (Fig. (**4**)).

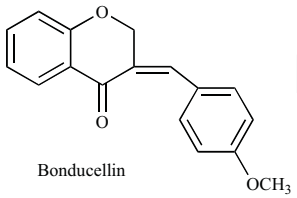
Within the various classes, further differentiation is possible, since other modifications of the flavonoid may occur resulting in: additional or reduced hydroxylation; methylation of hydroxyl groups or of the flavonoid nucleus; isoprenylation of hydroxyl groups or of the flavonoid nucleus (Fig. (**4**)); methylenation of *ortho*-dihydroxyl groups; dimerization to produce biflavonoids (Fig. (**4**)); bisulfate formation; and most importantly, glycosylation of hydroxyl groups to produce flavonoid *O*-glycosides or of the flavonoid nucleus to produce flavonoid *C*-glycosides [12]. Many flavonoids are polymerized into large molecules called tannins, either by the plants themselves or as result of food processing. Tannins consist of monomeric units of flavans linked by carbon-carbon and ether linkages [11].

Except for catechins, flavonoids do not occur in plants as aglycones and the most frequently occurring forms are the flavonoid *O*-glycosides, in which one or more of the flavonoid hydroxyl groups

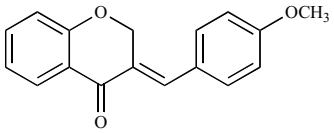
Isoflavones

	5	7	4'
Daidzein	OH	OH	
Genistein		OH	OH

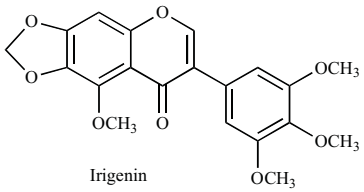




Bonducellin



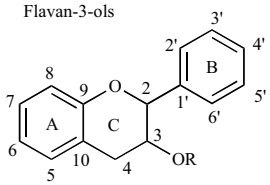
Isobonducellin



Irigenin

Flavan-3-ols

	R	5	7	3'	4'	5'
Catechin	H	OH	OH	OH	OH	
Epicatechin	H	OH	OH	OH	OH	
Epigallocatechin-3-gallate (EGCG)	gallate	OH	OH	OH	OH	OH



Anthocyanidins

	3	5	7	3'	4'	5'
Cyanidin	OH	OH	OH	OH	OH	
Pelargonidin	OH	OH	OH		OH	
Peonidin	OH	OH	OH	OCH_3	OH	
Malvidin				OCH_3		OCH_3
Delphinidin	OH	OH	OH	OH	OH	OH

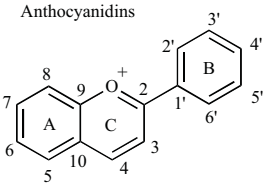


Fig. (3). Isoflavones, flavan-3-ols and anthocyanidins.

is bound to sugar(s) by an acid-labile hemiacetal bond [12]. The preferred glycosylation site on flavones, isoflavones, and flavanones is the 7-OH group, on flavonols is the 3-OH group (less frequently the 7-OH group) and on anthocyanidins is the 3- and 5-OH groups. D-glucose is the most usual sugar residue, but other carbohydrate substitutions include arabinose, galactose, glucorhamnose (rhamnose-glucose disaccharide), lignin, L-rhamnose, and xylose [13]. The most commonly studied flavonoid glycosides are rutin, a quercetin-3-*O*-glucose-rhamnose (also known as quercetin-3-rutinoside), and quercitrin (quercetin-3-*O*-rhamnose). In addition, occasionally, one or more of the sugar hydroxyl groups of glycosides can further be derivatized with acetic or ferulic acid (acylated glycosides).

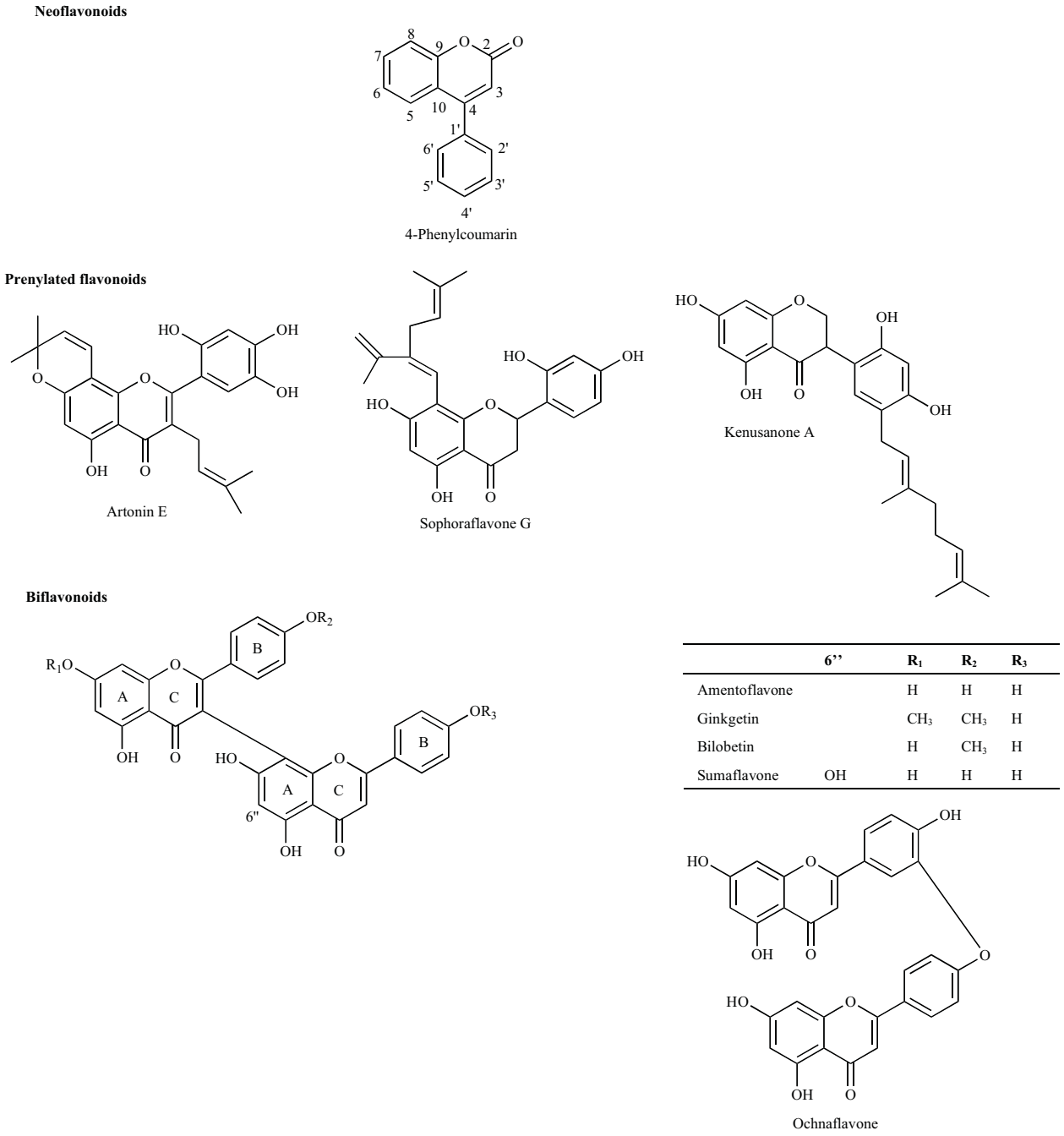
On flavonoid *C*-glycosides, the sugars are *C*-linked to the benzene nucleus, at the 6- and 8-positions, by a carbon-carbon bond that is acid resistant. Glucose is the most common sugar and flavone-*C*-glycosides are definitely the most ubiquitous [12].

2.2. Food Sources

Flavonoids are widely distributed in foods and beverages of plant origin, such as fruits, vegetables, tea, cocoa and wine. The intake of dietary flavonoids has been considered to provide several heath benefits (see [14] for a recent review). As a consequence, there has been a growing interest in knowing the content of flavonolic compounds in food, originating a large number of studies in which many compounds have been identified and quantified in several foods and beverages. Thus, the information related to the subject is vast and, many times, not consensual due to the variety of factors that can affect the flavonoid content in food. Firstly, the flavonoid content in plants is sensitive to many factors, including species, cultivars, fertility, season, climate, and degree of ripeness. Secondly, the food preparation and processing is also a cause of variability [13]. Additionally, the results of the quantification assays may vary according to the analytical procedure used. Nevertheless, it is possible to obtain very reliable and complete information concerning the flavonoid content in food in three databases created by the United States Department of Agriculture (USDA): "USDA – Iowa State University Database on the Isoflavone Content of Foods", release 1.3 - 2002, "USDA Database for the Proanthocyanidin Content of Selected Foods", 2004, and "USDA Database for the Flavonoid Content of Selected Foods" release 2 - 2006, which is more complete than the former two. This last database encloses information from five classes of flavonoids (flavones, flavonols, flavan-3-ols, anthocyanidins, and flavanones). In these databases, the information is organized in tables that provide the content of a number of selected flavonolic compounds in several food sources along with additional relevant details. Table **1** summarizes the prevailing flavonoids in food, separated in the different classes discussed in the present review, as well as their main food sources.

2.3. *In Vivo* Fate

In order to evaluate or to understand the potential beneficial effects of flavonoids in humans for their possible role in disease



	6''	R_1	R_2	R_3
Amentoflavone		H	H	H
Ginkgetin		CH_3	CH_3	H
Bilobetin		H	CH_3	H
Sumaflavone	OH	H	H	H

Fig. (4). Neoflavonoids, prenylated flavonoids and biflavonoids.

prevention (or treatment) it is crucial to know their *in vivo* fate. In the past decades, an increased number of studies on flavonoids absorption, metabolism, distribution and excretion has been published and will be briefly reviewed in this section.

2.3.1. Gastrointestinal Absorption and Metabolism

As stated before, most flavonoids occur in plants as flavonoid *O*-glycosides. Though most of the flavonoids metabolism occurs after absorption, extensive hydrolysis of glycosides undergoes in the gastrointestinal tract. Indeed, after oral administration, for flavonoids in general, it is assumed that they are absorbed after prior hydrolysis of the glycosides in the digestive tract [15]. Recently, Walle *et al*. [16,17] have shown that flavonoid glucosides can also be hydrolysed in the oral cavity by β–glucosidases derived both from bacteria and shedded oral epithelial cells to deliver the biologically active aglycones at the surface of the epithelial cells.

The fate of flavonoid *O*–glycosides in the stomach is not yet well known. Most of them, however, seem to resist to acid hydrolysis in the stomach and thus arrive intact to the duodenum [18].

The small intestine is one of the main organs responsible for flavonoid absorption and metabolization. Flavonoid aglycones are hydrophobic and can be transported across membranes by passive diffusion. However, the correspondent glycosides are more hydrophilic, decreasing this way the possibility of passive transport. Nevertheless, Hollman *et al*. [19] proposed that quercetin glucosides could be transported into enterocytes *via* interaction with epithelial brush border membrane transporters, such as the sodium-dependent

Table 1. Prevailing Flavonoids in Food

Flavonoid classes	Main food sources[1]	Prevailing flavonoids (aglycone designation)[1]
Flavones	Aromatic herbs (*e.g.* parsley)	Apigenin, luteolin
Flavonols	Fruits (*e.g.* apple), vegetables (*e.g.* onion), green tea	Quercetin, kaempferol, myricetin, isorhamnetin
Flavan-3-ols	Green tea, red grapes	Catechins and gallic acid esters of catechins
Isoflavones	Soy beans and some soy foods[2]	Daidzein, genistein[2]
Anthocyanidins	Berries, red grapes	Cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin
Flavanones	Citrus fruits	Naringenin, hesperidin, eriodictyol

[1]Source of information (except for isoflavones): "USDA Database for the Flavonoid Content of Selected Foods" release 2 – 2006. Web site: http://www.ars.usda.gov/is/pr/2007/070110.htm.
[2]Source of information: "USDA – Iowa State University Database on the Isoflavone Content of Foods", release 1.3 – 2002. Web site: http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html.

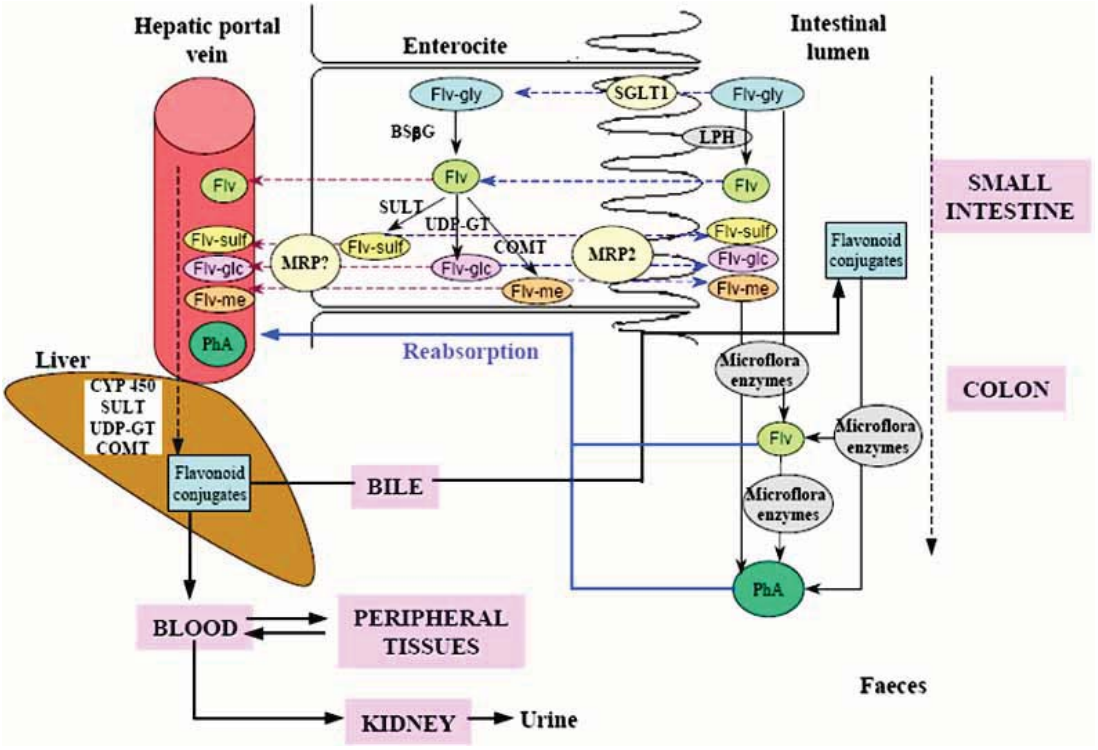


Fig. (5). Possible metabolic routes for flavonoids after oral ingestion. Flv, flavonoid aglycones; Flv-gly, flavonoid glycosides; Flv-sulf, flavonoid sulfates; Flv-glc, flavonoid glucoronates; Flv-me, flavonoid methylates; PhA, phenolic acids; LPH, lactase phloridzin hydrolase; BSβG, broad-specific β-glucosidase; SGLT1, sodium-dependent glucose transporter 1; MRP, multidrug resistance-associated proteins; UDP-GT, uridine diphosphate-glucuronosyltransferases; SULT, sulfotransferases; COMT, catechol-*O*-methyltransferases, CYP450, cytochrome P450.

glucose transporter SGLT1. The active transport hypothesis was later confirmed [20-22], but it was demonstrated that the efficiency of such absorption was considerably decreased due to the presence of other membrane transporters, mainly efflux transporters, through which a significant part of flavonoid conjugates absorbed by the intestinal mucosa are secreted back to intestinal lumen [23,24]. This efflux seems to involve the apical transporter multidrug resistance-associated protein 2 (MRP2) [15,25,26], an ATP-dependent export pump for conjugates with glutathione, glucuronate or sulfate [27] (Fig. (**5**)).

It was also demonstrated that after uptake of flavonoid glycosides into enterocytes, these conjugates could be hydrolysed by a broad specific cytosolic β-glucosidase (BSβG) [28,29]. In addition, lactase phloridzine hydrolase (LPH), a β-glycosidase present in the brush border membrane of the small intestine, can catalyze the extracellular hydrolysis of some glycosides, which is followed by diffusion of the aglycone across the brush border [30]. The relative role of both enzymes on the hydrolysis of glycosides is not well known. Therefore, with the exception of anthocyanins, most of flavonoid glycosides are hydrolysed before absorption, either in the lumen or in the cells of the gut (Fig. (**5**)).

After hydrolysis, and before reaching the liver, aglycones may undergo conjugation reactions in the small intestine, namely glucuronidation, sulfation, and *O*-methylation [31], forming glucuronidated, sulfated, and methylated (simple or mixed) conjugates, which enter systemic circulation (Fig. (**5**)).

Perfusion studies performed with rat intestine indicate that flavone and flavonol aglycones become glucuronidated, on absorption through the jejunum and ileum [32], by the action of uridine diphosphate (UDP)-glucuronosyltransferase (UDP-GT). These transferases are membrane-bound enzymes that are found in the endoplasmic reticulum in many tissues and that catalyze the transference of glucuronic acid from UDP-glucuronic acid to several

endogenous and exogenous substrates, including polyphenols. Most UDP-GT isoforms appear to have a distinct hepatic and/or extrahepatic expression. The gastrointestinal tract possesses a complex expression pattern, largely containing members of the UDP-GT1A subfamily [33].

Sulfation, or more correctly, sulfonation, occurs by the action of cytosolic sulfotransferases [34,35]. These enzymes, with broad-substrate activity, catalyze the transfer of a sulfate moiety from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to an hydroxyl group (phenolic group) on many different compounds.

Flavonoids possessing a 3',4'-*o*-dihydroxyl (or catechol) group in the B-ring are predictably prone to *O*-methylation by catechol-*O*-methyl transferases (COMT), which catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to the 3',4'-*o*-dihydroxyl group, though predominantly in the 3' position [36,37]. Catechol-*O*-methyl transferases activity is highest in the liver and the kidneys [35,38], but is also present in the gut jejunum enterocytes [31,39].

Flavonoid metabolism in humans also depends on the participation of intestinal microbiota. Pioneering studies developed by Booth *et al.* in the 1950s [40,41] and later in the 1980s by Hackett *et al.* [42] demonstrated that flavonoids that are not absorbed in the small intestine, such as polyphenols linked to rhamnose, arabinose, or xylose [43,44], can be metabolized by colonic microbiota into aglycones, which are broadly metabolized into phenolic acids. These aromatic compounds may be further absorbed from the colon and can be conjugated with glycine, glucuronic acid, or sulfate [37] (Fig. (**5**)).

Phenolic acids are formed from the aglycone by the opening of the heterocyclic C-ring at different points depending on their chemical structure. Scission of the flavonoid structure depends on their hydroxylation pattern as well as on the type and extent of oxidation of the carbon atoms of the heterocyclic ring (reviewed in [45]). While flavonols mainly generate hydroxyphenylacetic acids, flavones and flavanones are degraded to hydroxyphenylpropionic acids, and flavanols to phenylvalerolactones and hydroxyphenylpropionic acids. Phenylpropionic acids are further metabolized to benzoic acid derivatives. In addition, the fact that a high percentage of the quercetin dose was recovered as $^{14}CO_2$ in expired air, after ^{14}C-labeled quercetin has been administrated, both orally and intravenously, to healthy volunteers, emphasizes the importance of bacteria from the lower part of the intestine as the final step of elimination of this, and likely, other flavonoids [15,46].

The balance between the absorption of some flavonoids, as specific conjugated derivatives, and their metabolism through colonic degradation showed that the majority of the *in vivo* forms results from cleavage products of the action of colonic bacterial enzymes and subsequent metabolism in the liver [47]. Taking as example the flavonol quercetin-3-rutinoside (rutin), it was confirmed that the colon is the major site of rutin metabolism and absorption, principally towards catabolic pathways (production of phenolic catabolites), with the production of conjugated quercetin metabolites being a minor route [48]. These results suggest that only a small proportion of ingested flavonols is absorbed with an intact flavonol structure.

Metabolism and ring scission of flavonoids, by the colon microbiota, were established from animal studies. Until now, few data exist for humans but increasing evidences indicate that human intestinal bacteria possess a large number of hydrolytic enzymes, which can degrade a wide range of flavonoid glycosides [49,50].

2.3.2. Systemic Metabolism

Absorbed flavonoids, after metabolism in the small intestine, leave the intestinal cell across the basolateral side for their transport, *via* portal vein, to the liver [51], where they can be further metabolized. In the liver, the chief organ involved in flavonoid metabolism, flavonoids are substrates for a number of phase I and II enzymes. Flavonoid aglycones and their conjugated derivatives may undergo reactions such as hydroxylations, methylations, and reductions but, overall, new conjugation reactions with glucuronic acid and/or sulfate, leading to an increase of their degree of glucuronidation, sulfation and methoxylation. These metabolic processes are common to many xenobiotics. They usually decrease their potential toxic effects, as well as their biological activities, and increase their hydrophilicity, making possible their biliary and urinary elimination (Fig. (**5**)).

The relative importance of the 3 types of conjugation (methylation, sulfation, and glucuronidation) appears to vary according to the nature of the substrate and the dose ingested. Comparing with glucuronidation, in liver, sulfation is generally a higher-affinity, but lower-capacity pathway, so that when the ingested dose increases, a shift from sulfation toward glucuronidation occurs [52]. In a recent study, these facts were confirmed with the flavonoid baicalein [53].

Besides information regarding the mechanisms underlying the absorption and metabolism of flavonoids, a fundamental point for the understanding of their bioactivity and potential health effects is the knowledge on the nature and concentration of their plasma metabolites. Since the metabolic pathways for flavonoid conjugation are highly efficient, the corresponding aglycones either do not exist in plasma or exist only in very small amounts, when the quantities ingested correspond to those usually present in the diet.

A number of *in vivo* studies, performed in humans and rats, have conducted to the identification of quercetin metabolites in plasma, namely methylated, glucuronidated, and sulfated mono or mixed conjugates [54-59]. In human plasma, the major circulating metabolites identified, after consumption of quercetin glucoside-rich onions, were quercetin-3-glucuronide, 3'-methylquercetin-3-glucuronide (isorhamnetin-3-glucuronide) and quercetin-3'-sulfate [57]. In rats, when quercetin was administrated orally in a single dose, the major plasma metabolites were identified as quercetin mono and diglucuronides, quercetin glucuronides sulfates and methylquercetin glucuronides sulfates [36,55,59]. The most plausible positions for glucuronidation and sulfation were identified as being the hydroxyl groups located at positions 5 and 7 [36], but the 3-*O*-β-glucuronide and 4'-*O*-β-glucuronide of quercetin were also reported [58]. However, when rats were adapted, for 3 weeks, to a diet supplemented with quercetin, a very different metabolic pattern was obtained. The major part of the circulating metabolites (80-90%) was constituted by glucuronide and sulfate of isorhamnetin (3'-*O*-methyl quercetin) [54,60]. The most likely conjugation positions were at 3-OH, 5-OH, and 7-OH groups [60]. This is an important point, since the position at which conjugation occurs in flavonoids can change their biological activities. For example, when the *o*-catechol group is involved in conjugation reactions, the antioxidant activity of the resulting metabolites decreases significantly and they don't contribute to the antioxidant potential of plasma [36,60].

Regarding the circulating concentrations of flavonoid metabolites in humans, Erlund *et al.* [61] were able to detect quercetin glucuronide and/or sulfate conjugates (0.14-0.29 μmol/L) in human plasma, after oral administration of quercetin (8-50 mg), while no or barely detectable levels of quercetin aglycone were found. Moreover, after quercetin aglycone and rutin administration, it was observed that quercetin conjugate concentrations in plasma were similar, although the time to attain C_{max} was clearly delayed for rutin due to the hydrolysis of the glycoside that occurs in the small intestine or in the colon prior to its absorption.

Additional information can be obtained when absorption studies are performed using radiolabelled flavonoids. When the radiolabelled atom is in such a position that no change is observed when the molecule suffers metabolization, all metabolites can be measured in plasma, giving additional important information about their biological fate. In fact, after the oral administration of

[^{14}C]quercetin aglycone, the absorption was as high as 36–54%, when compared with intravenous administration. Interestingly, the plasma half-life for total radioactivity was very long, 20–72 h, which may in part be due to enterohepatic recirculation. This may include metabolites of quercetin with biological activity [46].

For other flavonols and flavones, the existent information on their absorption and bioavailability in humans is much more limited than for quercetin. Nevertheless, some studies have been made with a few of those compounds. Free luteolin and its monoglucuronide were detected in human serum after oral administration of this flavonoid [62]. The main metabolite in plasma was a monoglucuronide but the glucuronidation site was not defined [62]. Accordingly, free luteolin and glucuronide and sulfate conjugates were detected in rat plasma. In a recent study, DuPont *et al.* [63] could identify one conjugated kaempferol metabolite in human plasma, namely kaempferol-3-glucoronide, which accounted for 55-80% of total kaempferol. Although it is possible that the circulating compound resulted from absorption of the original glucuronide, since the majority of the kaempferol in the administrated food source (endive) is present as a glucuronic acid conjugate (kaempferol-3-glucuronide), the authors considered more likely that the compound had been deconjugated by gut microbiota β-glucuronidase in the colon prior to absorption and then the aglycone was reconjugated by UDP-GT in the small intestine or liver. On the other hand, a high level of non-conjugated kaempferol was detected in the plasma and urine [63]. Another study reported that following a single 400 mg oral dose of the aglycone the plasma concentration of unchanged chrysin was very low [64]. The only metabolites observed were conjugate derivatives; the mean plasma concentrations of chrysin sulfates exceeded that of chrysin approximately 30-fold while the concentrations of glucuronic acid conjugates were too low to be measured accurately. The overall recovery of the administrated chrysin dose in urine was only 1-7% of the dose. The excretion *via* feces may be the main route of elimination of chrysin and, in particular, its metabolites.

Studies with anthocyanins point to the low bioavailability of this class of flavonoids, although, according to Manach *et al.* [65], the methods used in the existing studies may not be the most adequate, leading to false conclusions. Anthocyanin glucuronide and sulfate metabolites have been recently identified in human urine [66,67], in contradiction with previous pharmacokinetic studies that could only identify the native forms of anthocyanin *i.e.* unchanged glycosides.

Flavan-3-ols are among the most well absorbed classes of flavonoids. The number of bioavailability studies in humans is vast, involving different compounds of this class, administrated either pure or in different food sources, and were reviewed recently by Manach *et al.* [65]. The galloylated compounds are, apparently, less efficiently absorbed than the non-galloylated. A very important metabolite of epigallocatechin is the 4'-*O*-methyl-epigallocatechin. Epigallocatechin gallate (EGCG) is also methylated, originating 4',4''-di-*O*-methyl-EGCG, although the free form of this compound is predominant. Methylated and glucuronidated metabolites of epicatechin have been identified. In addition, the microbial metabolism seems to be a relevant metabolic pathway in what concerns to catechin and epicatechin [65].

Unlike flavan-3-ols, human bioavailability studies with flavanones are still scarce. Plasma concentrations as high as 1.3–2.2 μmol/L of hesperetin metabolites (with an intake of 130–220 mg given in orange juice) and up to 6 μmol/L of naringenin metabolites (with 200 mg ingested in grapefruit juice) have been detected [68,69] but the metabolites haven't been identified yet. Nevertheless, in a recent pharmacokinetic study performed in rats, when naringenin was orally administrated, its sulfated and glucuronidated conjugates were found almost exclusively circulating in the bloodstream, whereas naringenin aglycon was negligible [70]. The systemic exposure of naringenin sulfates was 3 times higher than naringenin glucuronides. The first peaks of naringenin sulfates and glucuronides occurred at 5 min, indicating very rapid absorption of naringenin and simultaneous sulfation/glucuronidation. In addition, after intravenous bolus administration of naringenin, the serum profiles of naringenin, its sulfates and glucuronides indicated very rapid and extensive conjugation metabolism of naringenin to yield sulfates as the major metabolites in the bloodstream.

Isoflavones are the most well absorbed flavonoids [65]. Glycosides are not recovered in plasma and aglycones represent a very low percentage (< 5%) of the total metabolites. The main metabolites are 7-*O*-glucuronides and 4'-*O*-glucoronides, with small proportions of sulfate esters. Equol is a microbial metabolite of daidzein with clinical importance due to its estrogenic effects. However, not all individuals are able to produce equol. This phenomenon, which has led to subjects being described as equol "producers" and "non-producers", has been intensively studied, with factors like intestinal microbiota, gender, age, genetics, and background diet being pointed as relevant, yet it is not fully understood [65,71].

It has to be noted that the metabolic patterns observed may depend on the dose, due to possible saturation effects, route of administration, *i.e.*, intragastric or intravenous (in single or multiple doses) or in the diet, and in the latter case on the period of administration. On the other hand, the individual variation in the bioavailability of flavonoids has also to be considered. Such variation is due to both physiological (body weight, body composition, gastric motility, and disease state) and molecular factors (activity or synthesis of transporters or enzymes involved in biotransformation). In addition, the composition and metabolic activity of the gastrointestinal microbiota are likely determinants of the bioavailability of flavonoids absorbed from the distal parts of the gastrointestinal tract [72].

2.3.3. Tissue Distribution and Elimination

After absorption and metabolism, the availability of pharmacologically active flavonoids for the peripheral tissues is affected by their plasma binding and by the way they are eliminated from the body.

From the liver, flavonoid conjugated derivatives go into systemic circulation, where they may bind to plasma proteins, or be distributed to tissues. The decrease in flavonoids lipophilicity induced by their conjugation does not favour a passive diffusion of the conjugates through the membrane. However, it is quite probable that their uptake by the target cells involves specific transport systems, as it has already been shown for estrogen conjugates [73].

The binding to plasma proteins, especially to albumin, is particularly high in quercetin (> 99%), which may result in poor cellular availability [74]. However, the binding of quercetin metabolites to plasma albumin may also delay their clearance from plasma and, therefore, provide a depot, from which they may be distributed to target organs [75]. In fact, the elimination of quercetin metabolites in humans is quite slow, with reported half-lives raging from 11 to 28 h. This could favour accumulation in plasma with repeated intakes [65].

Flavonoid metabolites, resulting from the conjugative activities of both liver and intestine, such as methylated and/or glucuronidated and/or sulfated metabolites are polar, water-soluble compounds that can be excreted in the urine and bile. The nature of the conjugation influences the elimination pathway of the circulating metabolites: the glucuronides are excreted in the bile to a much higher extent than the sulfates, which are preferentially eliminated in urine [73]. When excreted in bile, flavonoid metabolites are secreted *via* the biliary route into the duodenum and further metabolized by bacteria in the large intestine, which results in the scission of the flavonoid structure and/or the hydrolysis of glucuronide and sulfate conjugates. The resultant products may be reabsorbed and undergo an enterohepatic cycling [51].

The activity of the biliary secretion strongly depends on the nature of the compounds. According to Crespy *et al.* study [73], performed in a rat intestinal perfusion model, catechin is sparingly secreted in the bile (1%), whereas genistein and eriodictyol are extensively recycled (32 and 23% of the perfused dose, respectively). The biliary secretion of quercetin is quite limited, indicating that the biliary pathway probably plays a minor role in its elimination.

A recent compilation of several human bioavailability studies [65] shows that the elimination half-lives of isoflavones, rutin and quercetin derivatives are quite slow, indicating their probable accumulation in plasma after repeated intakes. On the contrary, flavanones and catechins have fast elimination half-lives, although the half-lives of their metabolites may be longer.

3. FLAVONOIDS AND INFLAMMATION

The inflammatory response encompasses a wide range of activities, including increased permeability of endothelial lining cells and influxes of blood leukocytes into the interstitium, oxidative burst, release of cytokines [*e.g.* interleukins, and tumor necrosis factor-α (TNF-α)]. In this process, various enzyme activities are also induced (oxygenases, nitric oxide synthases, peroxidases), as well as the arachidonic acid (AA) metabolism, and the expression of cellular adhesion molecules, such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM).

The anti-inflammatory properties of flavonoids have been attested through a vast number of assays applied on diverse molecules from the different flavonoid classes, to explore their effects on various pathways involved in the inflammatory process. Several reviews on the subject have also emerged in the last years. Therefore, the purpose of the present chapter is not to make an exhaustive review of all the information related to the anti-inflammatory capacity of flavonoids but, instead, to highlight the main cellular mechanisms conducting to inflammatory response in which flavonoids were shown to interfere and, mainly, to reunite the most recent data on the subject, relating it to previous studies when opportune.

3.1. Antioxidant Activity of Flavonoids

During the inflammatory process, a stress oxidative condition results from the activation of phagocyte cells (phagocytotosis) once they have arrived at the site of inflammation by chemotaxis.

Phagocytosis, which occurs during inflammation in a variety of cells, namely monocytes, neutrophils, eosinophils, and macrophages, is accompanied by a dramatic increase in oxygen consumption (oxidative burst) resulting firstly in the formation of superoxide radical ($O_2^{\cdot-}$) through the activity of a plasma-membrane NADPH oxidase [76,77]. $O_2^{\cdot-}$ produced *in vivo* is quickly converted to hydrogen peroxide (H_2O_2) spontaneously or by the enzyme superoxide dismutase (SOD) [78,79]. Although being a rather unreactive molecule, H_2O_2 can be reduced by transition metal ions generating the hydroxyl radical ($HO^{\cdot}$). This is one of the strongest oxidizing agents, producing severe damage in its vicinity and initiating lipid peroxidation due to its well-known reactivity with polyunsaturated fatty acids, which results in the production of peroxyl radical ($ROO^{\cdot}$) and other cytotoxic agents [79-81]. In addition H_2O_2 can oxidise halide ions such as Cl^- (the most abundant anion in biological fluids) to hypochlorous acid (HOCl). This reaction is catalysed by myeloperoxidase (MPO), a hydrogen peroxide oxidoreductase specifically found in mammalian granulocytic leukocytes, including neutrophils, monocytes, basophils, and eosinophils. HOCl is a strong oxidant and can react with amines producing chloramines, oxidizing species some of which being very toxic. Moreover, singlet oxygen ($^1O_2$), a highly reactive form of O_2, can be produced *in vivo* by a range of peroxidase enzymes (*e.g.*, MPO, lactoperoxidase) and during lipoxygenase-catalyzed reactions. $^1O_2$ can also be generated during lipid peroxidation [82].

During the inflammatory process, reactive nitrogen species (RNS) are also produced. Effectively, during the host defence against pathogenic microorganisms, nitric oxide ($^{\cdot}NO$) is produced by the inducible nitric oxide synthases (iNOS) existent in activated macrophages and neutrophils. Identified as a cytotoxic factor, $^{\cdot}NO$ produced by iNOS is involved primarily in the mediation of cellular immune response. Additionally, it is involved in the pathogenesis of some conditions including inflammation [83,84]. Besides its own pro-inflammatory effects, $^{\cdot}NO$ can exert its toxicity by generating more destructive reactive species, particularly the peroxynitrite anion ($ONOO^-$), by reacting with $O_2^{\cdot-}$ [85].

Due to their ability to kill microbial pathogens directly, reactive oxygen species (ROS) and RNS produced by phagocytes have an important role in the mechanism of host defence. Moreover, this reactive species have important roles in inflammation essentially by being trigger elements or by being signaling messengers molecules [86]. On the other hand, their overproduction may provoke or exacerbate damage in inflammatory sites [87,88]. This rationale clearly evidences the putative importance of the antioxidant activity of flavonoids in the therapy of inflammation.

This section presents the current knowledge on structural aspects and antioxidant properties of flavonoids, concerning their ability to scavenge free radicals, to protect against other oxidants, such as $ONOO^-$ and HOCl, and to chelate metal ions, for which structure-activity relationships have been well established.

3.1.1. Free Radical Scavenging Activity

For a compound to be considered a good free radical scavenger it must be highly reactive with different radicals and the stoichiometry of the radical scavenging process should be high. In addition, the resulting "antioxidant" radical must possess a high stability, that is, the "antioxidant" radical must interrupt (rather than propagate) a chain reaction. The selective generation of individual radical species by pulse radiolysis and photolysis allows the determination of rate constants for both "antioxidant" radical formation and decay [89-92]. The stoichiometry can only be obtained from product identification. Those techniques, however, are not available at the majority of research units and, therefore, alternative chemical and biochemical methods have been employed.

Numerous studies have described flavonoids as specific scavengers of $O_2^{\cdot-}$ [93-96], $HO^{\cdot}$ [90,93,97,98], and $ROO^{\cdot}$, both in aqueous and organic solutions [96,99,100], as well as of $^{\cdot}NO$ [96,101]. In order to elucidate their structure-activity relationships, some studies were conducted aiming to compare the antioxidant behaviour of some classes of flavonoids. Pioneer works using hydroxyl and azide radicals, generated by pulse radiolysis, and *tert*-butoxyl radicals, generated by photolysis, allowed the determination of rate constants of both flavonoid aroxyl radical formation and decay [89-92]. From the kinetic analysis and comparison of the spectral features of the aroxyl radicals produced, it was, for the first time, derived a structure-activity relationship for flavonoids [90].

Further studies were developed on structure-activity relationship for flavonoids and, since most antioxidants can behave in different ways *in vitro*, depending on the oxidant, the antioxidant properties of flavonoids were evaluated using different methods. Some of these methods are based on the scavenging of radicals such as the ferrylmyoglobin [102], a heme protein radical [103], hydrophilic $ROO^{\cdot}$, generated from 2,2'-azo*bis*(2-amidino-propane) dihydrochloride (AAPH) [96,102,104], and the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) [102,105,106]. Considering the relevance of lipid oxidation to biological systems, different methodologies have been used to evaluate the protection conferred by flavonoids against lipid peroxidation [102,107,108]. It must be pointed out, however, that the structural features of flavonoids re-

quired for inhibiting lipid peroxidation correspond to the sum of the structural features for the different processes involved. Besides the scavenging of lipid radicals, the inhibition of lipid peroxidation by flavonoids is also influenced by their lipophilicity, which affects their penetration into lipidic bilayers, and by their ability to scavenge the initiating radicals. In addition, for the lipid peroxidation induced by Fe^{3+}-EDTA-ascorbate, for example, synergistic effects between ascorbate and flavonoid radicals have been shown to occur [102].

Bors *et al.* [89-91] have proposed that three structural features should be responsible for the effective free radical scavenging by flavonoids: (1) the *o*-dihydroxyl or catechol group in the B-ring, which confers a high stability to the radical formed; (2) the conjugation of the B-ring with the 4-oxo group *via* the 2,3-double bond, which ensures the electron delocalisation from the B-ring and (3) the combination of 3- and 5-OH groups with the 4-oxo group, which allows electron delocalisation from the 4-oxo group to both substituents (Fig. (**6**)). The combination of all of these structural features enables a higher electron delocalisation conferring, therefore, a higher stability to aroxyl radicals. The *o*-catechol group confers a high stability to the resulting radical since, when the O-H bond is broken, a strong H bond is formed between the radical and the other OH group, which stabilizes the radical and decreases the O-H bond dissociation enthalpy [109,110]. In addition, when the B-ring possesses a pyrogallol (3',4',5'–OH) group, the central O-H bond is weaker due to the presence of two *ortho* groups which can form two hydrogen bonds with the radical [109].

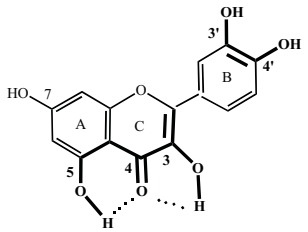


Fig. (6). Structural features responsible for the effective free radical scavenging by flavonoids (in bold).

In this context, and taking as examples some flavonoid aglycones, the flavone myricetin (3,5,7,3',4',5'-OH) is expected to be an excellent radical scavenger and more efficient than quercetin (3,5,7,3',4'-OH). In fact, this was observed in some studies [102,104,111]. Furthermore, one of these studies reported that the flavan-3-ol catechin and the flavanone taxifolin exhibited antioxidant capacities comparable to that of quercetin against different radicals [102]. These flavonoids have the same hydroxylation pattern of quercetin but catechin lacks the structural determinants (2) and (3) and taxifolin the determinant (2). This behaviour is in agreement with van Acker *et al.* hypothesis [112], according to which the B-ring determines the antioxidant activity whereas the basic structure has only a small influence. Indeed, for most flavonoids, the part of the polyphenol molecule with better electron-donating properties is the B-ring [113]. Moreover, Bors and Saran [89] also concluded that substances with a saturated heterocyclic ring are predominantly attacked at the *o*-dihydroxyl site in the B-ring and the semiquinones formed are quite stable.

Nevertheless, the flavonol kaempferol (3,5,7,4'-OH), in spite of bearing no catechol structure, also presents a high antioxidant activity. It seems that, for flavonoids lacking a catechol group, the basic structure becomes important, *i.e.*, the presence of both 2,3-double bond and 3-OH group is also important for conferring a high antioxidant activity. This difference in antioxidant activity, based on the presence of both 2,3-double bond and the 3-OH group, is probably a result of the enhanced planarity of the molecule. The 2,3-double bond in the C-ring confers higher rigidity to the ring and holds the A and C rings in a more coplanar position. In addition, the 3-OH moiety interacts with B-ring through an intramolecular hydrogen bond with the 2'- or the 6'-hydrogen and this conformational arrangement places the B-ring approximately in the same plane as rings A and C [102,107,112].

It can be concluded that the radical scavenging activity of flavonoids is therefore determined by the B-ring, the presence of *o*-catechol group being the most important factor for a high activity. Nevertheless, the basic structure of flavonoids (the presence of both 2,3-double bond and 3-OH group) becomes important when the antioxidant activity of B-ring is small.

3.1.2. Protection Against Hypochlorous Acid

It has been shown that some isoflavones, namely genistein, biochanin-A, and daidzein, react with HOCl to form stable mono and dichlorinated products, modified at the C_6 and C_8 sites of the A-ring [114]. Accordingly, quercetin and its derivative rutin also showed to suffer chlorination by HOCl in the same positions, which resulted in even more efficient scavengers of ROS and RNS than the parent compounds [115]. In a recent study, quercetin, luteolin, apigenin, and chrysin were demonstrated to have a strong HOCl scavenging effect [96]. All these compounds have the 5,7-dihydroxylated A-ring in common, indicating that this structural feature probably contributes to a higher reactivity with HOCl. The C_3-OH substitution seems to be another important feature to the HOCl scavenging activity, considering that quercetin showed the strongest effect [96]. Indeed, other authors [116] have previously discussed the importance of this substituent, based on the HOCl scavenging activities of several flavonoids from different classes.

3.1.3. Protection Against Singlet Oxygen

The $^1O_2$ scavenging activity of several flavonoids has been clearly demonstrated [96,117-119]. The efficiency of the chemical reaction between flavonols or flavones and $^1O_2$ is mainly controlled by the presence of an OH group in position 3 activating the C_2-C_3 double bond of C-ring towards $^1O_2$. The molecules that do not contain an OH group in position 3 (or those in which this group is substituted by a sugar) react much more slowly with $^1O_2$ than the corresponding flavonol does [96,117]. The influence of OH substituents on rings A and B is relatively small [117]. Nevertheless, the presence of a catechol structure in B-ring (3',4'-dihydroxy) favours its physical quenching on $^1O_2$ [117,118].

3.1.4. Protection Against Peroxynitrite

$ONOO^-$ is a reactive and short-lived species and its formation by phagocytes, for killing invading microorganisms, is an important event of host defense [120]. However, high concentrations and an uncontrolled generation of $ONOO^-$ can result in the oxidation and nitration of a wide variety of host biomolecules and the consequent damages have been proposed to contribute to the pathogenesis of several diseases (reviewed in [121]). The detection of 3-nitrotyrosine was considered a biomarker of $ONOO^-$ action *in vivo* and high levels of this molecule were detected in pathologies such as atherosclerosis, rheumatoid arthritis, neurodegenerative diseases and multiple sclerosis [121-123]. Flavonoids possess chemical features that may confer an efficient protection against $ONOO^-$ or their reactive intermediates. Indeed, a considerable number of studies have proved the interaction of flavonoids with $ONOO^-$ [96,114,124-131], although only some of these studies were carried out taking into account that CO_2/HCO_3^-, an important plasma buffer system, may significantly modify the reactivity of $ONOO^-$ [96,125,130-132]. The chemistry of $ONOO^-$ is complex and it has been shown that it reacts with CO_2 at a rate that is significantly higher than that observed with other biomolecules. The reaction with CO_2 is fast and yields a short-lived intermediate that rapidly homolyses to carbonate radical ($CO_3^{\cdot-}$), a relatively strong oxidant, and nitrogen dioxide ($^{\cdot}NO_2$), a more moderate oxidant and also nitrating agent [133].

The protection of flavonoids against $ONOO^-$ has been evaluated by their ability to inhibit the $ONOO^-$-mediated dihydrorhodamine 123 (DHR123) oxidation [96,124,128-130] tyrosine nitration [126,128,131,132], and α_1-antiproteinase inactivation [131,132], with or without physiological concentrations of bicarbonate. When the effect of flavonoids against $ONOO^-$ was evaluated by their ability to inhibit DHR123 oxidation, it was observed that flavones are the most effective [124,125]. Their effects depend mainly on the presence of a large number of hydroxyl groups, which must include either a catechol group in the B-ring or a hydroxyl group at the 3-position [96,124,125]. The important contribution of the 2,3-double bond towards the protection by flavonoids against $ONOO^-$ was also shown [125]; the flavanone taxifolin, with the same five hydroxyl groups as quercetin (3,5,7,3',4'–OH), but lacking the 2,3-double bond, presents a lower activity [125]. These data indicate that the ability of flavonoids to protect against $ONOO^-$ depends on some structural features, also important to scavenge free radicals.

Flavonoids were shown to be somewhat less effective at protecting against $ONOO^-$ when physiological concentrations of bicarbonate were present [125,131], suggesting that they are less competitive at reacting with secondary radicals formed from the reaction of $ONOO^-$ and CO_2, present in equilibrium with HCO_3^-. The most efficient flavonoids, such as myricetin and quercetin are effective at low concentrations, with IC_{50} of the same magnitude as ebselen [125], a selenocompound that has been reported to be excellent at protecting against $ONOO^-$ [134].

The mechanisms by which flavonoids protect against $ONOO^-$-mediated oxidation and nitration are hitherto not established. Previous studies conducted in the absence of added bicarbonate have shown that polyphenolic compounds may be both oxidized and nitrated by $ONOO^-$ [126,127]. Catechol-containing compounds have the ability to reduce $ONOO^-$ to nitrite, in a two-electron pathway, resulting in their oxidation to the corresponding *o*-quinones. Flavonoids are powerful electron-donating compounds, the presence of *o*-catechol group (3',4'-OH) in the B-ring being determinant for a high reducing activity [125]. The majority of nitration reactions will occur on B-ring at $C_{2'}$ and $C_{5'}$, for catechol flavonoids [126].

3.1.5. Chelating Properties

Besides the well-known radical scavenging activity of flavonoids, another antioxidant mechanism may result from the interactions between transition metal ions (especially iron and copper) and flavonoids to produce complexes that prevent the participation of these metal ions in free radical generating reactions [112,135-140]. In fact, iron and copper have a major role in the production of the very reactive $HO^{\cdot}$, through the Fenton and Haber-Weiss reactions [141-144]. In addition, through a Fenton type reaction, preformed lipid hydroperoxides (ROOH) are decomposed to form the alkoxyl radicals, strong oxidants, which can propagate the chain reaction of lipid peroxidation [145,146] or react with other cell constituents. In order to be an effective catalyst in those radical reactions, iron has to be present as an ionic or "free" form. Traces of iron salts are present in several body fluids, with the exception of blood plasma [147]. Usually iron is safely sequestered in proteins that normally bind iron, hindering or preventing its action in catalysing radical reactions. However, it can be released from those proteins at low pH [148], or as a result of protein damage produced by peroxides [148,149] or even by reductive mobilization by $O_2^{\cdot -}$ [150]. Most copper is "tightly bound" to the plasma protein caeruloplasmin, but some is attached to albumin and to aminoacids, such as histidine, in such a way that it still can catalyse free radical reactions [151,152]. Therefore the chelation of metals can be crucial in the prevention of radical generation and the subsequent damage of target biomolecules. Moreover the use of natural metal chelators, such as flavonoids, should be favoured against other synthetic chelators, which present associated toxicity problems [153].

The majority of the studies about iron and copper chelation by flavonoids have been performed by means of ultraviolet/visible (UV/Vis) absorption spectroscopy, analysing the bathochromic shifts of UV bands I and II, which characterize the flavonoid spectra [12]. UV spectroscopy measurements, however, give only indirect evidence for complex formation between flavonoid and metal ion. Direct evidence for flavonoids/transition metal complexation, however, can be achieved by means of mass spectrometry [139,140,154,155] and additionally by NMR as reported for diamagnetic transition metal ions [154,155].

In relation to the formation of chelates, and according to the literature, there are three possible metal-complexing (binding) sites within a flavonoid molecule containing hydroxyl groups at 3, 5, 3', and 4' positions (Fig. (**7**)). These sites are between the 3-OH group and the 4-oxo group, the 5-OH group and the 4-oxo group, and between the *o*-dihydroxyl groups in the B-ring [156].

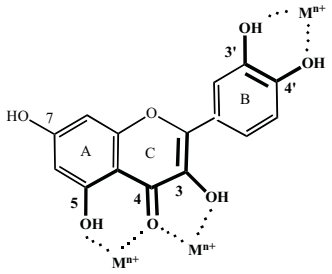


Fig. (7). Possible binding sites for trace metal ions (M^{n+}) to flavonoids (in bold).

Chelation studies performed with iron and copper ions at pH 7.4 and pH 5.5, by means of both UV/Vis and ESI-MS, showed that only flavones and flavonols (quercetin, myricetin, luteolin, kaempferol, rutin and apigenin) and the flavan-3-ol catechin interacted with metal ions [139]. The flavanones (taxifolin, naringenin and naringin) and isoflavones (daidzein and genistein), however, do not chelate metal ions. This can be explained by the fact that, in flavones and flavonols, the presence of the 2,3-double bond increases the planarity of the molecule. This double bond in the C-ring confers higher rigidity to the ring and holds the A and C rings in a more coplanar position, allowing the 3-OH/4-oxo groups and 5-OH/4-oxo groups to be closer. At pH 7.4 and pH 5.5, all flavones studied appear to chelate Cu^{2+} at the same site, probably between the 5-hydroxyl and the 4-oxo groups (not between the 3-hydroxyl group and the 4-oxo group), this probably being associated with the stability of the six-membered ring over that of the five-membered ring complexes [157]. Myricetin and quercetin, however, at pH 7.4, appear to chelate Cu^{2+} additionally at the *o*-catechol group, which is also the chelating site for catechin with Cu^{2+} at pH 7.4. Chelation studies of Fe^{3+} at pH 5.5 proved that only myricetin and quercetin interact strongly with Fe^{3+}, the complexation probably occurring again between the 5-hydroxyl and the 4-oxo groups. The metal chelating properties of flavonoids suggest that they may play a role in metal-overload diseases and in all oxidative stress conditions involving a transition metal ion.

Flavonoids have also been reported to show pro-oxidant effects, which have been related with their iron and copper reducing activities. These reduced metals can catalyse the production of $HO^{\cdot}$ through Fenton reaction [141-144] and lipid radicals through the decomposition of preformed lipid hydroperoxides [146,158]. Nevertheless, metal-chelating flavonoids remove "free" metal ions and can alter their redox potentials rendering those ions inactive in generating free radicals. Furthermore, if the metal-complex still undergoes redox reactions, the free radicals generated can be scavenged by the flavonoid itself.

3.1.6. Antioxidant Potential In Vivo

As it was previously described, flavonoids are extensively metabolized, *i.e.*, they undergo several chemical modifications in the gastrointestinal tract and in the liver and their metabolites are the potential bioactive *in vivo* forms. This point is crucial for the final therapeutic effect, since the modifications on the flavonoid structure can change their biological activity, including their redox potential.

In a recent study [36], after quercetin had been orally administrated to rats in a single dose, quercetin metabolites where identified and evaluated for their structure-antioxidant activity relationships. Major plasma metabolites were glucuronides and sulfoglucuronides and the most plausible positions for glucuronidation and sulfation were the hydroxyl groups located at positions 5 and 7, excluding the *o*-catechol group (3',4'-OH). These quercetin metabolites possessed antioxidant activity, since plasma antioxidant status was significantly higher in animals to which quercetin was administrated. These results suggest that quercetin metabolites can retain some antioxidant activity when the *o*-catechol group is not involved in conjugation reactions. Following this work, the same authors developed a more realistic approach to study *in vivo* quercetin metabolites through its administration under a low dose oral regime [60]. A completely different metabolic pattern was observed, 3'-methylquercetin (isorhamnetin) glucuronide sulfate conjugates being the major plasma metabolites, with most plausible conjugation positions at 3-OH, 5-OH, and 7-OH groups. Isorhamnetin conjugates are metabolites where the *o*-catechol group is methylated at 3'-OH, then diminishing quercetin antioxidant activity and, consequently, contributing weakly to the antioxidant potential of plasma. The comparison of the data obtained in both studies allows establishing structure–antioxidant activity relationships for quercetin metabolites, the antioxidant activity of which decreases significantly when the *o*-catechol group undergoes conjugation reactions. These data also emphasize how important quercetin administration mode is to metabolic patterns, to which different antioxidant profiles may correspond.

It has been suggested that the antioxidant protection of flavonoids and other phenolic compounds may occur before absorption, *i.e.*, within the stomach, intestines and colon, playing an important role in protecting the gastrointestinal tract itself from oxidative damage, and in delaying the development of stomach, colon and rectal cancer [159]. In fact, gastrointestinal tract is constantly exposed to reactive species generated by the gastrointestinal tract itself (*e.g.* $O_2^{\cdot-}$ and H_2O_2 produced by epithelial NADPH oxidases) or by chemical reactions of dietary components (iron, ascorbate, haem proteins, lipid peroxides and aldehydes, and nitrite) in the stomach [160].

On the other hand, it must be pointed out that the major part of ingested flavonoids is largely degraded by the intestinal microbiota, whose bacterial enzymes catalyse several reactions including cleavage of the heterocyclic C-ring. The resulting phenolic acids may account for a large fraction of the ingested flavonoids (30-60%) and, taking into account the fact that the antioxidant action is especially related to the catechol structure, only those with unconjugated catechol moiety may contribute to the plasma antioxidant potential [161]. This is consistent with a study where it was shown that chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized by colonic microbiota, which converts most of these dietary phenols into metabolites with lower antioxidant activity than their parent compounds [162].

Finally, it is important to note that the position at which conjugation occurs is also significant for the pro-oxidant capacity of the flavonoids which seems to be directly proportional to the total number of hydroxyl groups. Similarly to the antioxidant activity, the pro-oxidant behaviour of flavonoids *in vivo* may be reduced by conjugation and methylation of OH groups, particularly by catechol-*O*-methyltransferases [163].

It is worth mentioning that, independently of the mechanisms underlying the action of flavonoids, the biological effects of flavonoids rely in the activity of their metabolites due to rapid and extensive biotransformation.

3.2. Effect of Flavonoids on the Production of Pro-Inflammatory Mediators and on the Expression of Pro-Inflammatory Genes

The anti-inflammatory properties of flavonoids may be attributed not only to their antioxidant activity, but also to their interactions with several key enzymes, signaling cascades involving cytokines and regulatory transcription factors, and on the expression of pro-inflammatory genes.

It is presently known that flavonoids modulate the enzyme activities of AA metabolizing cascade. This polyunsaturated fatty acid is a second-messenger molecule that is released by phospholipase A_2 (PLA_2) in stimulated cells and further metabolized by the cyclooxygenase (COX) and lipoxygenase (LOX) pathways in different eicosanoids. The inhibition of these enzymes reduces the production of prostaglandins (PGs), and leukotrienes (LTs) in diverse cells and thromboxane A_2 in platelets.

It has to be noted that, a significant role has been attributed to ROS and RNS in the regulation of intracellular signalling pathways in leucocytes and consequently in the regulation of the immune and inflammatory responses [86]. ROS and RNS act as modulators of protein and lipid kinases and phosphatases, membrane receptors, ion channels, and transcription factors, including nuclear factor-κB (NF-κB), which regulate the expression of key cytokines [86]. Furthermore, iNOS are highly expressed by inflammatory stimuli in certain inflammatory cells. Several studies have been performed to identify flavonoids that could reduce the ˙NO production by these isoenzymes. However, these compouds must not affect the constitutive enzymes, endothelial NOS (eNOS) and neuronal NOS (nNOS) as ˙NO production is crucial to maintain homeostasis. Nevertheless, most flavonoids are not described as being efficient iNOS inhibitors and instead they can down-regulate iNOS expression [164].

During inflammation, the expression and activation of NADPH oxidase in mast cells, macrophages, eosinophils, and neutrophils also plays an important role in healing inflammation, though its uncontrolled activity may turn out to become deleterious. Thus, the putative inhibition of NADPH oxidase by flavonoids may constitute an advantage, although such an effect must be dealt with the awareness that NADPH oxidase expressed at other tissues, namely in the cardiovascular system, may also be affected by flavonoids [165].

In the present section, the effect of flavonoids on cytokines production, AA metabolizing enzymes PLA_2, COXs, LOXs, as well as on NADPH oxidase activity will be focused. A description on the modulation of pro-inflammatory gene expression and on the modulation of peroxissome proliferator activated receptors will also be given. Bearing in mind that this subject is not exhausted in the present review, we hope to contribute for an extended and fruitful discussion on the anti-inflammatory role of flavonoids.

3.2.1. Effects on Cytokines Production

Interleukin-1 (IL-1) and TNF-α are two important proinflammatory cytokines, with a prominent role in chronic inflammatory disorders. The administration of their antagonists, such as IL-1ra (IL-1 receptor antagonist), soluble fragment of IL-1 receptor, monoclonal antibodies to TNF-α, and soluble TNF receptor, has been shown to improve the symptoms of rheumatoid arthritis [166,167].

Certain anti-inflammatory drugs exert their effects by interfering with the secretion of pro-inflammatory cytokines. Indeed, steroidal anti-inflammatory drugs (SAIDs) such as prednisolone and dexamethasone are known to reduce the production of TNF-α and IL-1β [164]. Several flavonoids have also shown to be capable of

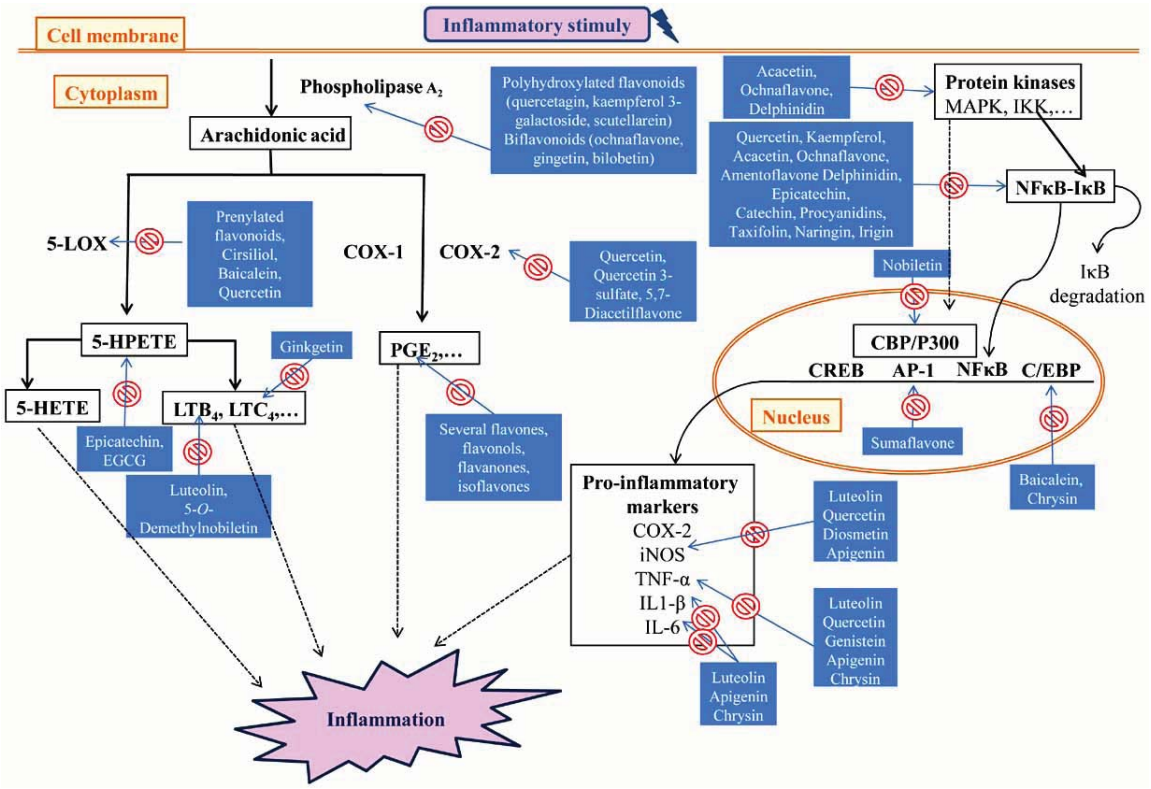


Fig. (8). Flavonoids targets in the modulation of the inflammatory response. Inflammatory stimuli induce inflammation by different pathways. Arachidonic acid is released by PLA_2 and further metabolized by the COX and LOX pathways in different eicosanoids some of which being responsible for inflammatory responses *e.g.,* PGE_2, 5-HETE, LTB_4 and LTC_4. On the other hand, protein kinases regulate transcription factors such as CREB, AP-1, NF-κB, and C/EBP that modulate the expression of pro-inflammatory markers such as COX-2, iNOS, TNF-α, IL-1β, and IL-6.

inhibiting the production of cytokines in different cell types (previously reviewed in [164] and [168]) (Fig. (**8**)).

Recently, flavonoids from different classes, with prominent presence in human diet (luteolin, quercetin and genistein), were shown to inhibit TNF-α secretion in a concentration-dependent manner on lipopolysaccharide (LPS)-induced mouse macrophage activation [169]. Accordingly, genistein was previously found to reduce TNF-α production in human mononuclear cells and peripheral blood leukocytes [170]. Comalada *et al.* [169] found that flavonoids with 3',4'-dihydroxyl substituents showed the highest degree of inhibition of TNF-α secretion, followed by those with only one hydroxyl group on the B-ring, regardless of the presence of a double bond in C_2–C_3, 3-hydroxylation or iso position of the B-ring. Absence of hydroxyl groups in the B-ring virtually abolishes this inhibitory activity [169]. In a previous study [171], however, in which various flavones, flavonols and one flavanone were tested for their ability to inhibit TNF-α, IL-1β, and IL-6 production, in LPS-stimulated peripheral blood mononuclear cells (PBMC), the hydroxylation pattern of the B-ring seemed not to be decisive for the inhibitory effect. Indeed, flavonoids apigenin and chrysin showed to be more effective than quercetin or kaempferol. The fact that different cells had been used in the present and the previous study may justify the contradictory results in what concerns to inhibition of TNF-α secretion. However, the flavonoid-dependent decrease in the levels of cytokines observed in LPS-stimulated peripheral PBMC [171] might be due to cytotoxic effects, since the most active compounds provoked a reduction in the metabolic activity of PBMC and specifically eliminated monocytes. Still, the *in vivo* relevance of the observed cytotoxic effects needs further clarification.

3.2.2. Effects on Arachidonic Acid Metabolism

3.2.2.1. Inhibition of Phospholipase A_2

Three major classes of PLA_2 were found in mammalian systems, secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), and calcium independent PLA_2 ($iPLA_2$). The sPLA2-IIA and cPLA2α subtypes of these classes are involved in the inflammatory process, which makes them suitable targets for anti-inflammatory therapy [172].

Several polyhydroxylated flavonoids, especially, quercetagetin, kaempferol-3-galactoside, and scutellarein were shown to be potent inhibitors of $sPLA_2$-IIA [173]. Additionally, this PLA_2 isoform was efficiently inhibited by some biflavonoids, such as ochnaflavone, ginkgetin and bilobetin [174,175] (Fig. (**8**)).

3.2.2.2. Inhibition of Cyclooxygenases

In mammalian cells, COXs exist in at least two isoforms (COX-1 and COX-2). COX-1 is a constitutive enzyme, expressed in almost every cell type, responsible for the physiological production of PGs in diverse organs and thromboxane A_2 (TXA_2) in platelets. COX-2 is highly expressed in the inflammation-related cell types including macrophages and mast cells when stimulated by LPS, phorbol esters, cytokines, or growth factors, producing large amounts of PGs, in particular the pro-inflammatory mediators PGE_2 and PGI_2. This isoform is associated with acute and chronic inflammatory disorders [176].

Several studies regarding the effects of flavonoids on COXs activity have been published so far (see [7,164,177] for reviews). Yet, most of them refer only to COX-1. In fact, many of the existing studies were performed before the inducible isoenzyme was known.

Until recently, the capacity to inhibit COX-2 activity had only been detected in prenylated flavonoids, gallated catechins, and some anthocyanidins. Additionally, only wogonin had been referred as a selective COX-2 inhibitor ([164] and references therein). Some new studies focusing on the influence of flavonoids on PGE_2 production by stimulated inflammatory cells have lately emerged (Fig. (**8**)). Takano-Ishikawa *et al.* [178] tested several flavonoids from different classes for the inhibitory effects on LPS-induced PGE_2 production and COX-2 expression in rat peritoneal macrophages. Various flavonoids from the classes of flavones, flavonols, flavanones, and isoflavones were able to inhibit PGE_2 production, while compounds from flavan-3-ol and anthocyanidin groups were ineffective. The most obvious conclusions that can be taken from these results are that the 4-oxo functional group is essential to a high inhibitory activity and that glycosides are less active than their corresponding aglycones, probably due to different cell membrane permeability. For apigenin [178] and luteolin [179], the flavonoid-dependent reduced production of PGE_2 was attributed to the inhibition of COX-2 expression. Nevertheless, some of the tested flavonoids may act by a different mechanism (*e.g.,* inhibition of COX-2 or PLA_2 activity) [178]. Inhibition studies of COX-2 activity by quercetin and its conjugates found in human plasma (quercetin-3-glucuronide, quercetin-3'-sulfate, and 3'-methylquercetin-3-glucuronide) showed that only quercetin and quercetin-3'-sulfate could inhibit COX-2 activity, although the second in a smaller extend [180]. Nevertheless, all the tested compounds were able to reduce COX-2 expression. 5,7-Diacetylflavone, a chrysin derivative, was shown to inhibit COX-2 activity with the same potency of indomethacin, a non-steroidal anti-inflammatory drug (NSAID) widely used in therapeutics [181] (Fig. (**8**)). Based on three-dimensional modelling studies, the authors suggest that there might be a hydrogen bond between the oxygen of the oxo group, at the 7-position of the compound, and the hydroxyl group of Tyr355 in COX-2. Additionally, this compound showed a selectivity of COX-2 over COX-1 by more than 10 fold. Chrysin and its other derivatives were ineffective as COX-2 inhibitors.

Although the role of flavonoids on COX-2 inhibition is far from being conclusive, the evidences of a suppressive effect on COX-2 expression by this group of compounds are constantly increasing (see 3.2.4). It is known that COXs inhibitors show stronger effects in intact cells than against purified enzymes or enzymes originating from broken cells [182]. Thus, there is a great probability that, in many cases, the interference of flavonoids with the production of eicosanoids by COX-2 does not involve direct enzymatic inhibition, but the reduction of the enzyme expression instead.

3.2.2.3. Inhibition of Lipoxygenases

Lipoxygenases produce hydroxy acids and leukotrienes (LTs) from arachidonic acid. From the LOXs present in human tissues, 5- and 12-LOX are those implicated in inflammatory and allergic disorders. 5-LOX is generated in neutrophils and produces 5-hydroxyeicosatetraenoic acid (5-HETE) and various LTs (LTA_4-LTB_4). 5-HETE and LTB_4 are potent chemoattractant mediators of inflammation. LTC_4, LTD_4 and LTE_4, have shown to be essential mediators in asthma pathophysiology. 12-LOX synthesizes 12-HETE, which aggregates platelets and induces the inflammatory response.

Several suggestions on the structure-activity relationships have already been made in what concerns to flavonoids-dependent 5-LOX inhibition. The importance of the C_3-OH structural feature has been controversial [183,184] but the presence of a catecholic B-ring seems to provide a superior 5-LOX inhibitory capacity [185]. The isoprenyl group seems to contribute to the inhibitory effect on 5-LOX activity, since a number of prenylated flavonoids, especially sophoraflavanone G, kenusanone A [186] and artonin E [187] were shown to be potent inhibitors. Some of these compounds were also shown to inhibit 12-LOX, although being less effective with this enzyme than with 5-LOX [186].

In a recent theoretical study, Redrejo-Rodrigues *et al.* [188] referred the planarity of the molecule as a characteristic that favours the lipoxygenase inhibitory capacity of flavonoids. Taking into account that the presence of a C_2-C_3 double bond contributes to the planarity of the molecules, the classes of flavonoids which lack this structural feature (flavanones and flavan-3-ols) might be, generically, less effective than those that include it (flavonols, flavones and isoflavones). Accordingly, some flavones with different patterns of substitution, such as the cirsiliol and its analogues [184,185,189] and baicalein [190,191], as well as the flavonol quercetin [185,186,192-194], can be detached for their high potency in inhibiting 5-LOX (Fig. (**8**)). Likewise, the flavone luteolin almost completely inhibited LTB_4 production in rat peritoneal leukocytes, while its glycosides (probably due to a lower cell membrane permeability) were all less effective [195] and the biflavone ginkgetin was able to efficiently suppress LTC_4 biosynthesis in stimulated bone marrow-derived mast cells [196]. The flavan-3-ols epicatechin and EGCG were shown to reduce the formation of the human 5-LOX product 5-hydroperoxyeicosatetraenoic acid (5-HPETE) [193]. These compounds, however, when esterified by gallic acid, which possesses a pyrogallol group, were shown to be potent and selective inhibitors of human platelet 12-LOX.

Recently, the polymethoxyflavone 5-*O*-demethylnobiletein was shown to inhibit the synthesis of 5-LOX product LTB_4 (Fig. (**8**)). This compound is structurally related to cirsiliol, although lacking the catechol group on the B-ring, suggesting that this molecular feature is not essential for the activity of cirsiliol analogous compounds, contrarily to what has been stated before [197]. Therefore the planarity of the molecules conferred by the C_2-C_3 double bond may be the structural feature that contributes most to the LOX inhibitory capacity of flavonoids.

3.2.3. Inhibition of NADPH Oxidase

The activity of NADPH oxidase and consequent production of $O_2^{\cdot-}$ may be reduced by decreasing its expression, by preventing translocation of cytosolic subunits into membrane and by direct inhibition of the activated enzyme. Flavonoids have been shown to act on these three pathways. In fact, the expression of the NADPH oxidase subunit $p22^{phox}$ in aortic endothelial cells from stroke-prone spontaneously hypertensive rats was inhibited by genistein [198], and the translocation of cytosolic subunits into membrane was demonstrated for (−)-epigallocatechin gallate [199]. Noteworthy, was the structure-activity relationship of flavonoids as NADPH oxidase inhibitors, as disclosed by Steffen and co-workers [165]: (i) flavonoids containing an unsubstituted catechol B-ring (epicatechin, catechin, quercetin, luteolin, fisetin) do not inhibit NADPH oxidase; (ii) *o*-methylation of the catechol arrangement in the B-ring (cf. isorhamnetin *vs*. quercetin; diosmetin *vs* luteolin; 3'-*O*-methylepicatechin *vs* epicatechin) or omission of one OH group (cf. kaempferol *vs* quercetin) or an additional vicinal OH group (cf. epigallocatechin *vs* epicatechin) converts the flavonoid to an NADPH oxidase inhibitor; (iii) among flavonoids of non-catechol-type, those possessing a 4'-OH group at the B-ring are NADPH oxidase inhibitors (cf. apigenin and kaempferol *vs* chrysin); (iv) hydrogenation of the C_2–C_3 double bond in the C-ring reinforces the NADPH oxidase-inhibitory potency (cf. taxifolin *vs* quercetin; dihydrokaempferol *vs* kaempferol; dihydrotamarixetin *vs* tamarixetin as well as activities of the flavanones naringenin and hesperetin); (v) the presence of a vicinal hydroxy-methoxy arrangement at an aromatic ring generally enhances the inhibitory potency, but is not essential for the inhibitory capacity (cf. inhibitory potencies of kaempferol, apigenin, procyanidin B2, resveratrol, tyrosol and hydroxytyrosol).

3.2.4. Modulation of Pro-Inflammatory Gene Expression

The role of flavonoids on the modulation of COX-2 and iNOS expression is becoming evident from the several studies that have recently emerged [164 and references therein]. In the COX-2 gene,

four transcription factors have been identified to bind the *cis*-acting elements in the promoter region and regulate COX-2 transcription, namely NF-κB, CCAAT/enhancer-binding protein (C/EBP), activator protein 1 (AP-1), and CRE-binding protein (CREB) [200]. On the other hand, iNOS gene expression is also regulated by transcription factors like NF-κB, AP-1, and C/EBP, which bind to several homologous consensus sequences in the promoter region [201]. Transcription co-activators, including CREB-binding protein (CBP) and its homologue p300 were shown to potentiate the transcriptional activities of AP-1, NF-κB, and CREB [202]. Furthermore, protein kinases including protein kinase B or Akt kinase and mitogen-activated protein kinases (MAPKs), *i.e.* extracellular signal related kinase (ERK), p38 MAPK and Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) are involved in the regulation of transcription factors and in the expression of pro-inflammatory enzymes such as COX-2 and iNOS [202,203]. Thus, the expression of these enzymes can be directly or indirectly inhibited by different mechanisms involving one or more of the aforementioned pathways.

NF-κB is one of the most important inducible transcription factors whose modulation triggers a cascade of signaling events, some of which are potential key targets for intervention in the treatment of inflammatory conditions. A number of pro-inflammatory stimuli (cytokines such as TNF-α or IL-1, oxidative stress, infectious agents) can activate NF-κB in different cell types. NF-κB can in turn activate the expression of many mediators of the inflammation including, cytokines, enzymes, chemokines, and adhesion molecules. In resting cells, NF-κB, which is composed mainly of two proteins, p50 and p65, is present within the cytoplasm in an inactive state, bound to its inhibitory protein, IκB (α or β). However, an inflammatory stimulation initiates an intracellular signaling cascade leading to IκB phosphorylation by IκB kinase (IKK), with its subsequent dissociation from NF-κB. Once liberated from its inhibitory protein, NF-κB translocates to the nucleus, where it orchestrates the transcription of a number of pro-inflammatory genes by binding to κB motifs found in the promoter or enhancer region [204]. In the recent years, the influence of flavonoids on NF-κB pathway has been deeply explored (see [164,177,205-207] for reviews).

Several studies were performed concerning the effects of flavonoids on protein expression of iNOS and COX-2 through mechanisms interfering with NF-κB activation. Quercetin and kaempferol were shown to inhibit iNOS and COX-2 as well as C reactive protein expression in Chang liver cells, apparently, *via* mechanisms involving blockade of NF-κB activation [208]. Results from this study indicate that both quercetin and kaempferol decrease IκBα degradation by inhibiting upregulation of members of the IKK complex. In a recent study using LPS-activated macrophages and flavonoids from different classes, a good correlation was found between the inhibition of iNOS expression and the interference with NF-κB pathway [169]. The most active compounds were quercetin, apigenin, luteolin and diosmetin, which downregulated iNOS expression more efficiently than kaempferol and chrysin. The assumptions made by the authors on the structure-activity relationships corroborate what has been previously stated [209], *i.e.*, the C_2–C_3 double bond plays a determinant role in the iNOS inhibitory effect and flavones appear to be more active than flavonols. Additionally, Comalada *et al.* [169] considered that the position of the B-ring is important to the grade of activity, since isoflavones were shown to be essentially ineffective, contrarily to flavones. Apparently, the inhibitory effect is improved when the molecule has a high number of hydroxyl substituents.

Compounds from minor classes of flavonoids such as, flavan-3-ols, flavanones, and isoflavones were also shown to modulate NF-κB signaling pathway. Hence, epicatechin, catechin, and dimeric flavan-3-ols (procyanidins) were able to inhibit phorbol myristate acetate-induced NF-κB activation at multiple steps in specific experimental settings [210]. These compounds inhibited NF-κB-DNA binding activity, reduced intracellular oxidants, an important event in the initiation of NF-κB activation, and prevented the phosphorylation of IκB, avoiding the release of activated NF-κB. On the other hand, the wide range of anti-inflammatory effects shown by taxifolin [211], results, at least in part, from the reduced activation of NF-κB caused by this flavonoid. The glycosylated flavanone naringin was able to significantly prevent the LPS-induced endotoxin shock in mice and the production of ·NO in RAW 264.7 macrophage cells. Again, this may be related to naringin's inhibitory effect on the activation of NF-κB [212]. The inhibition of LPS-induced mRNA and protein expression of iNOS and COX-2 by the isoflavone irigenin is likely to be related to the reduction of NF-κB activation [213] (Fig. (**8**)).

Other mechanisms of inhibition of COX-2 and iNOS expression beyond the NF-κB pathway have been identified for flavonoids (Fig. (**8**)). Woo *et al.* [214] proposed that the baicalein-dependent reduction of COX-2 gene expression, in LPS-stimulated macrophages, was mediated by the inhibitory effect of this flavone on the transcription factor C/EBPβ binding activity. Chrysin, also a flavone, was able to reduce COX-2 gene expression probably by inhibiting the binding and activation of another member of the C/EBP transcription factor family, NF-IL6. Acacetin was able to inhibit LPS-stimulated expression of COX-2 and iNOS in RAW 264.7 macrophage cells by interfering in the activation of phosphoinositide-3 kinase/Akt, p44/42 MAPK, the degradation and phosphorylation of IκBα, and the translocation of NFκB. Interestingly, acacetin was shown to be more potent than the intensively studied wogonin, demonstrating that the position of methoxyl on the B-ring strongly influences the conformation of the molecule and modulates its inhibitory effect [215]. The inhibition of transcription co-activators seems to be involved in the suppressive effect of nobiletin on COX-2 protein production [202]. This flavone was thought to disrupt the binding of CBP and p300 since it suppressed NF-κB, AP-1, and CREB activation, without affecting MAPK and Akt-NF-κB pathways. Delphinidin suppressed LPS-induced COX-2 expression by blocking MAPK pathways (JNK, ERK and p38 kinase) with the attendant inhibition of NF-κB, C/EBPα, and AP-1 activation. It is noteworthy that the number of hydroxyl groups on the B-ring might be associated with the effects of anthocyanidins. Compounds that contain a single hydroxyl group on the B-ring such as pelargonidin, peonidin and malvidin showed no inhibitory effect. Cyanidin, with two hydroxyl groups on the B-ring, showed stronger inhibition. Delphinidin, which contains three hydroxyl groups on the B-ring, exhibited the strongest inhibition of COX-2 expression [200]. Recent studies mention the ability of certain biflavonoids to downregulate iNOS and clarified their mechanisms. Hence, ochnaflavone was shown to efficiently inhibit LPS-induced ·NO production in mouse RAW 264.7 macrophage cells, with a concentration-dependent inhibitory effect on iNOS mRNA and protein expression. Additionally, the authors concluded that ochnaflavone exerts its inhibitory effect on iNOS induction by affecting different pathways, being capable of interfering with ERK, p38 kinase, JNK, NF-κB, and AP-1 [203]. On the other hand, the biflavonoid amentoflavone was shown to selectively inhibit NF-κB activation by LPS treatment [201]. According to the authors, the phosphorylation and the subsequent degradation step of IκBα is a pharmacological target of this compound. Conversely, MAPKs and AP-1 are unlikely to be responsible for the suppressive effect of amentoflavone on iNOS induction. Despite the structural similarity of sumaflavone and amentoflavone, only differing by the additional existence of a hydroxyl group at $C_{6''}$ in sumaflavone, the mechanism by which these compounds exert their blocking effects on LPS-dependent iNOS induction is, apparently, different. In a study performed by Yang *et al.* [216], sumaflavone was shown to suppress AP-1 activation but it was unable to affect NF-κB activation by LPS.

3.2.5. Modulation of Peroxissome Proliferator Activated Receptors

Peroxissome proliferator activated receptors (PPARs) are nuclear hormone receptors that are activated by specific endogenous and exogenous ligands. Once activated, PPARs bind to specific response elements as heterodimers with the retinoid X factor and exert regulatory functions. Some NSAIDs are able to activate PPARγ isoforms. Additionally, PPARγ ligands have been shown to hinder the production of pro-inflammatory cytokines and also to inhibit iNOS expression partially by antagonizing the activities of transcription factors such as AP-1 and NF-kB [217,218]. Thus, PPAR activation is one possible way for achieving anti-inflammatory effects.

The biflavonoid amentoflavone was shown to up-regulate PPARγ expression in TNF-α-activated A549 human lung epithelial cells, which might be the reason for the observed inhibition of NF-κB pathway, resulting in COX-2 reduced expression [219]. Liang *et al.* [220] studied the putative activation of PPARγ by various flavonoids. Flavanones and flavan-3-ols were inefficient in activating PPARγ, indicating that C_2-C_3 double bond of the C-ring was essential for this effect. The flavones, flavonols, and isoflavones were able to activate PPARγ, but this activation was dependent on the hydroxyl substituents at positions 5 and 7 in the A-ring and 4' in the B-ring such as in kaempferol, chrysin and apigenin. These compounds were also shown to inhibit COX-2 and iNOS promoters' activities in LPS-stimulated macrophages partially through PPARγpathways. The presence of the 3'-OH group on the B-ring resulted in a decreased PPARγ activation such as in luteolin and quercetin.

3.3. *In Vivo* Anti-Inflammatory Activity of Flavonoids

Animal models of inflammation have been widely used to test potential anti-inflammatory drugs. In the last two decades (particularly since the mid 90s) several flavonoids have been tested in this kind of experiments providing, in some cases, promising results. In this section studies regarding *in vivo* anti-inflammatory effects of flavonoids, using different experimental designs, summarized in Table **2**, are described. The most widely used models are the adjuvant-induced arthritis (collagen-, cell wall-, carrageenan-induced arthritis) in rats or ear edema in mouse. All of these models share key features related to human inflammation that make them critical tools in drug development. The onset of ankle/ear swelling, concomitantly with other biomarkers (protein and molecular mediators) is a robust marker able to monitor the course of the disease allowing the assessment of therapeutic agent progress.

A large range of flavonoids have been studied, quercetin and their derivatives in particular. Indeed, quercetin is the most common flavonoid in human diet and is present in high concentrations in various fruits and vegetables.

Quercetin-3,7-*O*-alpha-dirhamnoside and kaempferol-3,7-*O*-alpha-dirhamnoside two main flavonoid glycosides isolated from the leaves of *Tilia argentea*, where shown to possess potent anti-inflammatory activity in the mice carrageenan-induced hind paw edema model when orally administrated in a 50 mg/Kg dose, without inducing any apparent acute toxicity or gastric damage [221]. In a subsequent work, some of the authors of the last study observed that 3-*O*-methylquercetin (147.1 mg/Kg), 3,7-*O*-dimethylquercetin (113.6 mg/Kg), and 3,7-*O*-dimethylkaempferol (98.1 mg/Kg), the main active ingredients from the ethanol extract of *Cistus laurifolius*, possessed anti-inflammatory effect when tested by the aforementioned inflammation model. These compounds were active in both earlier and delayed stages of inflammation and their potency was found to be equal to the NSAID indomethacin (10 mg/Kg), without inducing any apparent acute toxic or gastric damage [222]. Oral administration of other quercetin glycoside, rutin (100 mg/Kg), reduced significantly the carrageenan-induced rat paw edema [223]. Moreover, this quercetin glycoside reduced significantly, in a dose-dependent manner, the polymorphonuclear neutrophils chemotaxis to fMet-Leu-Phe. Furthermore, elastase exocytosis, induced by phorbol myristate 13-acetate or fMet-Leu-Phe, was partially inhibited by rutin up to 25 μM. These authors suggest that rutin may exert its anti-inflammatory effect by inhibiting enzyme(s) involved in neutrophils response.

Quercetin (10 mg/Kg) was shown to reduce the carrageenan-induced acute inflammation in the rat air pouch model, when locally injected in the site of inflammation [224]. Additionally, there has also been a reduction on the levels of inflammatory mediators such as TNF-α, mRNA for COX-2, PGE_2, and chemoattractant chemokines in the quercetin-treated animals. Of note, quercetin was effective when administered simultaneously with or one hour after the carrageenan challenge, which suggests that it may prevent both early and late phase of the inflammatory response. Two quercetin related compounds, isoquercetin and rutin, were also evaluated in this study. Both compounds displayed similar level of suppressive effects with quercetin, indicating no influence of the sugar moiety on the anti-inflammatory effect.

Ueda *et al.* [225] studied the inhibitory effect of some flavonoids on serum TNF-α production and on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema, when orally administrated to mice (1 mg/mouse x 2). Only quercetin and luteolin were able to inhibit the TPA-induced ear edema. Additionally, luteolin and apigenin where shown to inhibit the systemic TNF-α production, while the LPS-induced TNF-α production from macrophages was also inhibited by quercetin, chrysin and myricetin. This proves that compounds showing *in vitro* activity are not necessarily effective *in vivo*, particularly when the oral route is chosen due to bioavailability-related issues. Noticeably, orally administrated luteolin was previously shown to inhibit inflammation (TPA- and AA-induced ear edema) and allergy (oxazolone-induced ear edema) [226].

In a study that included quercetin and rutin, along with the flavonol morin and the flavones hesperidin and hesperetin, animal models of acute and chronic inflammation were used and the flavonoids were administrated by intraperitoneal injection [227]. It was observed that the most active compound in acute inflammation, when studied by the carrageenan-induced paw edema in mice, was quercetin (75 mg/Kg), showing an activity superior to that of phenylbutazone (80 mg/Kg), a NSAID used to relieve rheumatic pain. On the other hand, only the flavanones hesperidin and hesperetin (0.25 M/Kg) showed anti-inflammatory effect on the ear edema induced by xylene, which causes neurogenic inflammation. All of the studied flavonoids were capable of inhibiting the development of cotton pellet induced granuloma, used as a model of proliferative phases of inflammation, morin (25 mg/Kg) being the most effective of all. On the other hand, rutin (75 mg/Kg) was the only significantly active compound in the chronic phase of adjuvant arthritis. This quercetin glycoside was previously shown to inhibit both acute and chronic phases of this experimental model of inflammation, after intraperitoneal administration of daily doses equivalent to 80 mg/Kg [228]. In the same study, quercetin and hesperidin were also tested, however both of them were found to be less effective than rutin, especially in the chronic phase. The authors proposed that pharmacokinetic factors contribute to the higher activity of rutin over quercetin, since these two compounds only differ in the sugar rutinose in position C_3. Even so, in a recent study [229], quercetin was shown to significantly reduce the severity of adjuvant arthritis in rats, when administrated either by oral route (150 mg/rat) or intracutaneous route (50 mg/rat). Additionally, this flavonol was able to prevent adjuvant arthritis development when intracutaneously injected at a dose of 25 mg/rat. Further *ex vivo* analysis of freshly isolated rat macrophages pointed to quercetin-dependent

lowering of their activation markers (TNF-α and nitrites), suggesting that the macrophage-mediated inflammatory response is a possible target for quercetin *in vivo*.

Inflammatory bowel disease is a chronic disease of the digestive tract that refers mainly to ulcerative colitis and Crohn's disease. Currently, trinitrobenzene sulfonic acid (TNBS) - and dextran sulfate sodium (DSS)-induced colitis animal models are the most widely used for the study of inflammatory bowel disease [230]. Comalada *et al.* [231], verified that 1 mg/Kg daily oral doses of quercitrin reduced the inflammatory process in a DSS-induced colitic rat model. The results from experiments using colon homogenates from non-colitic, colitic untreated and colitic treated rats evidenced that this flavonoid significantly reduced colonic MPO activity, an enzymatic marker of neutrophil infiltration; almost completely inhibited the production of the pro-inflammatory cytokines TNF-α and IL-1β; inhibited the iNOS expression; and down-regulated the activated NF-κB molecules. When quercetin was used in the same experiments, no anti-inflammatory activity was verified. However, this compound has already shown to be active when tested by *in vitro* assays. The authors justify these paradoxical results on the basis of the bioavailability of flavonoids. Flavonoid aglycones, like quercetin, can be absorbed in the small intestine, preventing them from a local beneficial effect while the glycosides, like quercitrin, are not well absorbed in the upper segments of the gastrointestinal tract, thus reaching the colon, where they should be hydrolysed by the intestinal microbiota releasing the active aglycone. This may also be the explanation for the results obtained in a similar study [230] where rutin, a quercetin glycoside, was shown to attenuate the DSS-induced colitis in mice, in opposition to quercetin, which shown no effect. In this study, rutin (0.1 % included in the diet) was found to decrease the pro-inflammatory gene expression, especially that of IL-1β and IL-6. Additionally, this compound was shown to be able to ameliorate the colitis not only in a prophylactic protocol but also in a therapeutic protocol by reversing the pathology. Moreover, rutin had previously shown prevention in colonic damage (acute phase) and facilitation in colonic healing (chronic phase) in TNBS-induced rat colitis [232]. Galvéz *et al.* [233] assessed the effect of morin, a flavonol aglycone, in the late animal model. Daily oral 25 mg/Kg doses of morin facilitated the recovery of the damaged tissue in the chronic phase of the disease. Additionally, this flavonol reduced the production of some mediators involved in the inflammatory response of the intestine, such as free radicals, LTB_4, ·NO, and IL-1β.

Sala *et al.* [234], used different *in vitro* and *in vivo* inflammation models to evaluate the anti-inflammatory activity of three naturally occurring flavonoids: gnaphaliin, a methoxyflavone; pinocembrin, a flavanone; and tiliroside, a flavonol acyl-glycoside. Pinocembrin was shown to be the most active of the tested compounds in inhibiting TPA-induced ear edema, providing an ID_{50} lower than indomethacin (61 μg/ear vs 125 μg/ear). However, in the chronic model only tiliroside significantly reduced the TPA-induced ear edema at the dose assayed (0.5 mg/ear). The PLA_2-induced paw edema in mouse was most visibly diminished by tiliroside (ID_{50}=35.6 mg/Kg), although the other tested compounds were also effective.

The polymethoxyflavone 5-*O*-demethylnobiletein, when orally administrated in 100 mg/Kg doses, was capable of reducing, in some stages, the paw edema induced by carrageenan and PLA_2. Furthermore, a topical dose of this compound (0.25 mg/Kg) reduced the subchronic inflammation induced by repeated application of TPA to mouse ears, with additional inhibition of neutrophil infiltration. The authors point the modification of 5-LOX activity as a possible mechanism of the anti-inflammatory effects of 5-*O*-demethylnobiletein [197].

Studies concerning myricetin-3-*O*-β-D-glucoronide, the active principle of the medicinal plant *Epilobium augustifolium L.*, synthesized by Hiermann *et al.* [235] were performed in order to evaluate its anti-inflammatory activity. The aforementioned flavonol glycoside, when orally administrated, exhibited potent anti-inflammatory activity in the acute (carrageenan-induced paw edema) and chronic (adjuvant arthritis) rat models of inflammation, in a dose-dependent manner. Furthermore, for equipotent anti-inflammatory effects, myricetin-3-*O*-β-D-glucoronide was dosed much lower than the NSAID indomethacin, showing no ulcerogenic effect contrarily to this NSAID. A possible mechanism of the anti-inflammatory activity of this compound is the inhibition of the enzymes in the arachidonic acid pathway, since *in vitro* experiments shown that myricetin-3-*O*-β-D-glucoronide was able to moderately inhibit COX-1, COX-2 and 5-LOX. Recently, Meotti *et al.* [236] studied the effects of myricitrin, again a myricetin glycoside, on the adjuvant arthritis mice model. The intraperitoneal treatment with this flavonoid (30 mg/Kg) produced an anti-allodynic effect, when evaluated using a mechanical stimulus in the hindpaw. Additionally, myricitrin reduced the complete Freund's adjuvant-induced paw edema, MPO activity, and the subcutaneous morphological footpad alterations.

With the purpose of finding new anti-inflammatory agents, Park *et al.* [237] tested six synthetic C-C biflavonoids having different connecting linkages between flavone monomers. The biflavonoid having C_6-C_6 linkage showed a significant anti-inflammatory activity against rat carrageenan-induced paw edema when intraperitoneally administrated in a 5 mg/Kg dose. Additionally, this biflavonoid inhibited COX-2-mediated production of PGE_2 from LPS-treated RAW 264.7 cells, being the most potent among the tested compounds. Furthermore, other compounds such as alkoxyflavonols were shown to be very effective in the reduction of carrageenan-induced paw edema in rats, when administrated by the intraperitoneal route at a dose of 25 mg/Kg [238].

It is difficult to take general conclusions about the structure-activity relationships of flavonoids with basis on the results of different *in vivo* studies with animal models of inflammation, since each model represents a certain type of inflammation, having its own specific characteristics. Furthermore, the administration route that is chosen in a particular experiment will determine the pharmacokinetic of the compounds in study influencing, consequently, their effect. Nevertheless, that kind of assumptions can be made when the compounds are compared in the same conditions. Thus, according to Kim *et al.* [164] the C_2-C_3 double bond, the 5,7-hydroxylation on the A-ring and the 4'-hydroxylation on the B-ring seem to be favourable structural features to the inhibition of TPA-, croton oil-, and AA-induced mouse ear edema. However, the compounds having this kind of molecular structure seem to be good inhibitors when topically administrated but weak inhibitors when orally administrated. On the other hand, from the several intraperitoneally administrated flavanones, flavones and flavonols tested by Pelzer *et al.* [239] those carrying a 3',4'-dihydroxy, 3'-hydroxy-4'-methoxy or 3'-methoxy-4'-hydroxy B-ring were shown to be active in a chronic inflammation model (cotton pellet-induced rat granuloma). The activity of the tested compounds seemed to be independent of the C_2-C_3 double bond, the C_3-OH substitution, as well as its methylation or conjugation with a sugar moiety. Ferrándiz and Alcaraz [184], studied the anti-inflammatory activity of several flavones and flavonols (aglycones and glycosides) using the mouse carrageenan paw edema model. Apparently, the introduction of a hydroxyl group at position 3 caused a reduction in the anti-edematous activity of the flavonoid, while an introduction of a methoxyl group at C_8 had no influence. Glycosides were shown to be more potent than their corresponding aglycones.

In this section, it becomes clear that several flavonoids have potent *in vivo* anti-inflammatory activity, which is, in some cases, similar to that find in drugs used in therapeutics. In addition, those compounds are, apparently, less prone to cause gastric lesions than NSAIDs, the most commonly used anti-inflammatory drugs.

Table 2. Summary of *In Vivo* Anti-Inflammatory Effects of Flavonoids

Coumpound	Animal model	Route of administration/ doses	Reference
Quercetin- and kaempferol-3,7-*O*-alpha-dirhamnoside	Carrageenan- induced paw edema	Oral/ 50 mg/Kg	[221]
3-*O*-methylquercetin 3,7-*O*-dimethylquercetin 3,7-*O*-dimethylkaempferol		Oral/ 147.1 mg/Kg 113.6 mg/Kg 91.8 mg/Kg	[222]
Rutin		Oral/ 100 mg/Kg	[223]
C_6-C_6 biflavone		Intraperitoneal/ 5 mg/Kg	[237]
Several alkoxyflavonoids		Intraperitoneal/ 25 mg/Kg	[238]
Quercetin, isoquercetin, rutin	Carrageenan-induced air pouch	Local injection/ 10 mg/Kg	[224]
Luteolin, quercetin	TPA-induced ear edema	Oral/ 2x1mg/mouse	[225]
Luteolin	TPA- and arachidonic acid- induced ear edema	Oral 1mg/mouse	[226]
Quercetin Hesperetin, hesperidin Morin Rutin	Carrageenan-induced paw edema Xylene-induced ear edema Cotton pellet-induced granuloma Adjuvant arthritis	Intraperitoneal/ 75 mg/Kg IP/ 0.25 M/Kg IP/ 25 mg/Kg IP/ 75 and 80 mg/Kg	[227] [227,228]
Quercetin	Adjuvant arthritis	Intracutaneous/ 25 mg/rat	[229]
Quercitrin	DSS-induced rat colitis	Oral/ 1 mg/Kg	[231]
Rutin		Included in the diet/ 0.1%	[230]
Rutin	TNBS-induced rat colitis	Oral/ 5-25 mg/Kg	[232]
Morin		Oral/ 25 mg/Kg/daily	[233]
5-*O*-demethylnobiletin	Carrageenan- and PLA_2-induced ear edema TPA-induced ear edema	Oral/ 100 mg/Kg Topical/ 0.25 mg/Kg	[197]
Pinocembrin Tiliroside	TPA-induced acute ear edema TPA-induced chronic ear edema PLA_2-induced paw edema	Topical/ 15 to 500 µg/ear Topical (several aplications)/ 0.5 mg/ear Intraperitoneal/ 80 mg/Kg	[234]
Myricetin-3-O-β-D-glucoronide	Carrageenan-induced paw edema Adjuvant arthritis	Oral 1-300 µg/Kg Oral 5-150 µg/Kg	[235]
Myricitrin	Adjuvant arthritis	Intraperitoneal/ 30 mg/Kg	[236]

4. CONCLUSIONS

Disruption of cellular signaling pathways is considered a key factor in pathogenic events involving inflammatory processes. ROS and RNS have long been associated with inflammation, either as triggering elements or as signaling messengers, for its evolution and maintenance. Indeed, changes in the pattern of gene expression through ROS/RNS-sensitive regulatory transcription factors modulate the inflammatory response mediated by cytokines and other inflammatory biomarkers. Therefore, an anti-inflammatory therapy should take into account not only the standard blockage of pro-inflammatory enzymes, but also the re-establishment of cellular homeostasis. The data presented in this review, suggests that this goal may be achieved through the use of flavonoids, which may provide a highly favourable benefit/risk ratio. However, more studies on the structure-biological activity and metabolism must be further rationalised or streamlined. Hence, flavonoids seem to be a promising alternative in the treatment of inflammation, especially in chronic inflammatory diseases in which severe side effects are common due to the long treatment periods.

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ABBREVIATIONS

AA = arachidonic acid
AP-1 = activator protein 1
ATP = adenosine triphosphate
C_{max} = maximum concentration
$cPLA_2$ = cytosolic phospholipase A_2
CBP = CREB-binding protein
C/EBP = CCAAT/enhancer-binding protein
COX = cyclooxygenase
CREB = CRE-binding protein
DNA = deoxyribonucleic acid
DHR123 = dihydrorhodamine 123
DSS = dextran sulfate sodium
EGCG = epigallocatechin gallate
ERK = extracellular signal related kinase
GT = glucuronosyltransferase
HETE = hydroxyeicosatetraenoic acid
$HO^{\cdot}$ = hydroxyl radical
ID_{50} = 50% inhibitory dose

IκB = nuclear factor-κB inhibitory protein
IKK = IκB kinase
IL-1 = Interleukin-1
$iPLA_2$ = calcium independent phospholipase A_2
JNK/SAPK = Jun N-terminal kinase/stress activated protein kinase
LOX = lipoxygenase
LPS = lipopolysaccharide
LT = leukotrienes
MAPK = mitogen-activated protein kinase
mRNA = messenger ribonucleic acid
MPO = myeloperoxidase
iNOS = inducible nitric oxide synthase
NADPH = nicotinamide adenine dinucleotide phosphate
NF-κB = nuclear factor-κB
$^{\cdot}NO$ = nitric oxide
NSAID = non-steroid anti-inflammatory drug
$^1O_2$ = singlet oxygen
$O_2^{\cdot-}$ = superoxide radical
$ONOO^-$ = peroxynitrite anion
PBMC = peripheral blood mononuclear cells
PG = prostaglandin
PPAR = peroxissome proliferator activated receptor
RNS = reactive nitrogen species
ROS = reactive oxygen species
$ROO^{\cdot}$ = peroxyl radical
$sPLA_2$ = secretory phospholipase A_2
TNBS = trinitrobenzene sulfonic acid
TNF-α = tumor necrosis factor-α
TPA = 12-O-tetradecanoylphorbol-13-acetate
UDP-GT = uridine diphosphate-glucuronosyltransferase

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CHAPTER I

GENERAL INTRODUCTION

I.2. General and specific objectives of the dissertation

The general objectives of this dissertation were to evaluate the antioxidant and anti-inflammatory properties of several 2-SC in order to establish structure-activity relationships and to pursuit for potential therapeutic alternatives to the existing anti-inflammatory drugs.

The following specific objectives were established:

- To study the scavenging activity for ROS and RNS of 2-SC;
- To understand the mechanism underlying the scavenging activity of 2-SC through the study of their electrochemical behaviour by cyclic voltammetry;
- To test the inhibitory effect of 2-SC on the activity of the enzymes COX-1 and COX-2 in an *in vitro* non-cellular system and on the production of LTB_4 by human PMNL;
- To study the effect of 2-SC on the activation of NF-κB and on the production of pro-inflammatory cytokines in a human monocytic cell culture (THP-1).

CHAPTER II

ORIGINAL RESEARCH

II.1. 2-STYRYLCHROMONES: NOVEL STRONG SCAVENGERS OF REACTIVE OXYGEN AND NITROGEN SPECIES

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2-Styrylchromones: Novel strong scavengers of reactive oxygen and nitrogen species

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Abstract—2-Styrylchromones are a small group of naturally occurring chromones, vinylogues of flavones (2-phenylchromones). Natural and synthetic 2-styrylchromones have been tested in different biological systems, showing activities with potential therapeutic applications. In particular, the potential and hitherto understudied antioxidant behavior of these compounds has been raised as a matter of interest. Thus the present work consisted in the study of the in vitro scavenging activities for reactive oxygen species (ROS) and reactive nitrogen species (RNS) of various 2-styrylchromone derivatives and structurally similar flavonoids. Some of the studied 2-styrylchromones proved to be extremely efficient scavengers of the different ROS and RNS, showing, in some cases, IC_{50}s under 1 μM. The hydroxylation pattern of 2-styrylchromones, especially in the B-ring but also in the A ring, modulates the activity of these compounds, the catecholic derivatives being the most effective scavengers. The styryl pattern also contributes to their observed outstanding antioxidant activity. In conclusion, the scavenging activities for ROS/RNS of 2-styrylchromone derivatives, here shown for the first time, provide novel and most promising compounds to be applied as antioxidants.

1. Introduction

2-Styrylchromones are a small group of naturally occurring chromones, vinylogues of flavones (2-phenylchromones). Several analogues of these compounds have been synthesized and tested in different biological systems, showing different activities with potential therapeutic application, even before the first isolation of natural 2-styrylchromones from the green algae *Chrysophaem taylori* in 1986.[1,2] The natural derivatives were demonstrated to possess cytotoxic activity against leukemia cells,[1,2] and those obtained by synthesis exhibited antiallergic,[3] antiviral,[4] antitumor,[5] antagonism of A3 adenosine receptor,[6] xanthine oxidase inhibitor,[7] hepatoprotective against pro-oxidant agents,[8] and antioxidant properties.[9] The antioxidant behavior of these compounds is a matter of particular interest, as previously demonstrated by their strong protective effects against pro-oxidant agents observed in cellular systems[8] and in non-cellular systems.[9] The main contributing factor to the oxidative stress-related pathophysiologies is the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS). A good example of endogenous overgeneration of ROS and RNS occurs during inflammatory processes. Phagocytosis, which occurs during inflammation in a variety of cells, namely monocytes, neutrophils, eosinophils, and macrophages, is accompanied by a dramatic increase in oxygen consumption (respiratory burst) resulting firstly in the formation of superoxide radical ($O_2^{\cdot -}$) through the activity of a plasma-membrane NADPH oxidase.[10,11] $O_2^{\cdot -}$ produced in vivo is quickly converted to hydrogen peroxide (H_2O_2) spontaneously or by the enzyme superoxide dismutase (SOD).[12] Although being a rather unreactive molecule, H_2O_2 interacts with transition metal ions generating the hydroxyl radical ($HO^{\cdot}$). This is one of the strongest oxidizing agents, producing severe damage in its vicinity and initiating lipid peroxidation due to its well-known reactivity with polyunsaturated fatty acids, which results in the production of peroxyl radical ($ROO^{\cdot}$) and other cytotoxic agents.[12–14] Moreover, the

Keywords: 2-Styrylchromones; Reactive oxygen species; Reactive nitrogen species; Scavenging activity; Antioxidant activity.
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enzyme myeloperoxidase (MPO), a hydrogen peroxide oxidoreductase that is specifically found in mammalian granulocytic leukocytes, including neutrophils, monocytes, basophils, and eosinophils, catalyzes the reaction of chloride ions and H_2O_2 with the formation of hypochlorous acid (HOCl), a powerful bactericidal agent.[15] On the other hand, singlet oxygen ($^1O_2$), a highly reactive form of O_2, can be produced in vivo by a range of peroxidase enzymes (e.g., myeloperoxidase, lactoperoxidase, horseradish peroxidase, and chloroperoxidase) and during lipoxygenase-catalyzed reactions. $^1O_2$ can also be generated in reactions involving ozone and during lipid peroxidation.[16]

Nitric oxide (·NO) is produced in different cell types by a family of isoenzymes termed nitric oxide synthases (NOS) and is involved in various physiological functions. Using again the inflammatory process as example, during the host defense against pathogenic microorganisms, nitric oxide is produced by the inducible NOS existent in activated macrophages and neutrophils. Identified as a cytotoxic factor, ·NO produced in these conditions is responsible for immunity. Additionally it is involved in the pathogenesis of some conditions including inflammation.[17,18] Besides its own pro-inflammatory effects, ·NO can exert its toxicity by generating more destructive reactive species, particularly $ONOO^-$, by reacting with $O_2{\cdot}^-$.[19] Although the ROS and RNS produced by phagocytes represent an extremely important mechanism of host defense, their overproduction may provoke or exacerbate damage in inflammatory sites.[20,21]

Of note, 2-styrylchromones have structural similarities with flavonoids, particularly those belonging to the class of flavones. Flavonoids have been deeply studied and have shown to possess innumerous biological activities from which the antioxidant properties are the best-described.[22–27] Considering the structural similarities, some of these properties are likely to be shared with 2-styrylchromones, although it needs to be experimentally confirmed. Thus, the present work consisted in studying the scavenging activities for the above-referred ROS and RNS of various 2-styrylchromone derivatives (Fig. 1) in order to better understand their antioxidant potentialities. Simultaneously, five flavones and one flavonol (Fig. 1) were used as positive controls. This allowed to take advantage from the existing knowledge about flavonoids and helped to interpret the results. The ultimate aim of this investigation was to look up for structure–activity relationships concerning the scavenging studies and to select the most promising compounds as new antioxidant therapeutic agents.

2. Results

2.1. Superoxide radical scavenging activity

The $O_2{\cdot}^-$-dependent reduction of NBT was prevented by all 2-styrylchromones from group 1, with IC_{50}s varying from 48.9 to 80.4 μM. Compounds **1A** and **1B** were the most effective of the group. Compounds **2A** and **2B** were also able to scavenge $O_2{\cdot}^-$ and their IC_{50}s matched the range of values of group 1 (Table 1). The effect shown by **2D** is only slightly increased along the studied concentration range, reaching only a 32% effect at the highest tested concentration (125 μM). Compound **3A** reached a 27% effect at 31.2 μM, which did not increase

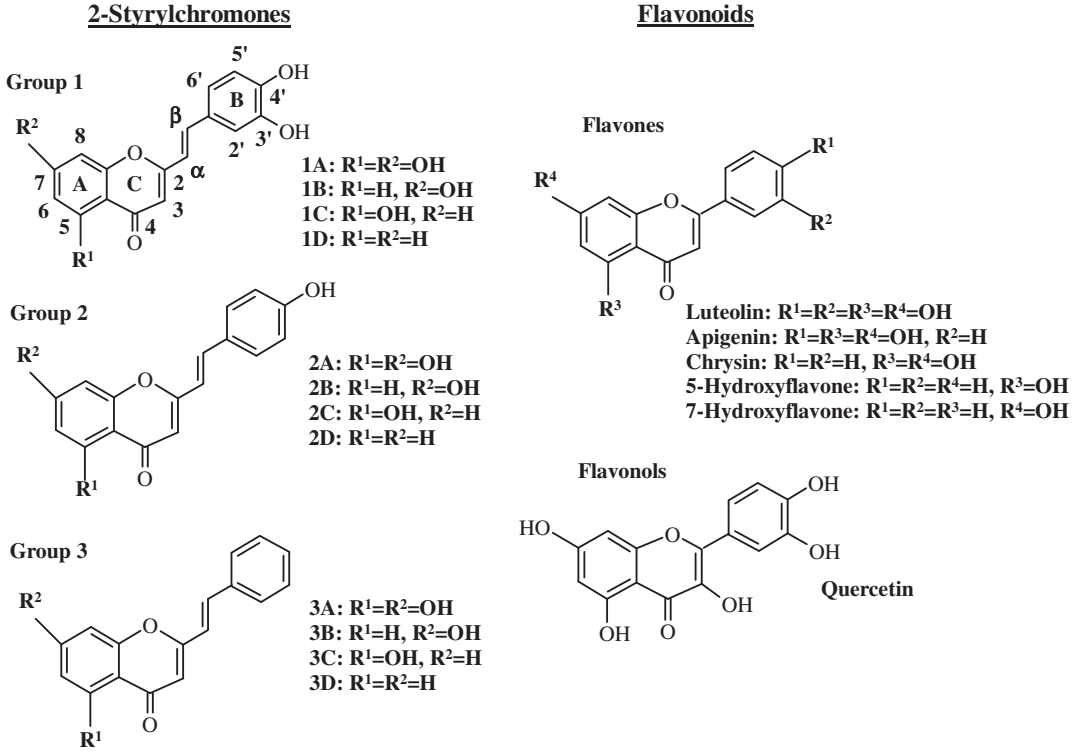

Figure 1. Chemical structures of the studied 2-styrylchromones and flavonoids.

Table 1. $O_2^{\cdot-}$, H_2O_2, HOCl, and $^1O_2$ scavenging activities (IC_{50}, mean ± SE) of the studied 2-styrylchromones, flavonoids, and positive controls

Compound	IC_{50} (μM)			
	$O_2^{\cdot-}$	H_2O_2	HOCl	$^1O_2$
2-Styrylchromones				
1A	**48.9 ± 1.2**	**50.3 ± 7.8**	**3.9 ± 0.2**	**6.5 ± 0.9**
1B	**51.0 ± 1.4**	**114.2 ± 19.1**	**18.0 ± 1.8**	**10.1 ± 1.3**
1C	**80.4 ± 7.1**	**48.9 ± 5.5**	**16.9 ± 1.1**	**4.9 ± 0.6**
1D	**71.3 ± 4.2**	**182.8 ± 3.2**	**32.1 ± 1.5**	**7.9 ± 0.9**
2A	**77.7 ± 5.5**	$36\%^{*250\mu M}$	**8.5 ± 0.3**	**182.8 ± 2.9**
2B	**66.2 ± 6.2**	$NA^{250\mu M}$	**92.0 ± 7.1**	**265.8 ± 27.5**
2C	$NA^{100\mu M}$	$40\%^{*250\mu M}$	$29\%^{*25\mu M}$	$29\%^{*40\mu M}$
2D	$32\%^{*125\mu M}$	$NA^{250\mu M}$	$34\%^{*100\mu M}$	**245.0 ± 6.3**
3A	$27\%^{*125\mu M}$	$NA^{250\mu M}$	**5.7 ± 0.7**	**162.6 ± 10.0**
3B	$40\%^{*125\mu M}$	$NA^{250\mu M}$	**72.3 ± 5.0**	NA^{1mM}
3C	$NA^{125\mu M}$	$NA^{250\mu M}$	$17\%^{*50\mu M}$	$NA^{100\mu M}$
3D	$NA^{125\mu M}$	$NA^{250\mu M}$	$10\%^{*125\mu M}$	$NA^{200\mu M}$
Flavonoids				
Quercetin	**47.3 ± 4.4**	**785.0 ± 45.0**	**1.1 ± 0.1**	**5.3 ± 0.3**
Luteolin	**58.5 ± 4.6**	**900.2 ± 42.1**	**7.1 ± 1.5**	**23.4 ± 2.4**
Apigenin	$35\%^{*100\mu M}$	$NA^{250\mu M}$	**3.9 ± 0.4**	$30\%^{*1mM}$
Chrysin	$NA^{125\mu M}$	$NA^{250\mu M}$	**3.9 ± 0.2**	$NA^{500\mu M}$
5-Hydroxyflavone	$NA^{125\mu M}$	$NA^{250\mu M}$	$17\%^{*50\mu M}$	$NA^{100\mu M}$
7-Hydroxyflavone	$NA^{125\mu M}$	$NA^{250\mu M}$	**146.5 ± 12.7**	NA^{1mM}
Positive controls				
Propyl gallate	**24.6 ± 3.2**	—	—	—
Ascorbic acid	—	**625.5 ± 49.6**	—	—
Lipoic acid	—	—	**2.4 ± 0.1**	—
Penicillamine	—	—	—	**4.9 ± 0.2**

^{NA}No activity was found up to the highest tested concentration (in superscript); *scavenging effect (mean %) at the highest tested concentration (in superscript).

with higher concentrations (up to 125 μM). Compound **3B** provided a 40% effect at the maximum tested concentration (125 μM). The other 2-styrylchromone derivatives were unable to scavenge $O_2^{\cdot-}$ within the studied concentration ranges.

Quercetin and luteolin could scavenge $O_2^{\cdot-}$ with an efficiency comparable to compounds **1A** and **1B** (Table 1). Apigenin reached a 35% steady-state effect along the studied concentration range (12.5–100 μM). No scavenging effect was observed for chrysin, 7-hydroxyflavone, and 5-hydroxyflavone. The IC_{50} of the positive control propyl gallate was 24.6 ± 3.2 μM.

2.2. Hydrogen peroxide scavenging activity

Only 2-styrylchromones from group 1 could scavenge H_2O_2 in a concentration-dependent manner. Compounds **1A** and **1C** presented a very similar activity, with IC_{50}s rounding the 50 μM. Compounds **1B** and **1D** were less efficient and provided IC_{50}s of 114.2 ± 19.1 μM and 182.8 ± 3.2 μM, respectively (Table 1). Compounds **2A** and **2C** could only reach a 36% and a 30% effect, respectively, at the highest tested concentration (250 μM). Compounds **2B** and **2D**, and the compounds from group 3 were completely ineffective up to the highest tested concentration (250 μM).

Among the tested flavonoids, only quercetin and luteolin were able to scavenge H_2O_2. However, these compounds were much less active than the 2-styrylchromones from group 1. The IC_{50} obtained for quercetin was 785.0 ± 45.0 μM, while for luteolin it was 900.2 ± 42.1 μM. The antioxidant ascorbic acid provided an IC_{50} of 625.5 ± 49.6 μM.

2.3. Hypochlorous acid scavenging activity

2-Styrylchromones from group 1 were able to efficiently scavenge HOCl, in a concentration-dependent manner (Table 1). Compound **1A** was considerably more efficient than the rest of the group, presenting an IC_{50} of 3.9 ± 0.2 μM, followed by **1B** and **1C**, with comparable effects (IC_{50}s of 18.0 ± 0.2 μM and 16.9 ± 1.1 μM, respectively), and by **1D** (IC_{50} = 32.1 ± 1.5 μM), which was the least potent of the group. From the group 2, only **2A** and **2B** have shown a concentration-dependent activity, although the first (IC_{50} = 8.5 ± 0.3 μM) was noticeably more potent than the second (IC_{50} = 92.0 ± 7.1 μM). Compound **2C** reached no more than a 29% effect, although it could only be tested up to 25 μM due to its low solubility. Compound **2D** reached a 34% effect at the highest tested concentration (100 μM). From group 3, only **3A** and **3B** showed an effect dependent on the concentration, with **3A** (IC_{50} = 5.7 ± 0.7 μM) being much more effective than **3B** (IC_{50} = 72.3 ± 5.0 μM) (Table 1). Compounds **3C** and **3D** presented only a slight effect of 17% and 10% at the maximum tested concentrations (50 and 125 μM, respectively). It should be noticed that **2A** and **3A** presented comparable effects, and their potencies were closer to **1A** than the other compounds from group 1 (Table 1). Quercetin was the most effective of the tested flavonoids (IC_{50} = 1.1 ± 0.1 μM), followed by apigenin and chrysin, with equal IC_{50}s (3.9 μM ± 0.4

and 3.9 μM ± 0.2), and luteolin (IC_{50} = 7.1 ± 1.5 μM). 7-Hydroxyflavone was also able to scavenge HOCl, although it has revealed to be much less potent than the above-referred flavonoids (IC_{50} = 146.5 ± 12.7 μM). 5-Hydroxyflavone revealed only a vestigial effect at the maximum tested concentration (17% at 50 μM). Lipoic acid efficiently scavenged HOCl (IC_{50} of 2.4 ± 0.1 μM).

2.4. Singlet oxygen scavenging activity

2-Styrylchromones from group 1 were shown to be potent scavengers of $^1O_2$ in a concentration-dependent manner and were effective in the low micromolar range (Table 1). In what concerns group 2, **2A** was the most potent if we consider the IC_{50} values (Table 1). However, the obtained IC_{20} for **2C** (28.9 ± 4.3 μM) was slightly lower than that of **2A** (35.1 ± 2.9 μM). Yet, it was not possible to test **2C** beyond the concentration of 40 μM due to the precipitation that occurred in the sample wells at higher concentrations. Compounds **2B** and **2D** presented a very similar effect, but were less potent than **2A** (Table 1). Compound **3A** was able to scavenge $^1O_2$ in a concentration-dependent manner, presenting an IC_{50} of 162.6 ± 10.0 μM. Compounds **3B**, **3C**, and **3D** did not show any scavenging activity up to the maximum tested concentrations (1000, 100, and 200 μM, respectively). In what concerns the tested flavonoids, quercetin showed an activity similar to the 2-styrylchromones from group 1, with an IC_{50} of 5.3 ± 0.3 μM, very close to the values obtained for **1A** and **1C** (Table 1). Luteolin revealed to be less potent than quercetin or any of the compounds from the referred group (IC_{50} = 23.4 ± 2.4 μM). Apigenin was able to scavenge $^1O_2$ although its effect had suffered only a slight increase along the studied concentration range (100–1000 μM), hence it could only reach a 30% effect at the maximum tested concentration (1 mM). The flavones chrysin, 5-hydroxyflavone, and 7-hydroxyflavone were not able to scavenge $^1O_2$ up to the maximum tested concentrations (500, 100, and 1000 μM, respectively). Penicillamine provided an IC_{50} of 4.9 ± 0.2 μM.

The studies related to possible interferences from each one of the tested compounds with the methodology revealed that compounds **1C** and **2A** react with rhodamine 123 in a concentration-dependent manner, in the range of concentrations used in this assay, which can contribute to a decrease of the fluorescence signal beyond that resulting from the scavenging effect. Thus, in what concerns to these two compounds, it is possible that the results do not correctly express their $^1O_2$ scavenging activity.

2.5. Peroxyl radical scavenging activity

The results from the Oxygen Radical Absorbance Capacity (ORAC) assay are listed in Table 2. All the compounds from group 1 were able to delay the $ROO^{\bullet}$-dependent oxidation of fluorescein. From group 2, only **2C** was not effective. Compound **3A** was the only active compound from group 3. In what concerns flavonoids, apigenin provided the highest ORAC value, followed by luteolin, quercetin, and chrysin. 5-Hydroxyflavone and 7-hydroxyflavone showed no effect in this assay.

Table 2. $ROO^{\bullet}$ scavenging activity of the tested 2-styrylchromones and flavonoids expressed as ORAC values (mean ± SE)

Compound	$ORAC_{ROO^{\bullet}}$ ± SE (μM trolox equiv/μM compound)	Concentration range (μM)
1A	9.15 ± 0.20	0.1–0.6
1B	7.17 ± 0.15	0.1–0.6
1C	6.23 ± 0.09	0.2–0.8
1D	5.57 ± 0.37	0.2–0.8
2A	8.99 ± 0.42	0.1–0.6
2B	6.17 ± 0.34	0.1–0.6
2C	NA	0.1–2.0
2D	5.84 ± 0.38	0.1–0.6
3A	3.77 ± 0.24	0.1–0.6
3B	NA	0.1–2.0
3C	NA	0.1–2.0
3D	NA	0.1–2.0
Luteolin	7.76 ± 0.38	0.1–0.8
Quercetin	5.98 ± 0.42	0.2–0.6
Apigenin	10.89 ± 0.60	0.05–0.5
Chrysin	4.66 ± 0.35	0.1–0.8
5-Hydroxyflavone	NA	0.1–2.0
7-Hydroxyflavone	NA	0.1–2.0

[NA]No activity was found within the assayed concentration range.

2.6. Peroxynitrite scavenging activity

The $ONOO^-$-induced oxidation of dihydrorhodamine 123 (DHR) was very efficiently prevented by the 2-styrylchromones from group 1 (0.26 ± 0.02 μM ⩽ IC_{50} ⩽ 0.30 ± 0.03 μM) and group 2 (1.04 ± 0.04 μM ⩽ IC_{50} ⩽ 1.40 ± 0.13 μM). From group 3, only **3A** was effective in a concentration-dependent manner (IC_{50} = 46.6 ± 7.0 μM). Compound **3D** reached a 27% effect at the highest tested concentration (150 μM). In the presence of 25 mM $NaHCO_3$, the same 2-styrylchromones that were active in its absence also managed to prevent DHR's oxidation. However, while the compounds from group 1 were visibly less efficient in the presence of $NaHCO_3$, the other 2-styrylchromones showed very similar effects in both circumstances. The relative order of potencies was the same either with or without $NaHCO_3$ (Table 3).

Quercetin and luteolin proved to be the most active flavonoids, with IC_{50}s very similar to those presented by 2-styrylchromones from group 1. This finding was confirmed in the presence of $NaHCO_3$ (Table 3). Apigenin and chrysin also prevented the $ONOO^-$-induced oxidation of DHR either in the presence or in the absence of $NaHCO_3$. Yet, the IC_{50}s of both compounds were lower in the presence of $NaHCO_3$, with a more noticeable difference for apigenin (Table 3). 5-Hydroxyflavone and 7-hydroxyflavone were ineffective in both assays. Ebselen, a selenium compound with well-known $ONOO^-$ scavenging activity, provided an IC_{50} of 0.91 ± 0.06 μM in the absence of $NaHCO_3$ and 4.63 ± 0.44 μM in its presence.

2.7. Nitric oxide scavenging activity

2-Styrylchromones from group 1 were able to inhibit the $^{\bullet}NO$-induced oxidation of 4,5-diaminofluorescein (DAF-2), in a concentration-dependent manner. The IC_{50}s obtained for this group were very low, varying

Table 3. ·NO and $ONOO^-$ (with and without 25 mM $NaHCO_3$) scavenging activities (IC_{50}, mean ± SE) of the studied 2-styrylchromones, flavonoids, and positive controls

Compound	IC_{50} (μM)		
	·NO	$ONOO^-$ without $NaHCO_3$	$ONOO^-$ with $NaHCO_3$
2-Styrylchromones			
1A	**0.51 ± 0.13**	**0.26 ± 0.02**	**0.63 ± 0.03**
1B	**0.29 ± 0.01**	**0.30 ± 0.03**	**0.61 ± 0.08**
1C	**0.24 ± 0.04**	**0.26 ± 0.04**	**0.51 ± 0.05**
1D	**0.34 ± 0.09**	**0.24 ± 0.02**	**0.44 ± 0.02**
2A	$21\%^{*250\mu M}$	**1.25 ± 0.14**	**1.21 ± 0.16**
2B	$23\%^{*250\mu M}$	**1.40 ± 0.13**	**1.65 ± 0.16**
2C	$NA^{50\mu M}$	**1.04 ± 0.04**	**0.98 ± 0.13**
2D	$46\%^{*250\mu M}$	**1.20 ± 0.17**	**1.23 ± 0.19**
3A	**73.9 ± 12.2**	**46.6 ± 7.0**	**40.6 ± 2.2**
3B	$NA^{250\mu M}$	$NA^{500\mu M}$	$NA^{500\mu M}$
3C	$NA^{50\mu M}$	$NA^{62\mu M}$	$NA^{62\mu M}$
3D	$NA^{100\mu M}$	$27\%^{*150\mu M}$	$27\%^{*150\mu M}$
Flavonoids			
Quercetin	**1.95 ± 0.06**	**0.26 ± 0.03**	**0.44 ± 0.02**
Luteolin	**2.61 ± 0.54**	**0.30 ± 0.05**	**0.64 ± 0.08**
Apigenin	$NA^{250\mu M}$	**76.1 ± 8.9**	**21.2 ± 4.7**
Chrysin	$20\%^{*250\mu M}$	**188.1 ± 8.2**	**140.0 ± 25.0**
5-Hydroxyflavone	$NA^{100\mu M}$	$NA^{62\mu M}$	$NA^{62\mu M}$
7-Hydroxyflavone	$NA^{250\mu M}$	$NA^{500\mu M}$	$NA^{500\mu M}$
Positive controls			
Rutin	**3.66 ± 0.43**	—	—
Ebselen	—	**0.91 ± 0.06**	**4.63 ± 0.44**

^{NA}No activity was found up to the highest tested concentration (in superscript); *scavenging effect (mean %) at the highest tested concentration (in superscript).

from 0.24 ± 0.04 μM to 0.51 ± 0.13 μM (Table 3). The compounds **2A**, **2B**, and **2D** were only able to scavenge ·NO at their maximum tested concentrations (250 μM) reaching the following effects: 21, 23, and 46%, respectively. Compound **2C** was completely ineffective, although it could only be tested up to 50 μM. Compound **3A** presented a concentration-dependent effect with an IC_{50} of 73.9 ± 12.2 μM. Compounds **3B**, **3C**, and **3D** could not show any effect up to the maximum tested concentrations (250, 50, and 100 μM, respectively). In what concerns flavonoids, quercetin and luteolin were the only compounds able to scavenge ·NO in a concentration-dependent manner (IC_{50}s of 1.95 ± 0.06 μM and 2.61 ± 0.54 μM, respectively). Chrysin provided a 20% effect at 250 μM, while the other flavonoids (apigenin, 5-hydroxyflavone, and 7-hydroxyflavone) showed no scavenging activity at all. The flavonoid rutin, which is a known ·NO scavenger, provided an IC_{50} of 3.66 ± 0.43 μM.

3. Discussion

The present study corroborates and extends previous findings indicating some styrylchromones as promising antioxidant pharmacophores. The scavenging activities for ROS/RNS of 2-styrylchromone derivatives are here shown for the first time, providing outstanding results, considering the nanomolar to micromolar range of the IC_{50} values found.

One of the main sources of $O_2{\cdot}^-$ is the enzyme xanthine oxidase (XO). A previous work from our group[7] has shown that the tested 2-styrylchromones have the ability to inhibit XO. In addition, several papers refer to the XO inhibitory capacity of different flavonoids, including quercetin, apigenin, chrysin, and 7-hydroxyflavone.[28–30] Thus, in the present work the $O_2{\cdot}^-$ scavenging activity was measured by a non-enzymatic methodology in order to avoid the confounding effects derived from the XO inhibition. 2-Styrylchromones from group 1 were all effective scavengers of $O_2{\cdot}^-$, indicating that the 3′,4′-dihydroxyl substitution on the B-ring plays an important role in what concerns $O_2{\cdot}^-$ scavenging activity of 2-styrylchromones. This comes in agreement with previous studies about the structure–activity relationships of flavonoids.[31,32] In addition, the number and position of the hydroxyl groups in the A-ring also seems to contribute to the effect of these compounds. Indeed, compounds **1A** and **1B**, with a 5,7-dihydroxyl and a 7-hydroxyl substitution in the A-ring, respectively, were noticeably more potent than **1C** and **1D** with a 5-hydroxyl in A-ring and no hydroxyl group at all, respectively. Moreover, compounds **2A** and **2B**, which have a phenolic instead of a catecholic B-ring, were still more active than **1C** and **1D**. Compounds from group 3 have shown almost no effect, indicating that an hydroxyl substituent in the B-ring is essential for the $O_2{\cdot}^-$ scavenging activity. From the studied flavonoids, quercetin was slightly more effective than luteolin. This confirms the previously referred importance of the hydroxyl group on C3, which distinguishes quercetin (a flavonol) from luteolin (a flavone), in their radical scavenging activity potencies. This structural feature provides the conjugation between B-ring and C-ring, and contributes to the stabilization of the phenoxy radical.[31,32] Of note, compound **1A**, which differs from quercetin by the presence of a styryl moiety and the absence of the hydroxyl group on C3, has shown an $O_2{\cdot}^-$ scavenging activity very similar to the referred flavonol. This fact points to a likely contribution of the styryl moiety to the molecular stabilization, increasing the compound's antiradical activity.

The results from the H_2O_2-scavenging assay once again show the importance of the 3′,4′-catechol in the B-ring for the antioxidant activity of 2-styrylchromones. In this case, the styrylphenol derivatives were almost ineffective, and none of the compounds from group 3, with no hydroxyl substituents in the B-ring, showed any activity. In contrast to what was observed for $O_2{\cdot}^-$, the 5-hydroxyl substituent seems to contribute to the H_2O_2 scavenging activity more effectively than the 7-hydroxyl substituent. In fact the presence of a 7-hydroxyl group in the benzopyrone moiety does not seem to bring any great advantage to the scavenging effect. The 2-styrylchromones from group 1 were much more effective than quercetin or luteolin, which is a very interesting aspect since it shows the major role of the styryl feature on the H_2O_2 scavenging activity. This group of compounds was also much more effective than ascorbic acid, which is a well-known important dietary antioxidant.

The HOCl scavenging activity of the tested 2-styrylchromones seems to be largely affected by the number and

position of the hydroxyl substituents on the A-ring. Although the catechol group on B-ring is indicative of a strong scavenging effect, as it happens with the above-mentioned ROS, this feature seems to be less important when a 5,7-dihydroxyl group is present in the A ring. This is clear by the analysis of the results, which shows that the most potent 2-styrylchromones are **1A**, **2A**, and **3A**. A mono- or a non-substituted A-ring greatly decreases the HOCl scavenging activity of the molecule. The same conclusion can be drawn from the results of the studied flavonoids. Quercetin, luteolin, apigenin, and chrysin all provided a strong HOCl scavenging effect even though apigenin and chrysin lack the catechol group on the B-ring. Yet, they all have in common the 5,7-dihydroxylated A-ring, which explains the high activity. The C3-OH substitution seems to be another important feature the HOCl scavenging activity, considering the very strong effect showed by quercetin. It has been previously referred that quercetin and its derivative rutin react with HOCl to form stable mono- and dichlorinated products, modified at the C6 and C8 sites of the A-ring, which results in even more efficient scavengers of ROS and RNS than the parent compounds.[33] Considering the structural similarity between quercetin and 2-styrylchromones, particularly the compound **1A**, it is tempting to admit that these compounds may also suffer chlorination upon reaction with HOCl and equally generate more active antioxidant products. This is an interesting point, although it needs to be confirmed with further studies.

The $^1O_2$ scavenging activity of 2-styrylchromones and flavonoids seems to depend particularly on the OH-substitution pattern in B-ring. Compounds with a 3′,4′-dihydroxyl pattern presented a considerably higher effect than those lacking this feature as it can be confirmed by the comparison of the results from group 1 with those from groups 2 and 3. Nevertheless, the number and position of the OH substituents in the A-ring also contributes to the $^1O_2$ scavenging activity of 2-styrylchromones. An OH group in C5 seems to positively affect the scavenging behavior of the antioxidant molecule, while this substitution in C7 appears to bring no advantage. The studied 2-styrylchromones were shown to be more efficient scavengers of $^1O_2$ than the flavones with comparable structures, which means that the styryl feature increases the scavenging activity for this reactive species. Quercetin was the most effective of the tested flavonoids, showing a much lower IC_{50} than luteolin, its corresponding flavone. These results are in agreement with the previously shown high chemical reactivity of flavonols with $^1O_2$, explained by the presence of the C3-OH substituent in the C-ring.[34] Nevertheless, the most effective 2-styrylchromones (**1A** and **1C**), although lacking this substituent, were quite as active as quercetin.

In the ORAC assay quercetin proved to be a good $ROO^{\cdot}$ scavenger, providing an ORAC value very close to those reported in previous similar studies.[35,36] Luteolin also behaved as an excellent $ROO^{\cdot}$ scavenger and apigenin, contrary to the expected, provided the highest ORAC value. Rice-Evans[25] considered flavonoids as ideal scavengers of peroxyl radicals and effective inhibitors of lipid peroxidation due to their favorable reduction potentials relatively to alkyl peroxyl radicals. Furthermore these compounds have the ability to interact with biomembranes,[37] occupying a favorable position to interrupt radical chain reactions. The results from the present study show that some 2-styrylchromones are as good $ROO^{\cdot}$ scavengers as the structurally similar flavonoids, or even better in some cases. Although the OH substitution pattern in the B-ring seems to exert a major influence on the activity of these compounds, a clear-cut structure–activity relationship is yet to be established.

The high $ROO^{\cdot}$ scavenging activity shown by some of the studied compounds may be of extreme value since, during its sustained overproduction, the walls of affected tissue cells involved become amenable to lipid peroxidation, explaining the increase of lipid peroxidation marker compounds in oxidative stress related diseases. For instance, a larger concentration of lipid peroxidation markers is detected in LDL samples of patients suffering from atherosclerosis rheumatic arthritis, after shock, or bacterial infection.[38] Noticeably, some of the tested 2-styrylchromones have previously shown to prevent *tert*-butylhydroperoxide (*t*-BHP) induced lipid peroxidation in rat hepatocytes.[8] Additionally, the compounds studied by Fernandes and coworkers reduced the pro-oxidant hepatotoxicity by preventing the depletion of reduced glutathione (GSH) and cell death. The results obtained in that same study showed that the B-ring catecholic derivatives were more effective hepatoprotectors than the phenolic derivatives.[8]

2-Styrylchromones from group 1 were the most effective derivatives to protect DHR against $ONOO^-$-induced oxidation. The different compounds from this group showed very similar activities, which indicates that the catecholic B-ring, a common feature in the group, is the main factor responsible for the scavenging activity. Moreover, the group 2 derivatives were visibly less active than the group 1 derivatives, although their IC_{50}s were still very low. Thus, the 4′-hydroxyl group in B-ring also seems to be of extreme importance for $ONOO^-$ scavenging activity of 2-styrylchromones. The absence of an OH substituent in the B-ring drastically decreases the scavenging effect, as it is evident by the analysis of the results from group 3. Nevertheless, **3A** was able to prevent $ONOO^-$-induced DHR oxidation, although to a smaller extent compared to the other active compounds, which indicates that the number of OH groups in the A-ring is an important factor to the scavenging effect when the B-ring lacks OH substituents. The results obtained from quercetin and luteolin are analogous to those of 2-styrylchromones from group 1. Apigenin was a much weaker protector of DHR oxidation than the above-referred flavonoids, confirming the requirement of the 3′,4′-hydroxylation pattern to achieve a strong effect. It should be noticed, however, that the 2-styrylchromones from group 2 were considerably more effective than apigenin, which means that the styryl link increases the $ONOO^-$ scavenging activity of the phenolic B-ring derivatives. A possible explanation for this fact might be on the contribution of the styryl moiety to the stabilization of the phenoxy radical that

is formed during the scavenging reaction, as it was previously suggested.[8] The comparison between the effects of chrysin and **3A** confirms the importance of the styryl feature to the $ONOO^-$ scavenging activity, since these two compounds only differ in this aspect, being the first considerably less effective than the second. The results provided by the flavonoids in this study, as well as the structure–activity relationships, are in agreement with other previous works.[39–42]

The results from the assays performed in the presence of 25 mM $NaHCO_3$ show a decrease in the effect of the catecholic B-ring-like compounds, either 2-styrylchromones or flavonoids. The same behavior was previously observed for quercetin and luteolin by other authors.[39] On the contrary, apigenin and chrysin were shown to be more effective in the presence of bicarbonate. These variations take place due to the formation of other $ONOO^-$ derivatives when bicarbonate is added to the reaction medium at physiological concentrations. The extremely fast reaction between $ONOO^-$ and CO_2 ($K = 3–5.8 \times 10^4\ M^{-1}\ s^{-1}$)[43,44] originates the nitrosoperoxycarbonate anion ($ONOOCO_2^-$), whose decomposition leads to the formation of different species including the highly reactive $^{\cdot}NO_2$ and $CO_3^{\cdot-}$ radicals.[45] The presence of these radicals in solution affects the reactivity of the $ONOO^-$ scavengers, either increasing or decreasing their effects. Nevertheless, the results of the assays in which $NaHCO_3$ was added give us, theoretically, a better approach to the developments in a biological system. In fact it is likely that many of the reactions of $ONOO^-$ in vivo are promoted by $ONOOCO_2^-$ derivatives, which are more efficient nitrating and oxidizing species than $ONOO^-$.[45,46]

The most studied $ONOO^-$-dependent effect in biological targets has been the nitration of tyrosine into 3-nitrotyrosine, which is a biomarker for RNS and has been associated with several diseases.[18,25] Luteolin and quercetin have been classified as excellent inhibitors of tyrosine nitration.[47] The catecholic B-ring, present in both referred flavonoids, plays an essential role in what concerns the inhibition process. This can be explained by the mechanism behind the reaction of catechol like compounds with peroxynitrite derivatives, which is likely to involve two electron transfer reactions with the formation of *o*-quinones.[47–49] Considering the structural similarity between the above-referred flavonoids and the 2-styrylchromones from group 1, it is very likely that these compounds can offer a biologically relevant protection against $ONOO^-$-induced deleterious effects.

In agreement with the results from other ROS/RNS, the $^{\cdot}NO$ scavenging activity of 2-styrylchromones depends mostly on the hydroxylation pattern of the B-ring. The compounds with a 3′,4′-catecholic group were shown to be very potent protectors of the $^{\cdot}NO$-dependent oxidation of DAF-2. The other 2-styrylchromones provided a much weaker effect or no effect at all. Strangely, compound **3A** was more effective than **2A**, regardless of the additional C4′-OH in the latter. Quercetin and luteolin, in spite of the high effects presented, were less potent scavengers than 2-styrylchromones from group 1. Here, as in other previously exposed cases, the styryl feature seems to improve the scavenging efficiency.

van Acker and coworkers[50] studied the $^{\cdot}NO$ scavenging activities of some therapeutically used flavonoids, which were shown to be very effective scavengers. These authors found a correlation between their scavenging potencies and the respective therapeutic efficacy in terms of protection against capillary permeability and fragility and anti-inflammatory and anti-odemic activities. Indeed, $^{\cdot}NO$ presents pro-inflammatory effects including augmentation of vascular permeability of inflamed tissues, the induction of cyclooxygenase as well as angiogenic and inflammatory cytokines, activation of matrix metalloprotease, induction of chondrocyte apoptosis, and the generation of $ONOO^-$ by reaction with $O_2^{\cdot-}$.[19] From this point of view, the high $^{\cdot}NO$ scavenging activity shown by some of the compounds tested in our work can possibly be of therapeutic interest.

In conclusion, the scavenging activities for ROS/RNS of 2-styrylchromone derivatives were investigated for the first time, in the present work, providing motivating results. Some of the studied compounds were shown to be remarkable scavengers of those reactive species. 2-Styrylchromones are, therefore, promising molecules with potential therapeutic value that should be further explored, particularly in what concerns their utility to prevent or control oxidative stress-related diseases whose actual therapeutic options are still unsatisfactory. Clearly, the therapeutic use of the tested 2-styrylchromones still requires a battery of in vitro and in vivo toxicological assays, to confirm their efficacy and assure their safety. Nevertheless, previous results obtained by our group give an excellent indication about their value, since, besides the protective effect against *t*-BHP, the tested 2-styrylchromones were not toxic at concentrations up to 200 μM.[8]

4. Experimental

4.1. Equipment

A microplate reader (Synergy HT, BIO-TEK), with spectrophotometric, fluorimetric, and chemiluminometric detection, plus temperature control capacity, was used for all ROS and RNS scavenging assays.

4.2. Chemicals

All the chemicals and reagents were of analytical grade. Dihydrorhodamine 123 (DHR), 4,5-diaminofluorescein (DAF-2), 30% hydrogen peroxide, ascorbic acid, sodium hypochlorite solution, with 4% available chlorine, lipoic acid, diethylenetriaminepentaacetic acid (DTPA), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), penicillamine, propyl gallate, rutin, lucigenin, ebselen, luteolin, apigenin, 5-hydroxyflavone, and

7-hydroxyflavone were obtained from Sigma–Aldrich (St. Louis, USA). α,α′-Azodiisobutyramidine dihydrochloride (AAPH), histidine, and trolox were obtained from Fluka Chemie GmbH (Steinheim, Germany). Fluorescein sodium salt, quercetin and chrysin were obtained from Aldrich (Milwaukee, USA). All the other reagents were obtained from Merck (Darmstadt, Germany).

2-Styrylchromones **1A–1D**, **2A–2D**, and **3A–3D** were synthesized according to procedures described in the literature by some of the authors.[51]

4.3. ROS and RNS scavenging assays

4.3.1. Superoxide radical scavenging assay. Superoxide radical was generated by the NADH/PMS system and the $O_2^{\cdot -}$ scavenging activity was determined spectrophotometrically in the microplate reader by monitoring the effect of the compound to be tested on the $O_2^{\cdot -}$-induced reduction of NBT at 560 nm for 2 min. The $O_2^{\cdot -}$ production was controlled by superoxide dismutase (SOD), which inhibited NBT reduction in a concentration-dependent manner.[52] The assay was performed at room temperature. The reaction mixtures in the sample wells contained the following reactants at the indicated final concentrations (in a final volume of 300 μL): NADH (166 μM), NBT (43 μM), the tested compounds at various concentrations, dissolved in DMSO, and PMS (2.7 μM). NADH, NBT, and PMS were dissolved in 19 mM phosphate buffer, pH 7.4. No direct effect was observed between DMSO and $O_2^{\cdot -}$ in the present assay conditions. The antioxidant propyl gallate was used as positive control. The effects were expressed as the inhibition (in percentage) of the NBT reduction to diformazan. Each study corresponds to four experiments, performed in triplicate.

4.3.2. Hydrogen peroxide scavenging assay. The H_2O_2 scavenging activity was measured by monitoring the H_2O_2-induced oxidation of lucigenin, using a previously described chemiluminescence methodology,[53] with modifications. Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 250 μL): 50 mM Tris–HCl buffer, pH 7.4, lucigenin (0.8 mM), dissolved in the buffer solution, the tested compounds at various concentrations, dissolved in DMSO, and 1% H_2O_2. The assays were performed at 37 °C. The chemiluminescence signal was detected in the microplate reader immediately after the plate introduction. The endogenous antioxidant ascorbic acid was used as positive control. The effects were expressed as the inhibition (in percentage) of the H_2O_2-induced oxidation of lucigenin. Each study corresponds to four experiments, performed in triplicate.

4.3.3. Hypochlorous acid scavenging assay. HOCl was measured by using a previously described fluorescent methodology,[54] based on the HOCl-induced oxidation of DHR to rhodamine 123, which has been adapted to a microplate reader. HOCl was prepared immediately before use by adjusting the pH of a 1% (m/v) solution of NaOCl to 6.2 with dropwise addition of 10% H_2SO_4. The concentration of HOCl was further determined spectrophotometrically[55] at 235 nm using the molar absorption coefficient of 100 M^{-1} cm^{-1} and the proper dilution was made in a 100 mM phosphate buffer at pH 7.4. A 2.89 mM stock solution of DHR in dimethylformamide was purged with nitrogen and stored at −20 °C. Working solutions of DHR were diluted in the phosphate buffer from the stock solution immediately before the determinations and placed on ice, in the dark. Reaction mixtures contained the following reactants at the indicated final concentrations (in a final volume of 300 μL): 100 mM phosphate buffer solution at pH 7.4, the tested compounds at different concentrations, dissolved in ethanol, DHR (5 μM), and HOCl (5 μM). The fluorimetric assays were performed at 37 °C, in the microplate reader, at the emission wavelength 528 ± 20 nm with excitation at 485 ± 20 nm. The fluorescence signal was measured immediately after the plate introduction. Lipoic acid was used as positive control. The results were expressed as the inhibition (in percentage) of HOCl-induced oxidation of DHR. Each study corresponds to four experiments, performed in triplicate.

4.3.4. Singlet oxygen scavenging assay. The $^1O_2$ scavenging activity was measured by monitoring the oxidation of non-fluorescent DHR to fluorescent rhodamine 123 by this ROS, according to a recently developed fluorimetric methodology.[56] $^1O_2$ was generated by the thermal decomposition of a previously synthesized water-soluble endoperoxide [disodium 3,3′-(1,4-naphthalene)bispropionate ($NDPO_2$)]. $NDPO_2$ working solutions, diluted in 100 mM phosphate buffer, pH 7.4, were prepared immediately before each assay. A 2.89 mM stock solution of DHR in dimethylformamide was purged with nitrogen and stored at −20 °C. Working solutions of DHR were diluted in the phosphate buffer from the stock solution immediately before the determinations and placed on ice, in the dark. Histidine solutions in phosphate buffer were daily prepared. Reaction mixtures contained the following reactants at the indicated final concentrations (in a final volume of 250 μL): Histidine (10 mM), the tested compounds at different concentrations dissolved in DMSO, DHR (50 μM), and $NDPO_2$ (1 mM). The fluorimetric assays were performed at 37 °C, in the microplate reader, using the emission wavelength 528 ± 20 nm with excitation at 485 ± 20 nm. The fluorescence was measured after a 30 min incubation period. Penicillamine was used as positive control. The results were expressed as the inhibition (in percentage) of $^1O_2$-induced oxidation of DHR. Each study corresponds to four experiments, performed in triplicate.

4.3.5. Peroxyl radical scavenging assay. The $ROO^{\cdot}$ scavenging activity was measured by monitoring the fluorescence decay resulting from $ROO^{\cdot}$-induced oxidation of fluorescein and expressed as the 'Oxygen Radical Absorbance Capacity' (ORAC), according to a previously described method.[38] $ROO^{\cdot}$ was generated by thermodecomposition of AAPH. Reaction mixtures in the sample wells contained the following reagents at the indicated final concentrations (in a final volume of 200 μL): fluorescein (61 nM), the tested compounds (0.1–0.8 μM), dissolved in acetone and subsequently di-

luted in 75 mM phosphate buffer, pH 7.4, and AAPH (19 mM). Working solutions of fluorescein were diluted in 75 mM phosphate buffer, pH 7.4, to 1/5000-fold, from a 1.53 mg/mL stock solution, which had been previously prepared and kept at ≈4 °C. AAPH was dissolved in phosphate buffer. The mixture was preincubated in the microplate reader for 15 min at 37 °C. The fluorescence signal was then monitored every minute at the emission wavelength 528 ± 20 nm with excitation at 485 ± 20 nm until the total decay of fluorescence. Trolox (1–6 μM) was used as a control standard in each assay. Each study corresponds to four experiments, performed in triplicate.

ORAC values were calculated according to a previous paper.[57] The net protection provided by a putative antioxidant sample was calculated using the difference between the area under the fluorescence decay curve in the presence of the sample (AUC_{sample}) and in its absence (AUC_{blank}). Regression equations between net AUC and the concentration of the sample were calculated for all the compounds. ORAC values were calculated by using the standard curve of each assay. Final results were expressed in micromole of Trolox equivalents/μmol of compound.

4.3.6. Peroxynitrite scavenging assay. The $ONOO^-$ scavenging activity was measured by monitoring the $ONOO^-$-induced oxidation of non-fluorescent DHR to fluorescent rhodamine 123, according to a described procedure.[58] $ONOO^-$ was synthesized as described before.[58] Briefly, an acidic solution (HCl 0.7 M) of H_2O_2 0.6 M was mixed with $NaNO_2$ 0.66 M in a Y junction and the reaction mixture was quenched with ice-cold NaOH 3 M. Residual H_2O_2 was removed by mixing with granular MnO_2 pre-washed with NaOH 3 M. The obtained $ONOO^-$ solution was filtered and then frozen (−80 °C). Prior to each experiment, the top layer of the stock solution was collected and the concentration of peroxynitrite was determined spectrophotometrically ($\varepsilon_{302nm} = 1670\ M^{-1}\ cm^{-1}$). Subsequently, the proper dilution was made in 0.05 M NaOH. A stock solution of 2.89 mM DHR in dimethylformamide was purged with nitrogen and stored at −20 °C. Working solutions of DHR, properly diluted with the buffer solution (90 mM NaCl, 50 mM Na_3PO_4, and 5 mM KCl, pH 7.4), were placed on ice, in the dark, immediately before the determinations. In the beginning of the experiments, 100 μM DTPA was added to the buffer. Reaction mixtures contained the following reactants at the indicated final concentrations (in a final volume of 300 μL): DHR (5 μM), the tested compounds at different concentrations, dissolved in DMSO, and $ONOO^-$ (600 nM). The assays were performed at 37 °C. The fluorescence signal was detected after a 2 min incubation period at the emission wavelength 528 ± 20 nm with excitation at 485 ± 20 nm, in the microplate reader. Ebselen was used as positive control. In a parallel set of experiments, the assays were performed in the presence of 25 mM $NaHCO_3$ in order to simulate the physiological CO_2 concentrations. This evaluation is important because, under physiological conditions, the reaction between $ONOO^-$ and bicarbonate is predominant, with a very fast rate constant ($K_2 = 3–5.8 \times 10^4\ M^{-1}\ s^{-1}$).[59] The results were expressed as the inhibition (in percentage) of $ONOO^-$-induced oxidation of DHR. Each study corresponds to four experiments, performed in triplicate.

4.3.7. Nitric oxide scavenging assay. The ·NO scavenging activity was measured by monitoring the ·NO-induced oxidation of non-fluorescent DAF-2 to the fluorescent triazolofluorescein (DAF-2T), according to a described procedure.[58] ·NO was generated by NOC-5. A stock solution of 2.76 mM DAF-2 in DMSO was purged with nitrogen and stored at −20 °C. Working solutions of DAF-2, diluted with a phosphate buffer solution (KH_2PO_4 50 mM, pH 7.4) to 1/368-fold from the stock solution, were placed on ice, in the dark, immediately before the determinations. The reaction mixtures in the sample wells contained the following reagents at the indicated final concentrations (in a final volume of 300 μL): DAF-2 (5 μM), the tested compounds at various concentrations, dissolved in DMSO, and NOC-5 (10 μM). The assays were performed at 37 °C. The fluorescence signal was detected after a 30 min incubation period at the emission wavelength 528 ± 20 nm with excitation at 485 ± 20 nm, in the microplate reader. Rutin was used as positive control. The results were expressed as the inhibition (in percentage) of ·NO-induced oxidation of DAF-2. Each study corresponds to four experiments, performed in triplicate.

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II.2. CYCLIC VOLTAMMETRIC ANALYSIS OF 2-STYRYLCHROMONES. RELATIONSHIP WITH THE ANTIOXIDANT ACTIVITY

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Cyclic voltammetric analysis of 2-styrylchromones: Relationship with the antioxidant activity

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ABSTRACT

2-Styrylchromones (2-SC) are a chemical family of oxygen heterocyclic compounds, vinylogues of flavones (2-phenylchromones), whose occurrence in nature has been reported. Recently, several 2-SC derivatives were demonstrated to have antioxidant properties, namely, xanthine oxidase inhibition, hepatoprotection against pro-oxidant agents in cellular and non-cellular systems and scavenging activity against reactive oxygen and reactive nitrogen species (ROS and RNS). Considering these antioxidant properties, it may be hypothesised that the electrochemical redox behaviour of 2-SC contributes significantly to their activity. To test this hypothesis, the electrochemical behaviour of different 2-SC was studied, together with a number of flavonoids with well-known antioxidant activities, by cyclic voltammetry, and the results correlated to their ability to scavenge ROS and RNS.

The results obtained showed that 2-SC with a catecholic B-ring have a low oxidation peak potential corresponding to the oxidation of the 3′,4′-OH (catechol) moiety. The compounds with a phenolic B-ring have a common peak, with oxidation potential values of about +0.4/+0.5 V versus Ag/AgCl, corresponding to the oxidation of the 4′-OH. The oxidation of the hydroxyl substituents in the A-ring generated peaks of higher potentials (+0.7/+0.8 V vs Ag/AgCl). The results from the scavenging assays were in agreement with those obtained from the cyclic voltammetry, that is, higher scavenging effects corresponded to lower values of oxidation potentials, with significant correlation coefficients. The values obtained for the studied flavonoids are in accordance with the literature, and reflect their relative antioxidant activity, when compared to the studied 2-SC. Thus, in this family of compounds, oxidation potentials obtained by cyclic voltammetry seem to be applicable as a general indicator of radical scavenging activity.

1. Introduction

2-Styrylchromones (2-SC) are a chemical family of oxygen heterocyclic compounds, vinylogues of flavones (2-phenylchromones), whose occurrence in nature has been reported. Only two natural 2-SC are known and they were extracted from the blue-green algae *Chrysophaeum taylori* in the 1980s.[1,2] Before and after the isolation of natural 2-SC, several analogues of these compounds have been synthesised and tested in different biological systems. The natural compounds were demonstrated to possess cytotoxic activity against leukaemia cells,[1,2] and those obtained by synthesis exhibited antiallergic,[3] antiviral,[4] antitumour,[5] and antagonism of A3 adenosine receptor[6] properties. Recently, these compounds have also demonstrated antioxidant properties, namely, xanthine oxidase inhibition,[7] hepatoprotection against pro-oxidant agents in cellular[8] and non-cellular systems[9] and scavenging activity against reactive oxygen and reactive nitrogen species (ROS and RNS).[10]

The capacity of phenolic compounds, especially the polyhydroxylated derivatives, to scavenge free radicals is intimately related to their effectiveness to donate hydrogen atoms (H). Electrochemical oxidation can conceivably be used as a model for the scavenging reaction, since both reactions involve the breaking of the same O–H bond and the donation of e^- and H^+.[11] Indeed, oxidation potentials have been correlated with the antioxidant power of phenolic compounds, particularly the flavonoids.[11–15]

Cyclic voltammetry is a widely used electroanalytic technique that allows determining the redox properties of molecules in a solution. Experimentally, the potential of a working electrode is linearly scanned (vs a reference electrode, typically Ag/AgCl) from an initial value to a final value and back. Thus, forward and backwards electrochemical reactions can be studied. This technique has been used to study the electrochemical oxidation mechanisms of flavonolic compounds.[16–18]

The purpose of the present study was to use cyclic voltammetry to assess the electrochemical behaviour of different 2-SC (Table 1)

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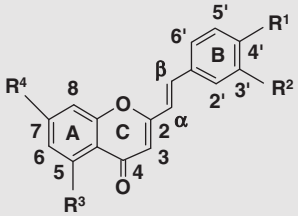
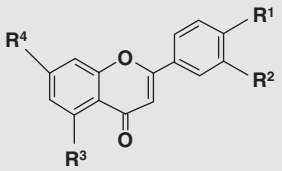
Table 1
Chemical structures and oxidation potentials of the tested 2-styrylchromones and flavonoids

Compounds	Chemical structure	R^1	R^2	R^3	R^4	$E_{p_{ox}}$ (V) versus Ag/AgCl	
						1st peak	2nd peak
2-Styrylchromones							
1A		OH	OH	OH	OH	+0.169	+0.762
1B		OH	OH	H	OH	+0.167	+0.806
1C		OH	OH	OH	H	+0.183	+0.819
1D		OH	OH	H	H	+0.173	–
2A		OH	H	OH	OH	+0.444	+0.777
2B		OH	H	H	OH	+0.485	–
2C		OH	H	OH	H	+0.424	–
2D		OH	H	H	H	+0.495	–
3A		H	H	OH	OH	+0.764	–
3B		H	H	H	OH	+0.787	–
3C		H	H	OH	H	+0.817	–
3D		H	H	H	H	–	–
Flavonoids							
Luteolin		OH	OH	OH	OH	+0.223	–
Apigenin		OH	H	OH	OH	[a]	
Chrysin		H	H	OH	OH	+0.767	–
5-OH Flavone		H	H	OH	H	+0.807	–
7-OH Flavone		H	H	H	OH	+0.797	–

[a] Not possible to determine $E_{p_{ox}}$.

in order to understand the mechanism behind the scavenging of ROS and RNS. Five flavones (luteolin, apigenin, chrysin, 5-hydroxyflavone and 7-hydroxyflavone) were also tested due to the similarities of their structures with those of 2-SC. This allowed to take advantage from the existing knowledge about flavonoids and helped to interpret the results.

2. Results

Cyclic voltammograms of 2-SC from group 1 (**1A**–**1D**) showed the permanent presence of a low potential oxidation peak (Table 1), with a correspondent reduction peak being detected in the reverse scan. Additionally, the compounds **1A**, **1B** and **1C** showed a second oxidation peak (see Fig. 1, for compound 1B, as a representative voltammogram).

The redox reaction that occurs at lower potential was studied in detail by analysing the change of the peak current (I_p) and the oxidation potential (E_p) with scan rate and pH. The ratio between the reduction and the oxidation peak currents ($I_{p_{red}}/I_{p_{ox}}$) tended to be smaller, less than unity, at low scan rate values, increasing at higher values, for compounds **1A** and **1B**, and was practically invariable, and always less than unity, along the scan rate interval for compounds **1C** and **1D**. When the potential scan was inverted before the second oxidation peak was shown, that is, at 0.4 V versus Ag/AgCl, that ratio became approximately 1 for all the compounds, indicating the reversibility of the electrochemical reaction (see Fig. 2, for compound **1A**, as a representative voltammogram). The oxidation and reduction peak potentials ($E_{p_{ox}}$ and $E_{p_{red}}$) were practically invariable along the studied scan rate interval.

The $E_{p_{ox}}$ of the first peak was shown to be dependent on the pH in the range of 3.8–11, that is, an increase of the pH was associated with a decrease of the $E_{p_{ox}}$, as it was demonstrated for compound 1B ($E_{p_{ox}} = -0.046\text{pH} + 0.539$). For higher pH values (>11) the electron transfer reaction was pH independent and the reduction peak tended to become smaller, being undetectable at the pH of 11.6 and beyond.

An oxidation peak with $E_{p_{ox}}$ values between +0.4 and +0.5 V versus Ag/AgCl was visible in the cyclic voltammograms of the compounds from group 2 (**2A**–**2D**). A second oxidation peak was observed at higher potentials for the compound **2A** (Fig. 1). The compounds **3A**–**3C** gave rise to an oxidation peak with $E_{p_{ox}}$ values comparable to those of the second peak of compounds **1A**–**1C** and **2A** (Fig. 1). These oxidation peaks appear to correspond to irrevers-

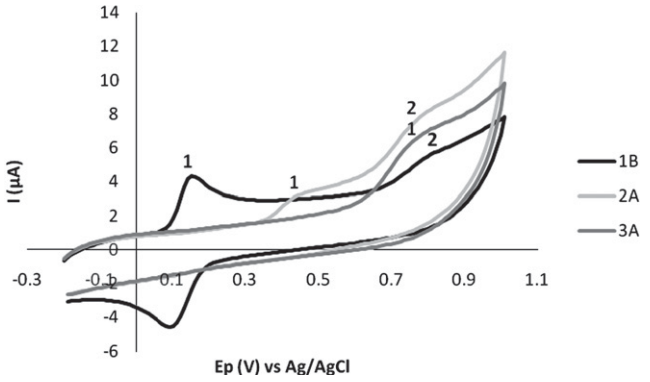


Figure 1. Cyclic voltammograms of compounds **1B**, **2A** and **3A** in pH 7.4 phosphate buffer. Scan rate 100 mV s^{-1}.

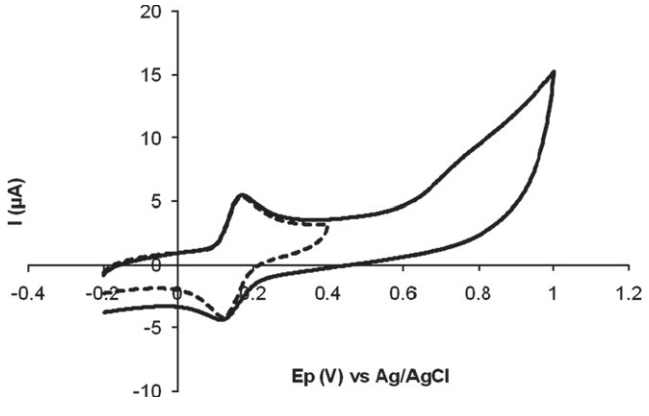


Figure 2. Cyclic voltammograms of compound **1A** in pH 7.4 phosphate buffer: (—) scan inversion at 1.0 V versus Ag/AgCl; (---) scan inversion at 0.4 V versus Ag/AgCl. Scan rate 100 mV s^{-1}.

ible processes, since no current was observed in the reverse scan. For compound **3D** there were no detectable peaks (Table 1).

The cyclic voltammogram of luteolin presented one oxidation peak and a correspondent reduction peak. The flavones chrysin, 5-OH flavone and 7-OH flavones revealed a common oxidation peak, which appeared at potentials around +0.8 V versus Ag/AgCl. Apigenin showed two possible oxidation peaks, although barely defined, not allowing the determination of the $E_{p_{ox}}$ values (Table 1).

3. Discussion

In the present study, the electrochemical behaviour of several 2-SC was evaluated for the first time. According to the literature related to studies with flavonoids, the first oxidation peak observed in the 2-SC **1A**–**1D** corresponds to the oxidation of the 3′,4′-OH (catechol) moiety in the B-ring.[16–18] The absence of a similar peak in the compounds lacking this catechol substituent supports this thesis. A detailed analysis of this peak indicated a reversible redox process with a coupled chemical reaction, also in accordance to previous reports for flavonoids.[16,19] The observed dependence of the $E_{p_{ox}}$ of the first oxidation peak on the pH (demonstrated for compound **1B**) shows that, during reaction, not only electrons but also protons are released from the molecule.[17,18] An increase in pH was associated with a decrease in the $E_{p_{ox}}$, meaning that at higher pH the compound is easily oxidised, thus, becoming a more efficient antioxidant. At pH > 11, this dependence no longer existed, indicating deprotonation of the molecule.[17,18] When Lemanska et al.[20] compared the calculated O–H bond dissociation energies (BDE) and ionisation potentials (IP) in the nondeprotonated and the protonated forms of various hydroxyflavones, they verified that, especially the parameter reflecting the ease of electron donation, that is, the IP, and not the BDE for hydrogen atom donation, is greatly influenced by deprotonation. Thus, electron donation becomes the dominant mechanism of antioxidant action of hydroxyflavones after deprotonation.

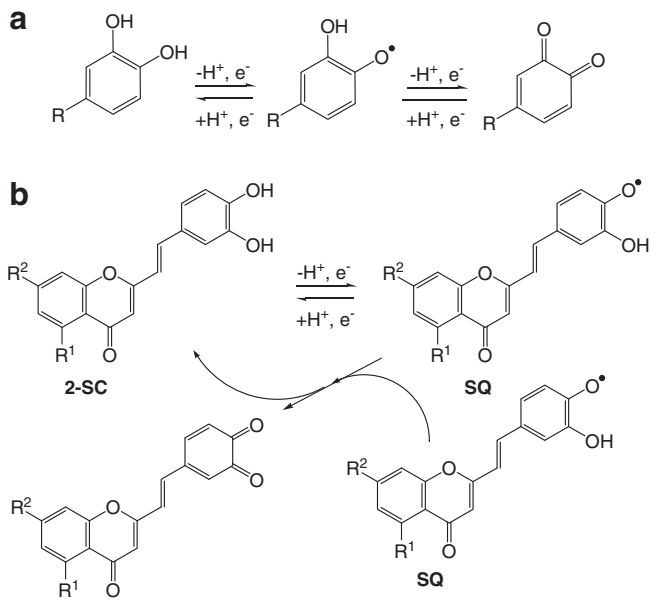


Figure 3. (a) Mechanism of oxidation of catechol-like compounds. (b) Proposed oxidation mechanism of 2-styrylchromones.

It is known that the antioxidant capacity is conceivably related to the electrochemical behaviour, a low oxidation potential being indicative of a high antioxidant power.[21] Accordingly, Firuzi et al.[15] found a good negative correlation between the 'ferric reducing antioxidant power' (FRAP) and the oxidation potential of several flavonoids. Moreover, flavonoids with low half peak oxidation potentials showed higher antioxidant activity (measured by the inhibition of microsomal lipid peroxidation) than those with high potentials.[11] Furthermore, it has been postulated that flavonoids, due to their lower redox potentials, are thermodynamically able to reduce highly oxidizing free radicals, such as superoxide, peroxyl, alkoxyl and hydroxyl radicals.[14,22,23]

The studied 2-SC had previously been tested for their antioxidant capacity by the determination of their scavenging activity against ROS and RNS, where some of them showed strong effects.[10] By comparing the results from that study and the present one, the compounds with the lowest oxidation potentials (**1A**–**1D**) were also the most effective scavengers of ROS and RNS. These compounds have in common a cathecol group in the B-ring, which is known to be an important structural feature to the antioxidant activity.[14,24] Of note, in the present study, luteolin showed an $E_{p_{ox}}$ slightly more positive than the catecholic 2-SC, corresponding, in theory, to a less efficient antioxidant activity than 2-SC. Indeed, in our previous work,[10] luteolin was always a worse scavenger than **1A**. Taking into account that these two compounds only differ in the styryl moiety, it is expected that this structural feature contributes positively to the reducing capacity of the molecule. The mechanism behind the reaction of catechol-like compounds with radical derivatives consists in a two electron two proton process with the production of an *ortho*-quinone (Fig. 3a).[15,16,18] However, our cyclic voltammetry results suggest an oxidation reaction involving only one electron process. This phenomenon can be explained by the formation of a semiquinone (SQ) (or to use the more generic term, an aryloxyl radical), which undergoes

Table 2
ROS and RNS scavenging activity by 2-SC[10]

	$O_2^{\cdot-}$ [a]	H_2O_2 [b]	HOCl [c]	$^1O_2$ [d]	$ONOO^-$ without $NaHCO_3$ [c]	$ONOO^-$ with $NaHCO_3$ [c]
1A	28.8 ± 0.9	66.7 ± 2.2	3.9 ± 0.2	2.3 ± 0.4	0.26 ± 0.02	0.63 ± 0.03
1B	18.9 ± 1.2	50.8 ± 2.6	18.0 ± 1.8	3.7 ± 0.4	0.30 ± 0.03	0.61 ± 0.08
1C	46.6 ± 5.5	67.9 ± 1.2	16.9 ± 1.1	1.9 ± 0.3	0.26 ± 0.04	0.51 ± 0.05
1D	42.4 ± 6.4	44.1 ± 2.8	31.2 ± 1.5	2.7 ± 0.4	0.24 ± 0.02	0.44 ± 0.02
2A		10.6 ± 1.7	8.5 ± 0.3	72.3 ± 2.3	1.25 ± 0.14	1.21 ± 0.16
2B	17.7 ± 1.4		92.0 ± 7.1	93.4 ± 18.3	1.40 ± 0.13	1.65 ± 0.16
2C		16.9 ± 1.6		40.0 ± 4.3	1.04 ± 0.04	0.98 ± 0.13
2D	56.0 ± 13.5			105.0 ± 5.1	1.20 ± 0.17	1.23 ± 0.19
3A	125.0 ± 2.9		5.7 ± 0.7	81.3 ± 4.8	46.6 ± 7.0	40.6 ± 2.2
3B	55.9 ± 2.3		72.3 ± 5.0			

[a] IC_{27} (μM).
[b] % Effect at 125 μM.
[c] IC_{50} (μM).
[d] IC_{30} (μM).

disproportionation leading to the *ortho*-quinone formation and to the regeneration of the starting 2-SC (Fig. 3b).[25,26]

The 2-SC **2A**–**2D** showed a common peak, with $E_{p_{ox}}$ values of about +0.4/+0.5 V versus Ag/AgCl, corresponding to the oxidation of the 4′-OH group in the B-ring. Although apigenin has a similar substituent, the presence of that peak was not clear in this flavone. Nevertheless, the comparison of the results obtained for **2D** ($E_{p_{ox}}$ = +0.495 V vs Ag/AgCl) and **3D** (no detectable peaks), which only differ in the 4′-OH group, prove that the observed oxidation peak results from the oxidation of this substituent. The difference in the behaviour of these phenolic 2-SC and apigenin is likely to lie on the styryl moiety, which facilitates the electron delocalization from the B-ring contributing for the stabilization of the phenoxy radical that is formed during the redox reactions.[23,27] Accordingly, our group had already shown that apigenin was a worse scavenger of ROS and RNS than the 2-SC **2A** and **2B**.[10]

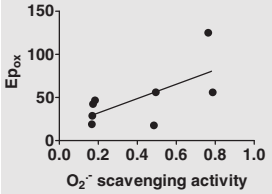
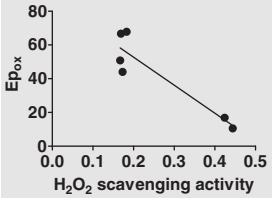
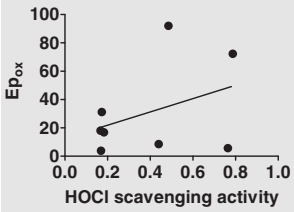
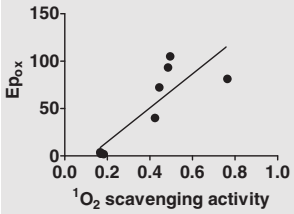
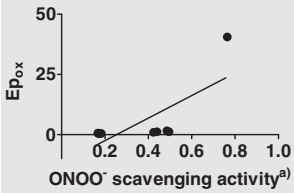
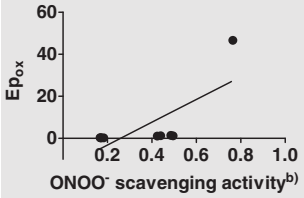
An oxidation peak with $E_{p_{ox}}$ values around +0.8 V versus Ag/AgCl was detected in the 2-SC **1A**–**1C**, **2A** and **3A**–**3C** as well as in the flavonoids chrysin, 5-hydroxyflavone and 7-hydroxyflavone (Table 1). This peak results from the oxidation of the 5-OH and/or 7-OH groups of the A-ring. The high $E_{p_{ox}}$ values point to a weak antioxidant capacity of the compounds that have hydroxyl substituents only in the A-ring. Indeed, the compounds **3A**–**3C**, chrysin, 5-hydroxyflavone and 7-hydroxyflavone have previously shown to be poor or even ineffective scavengers of ROS and RNS.

As expected, the compound **3D**, with no hydroxyl substituents, showed no redox peaks.

Correlations between the $E_{p_{ox}}$ of the first peak (Table 1) and the scavenging activity against ROS and RNS (Table 2) were analysed by using the Pearson correlation test. Significant correlations were found for H_2O_2, singlet oxygen ($^1O_2$) and peroxynitrite (Table 3), indicating that the scavenging mechanism against these reactive species is based on redox reactions. No significant correlation was found for superoxide radical ($O_2^{\cdot-}$) and hypochlorous acid (HOCl).

In conclusion, the scavenging effects of the studied 2-SC are related to their electrochemical behaviour. Oxidation potentials can be used as a general indicator of radical scavenging ability. The catecholic and phenolic 2-SC are more easily oxidisable than the corresponding flavones, probably due to the improvement of the electron-donating capacity of the molecule caused by the styryl moiety.

Table 3
Correlations between the $E_{p_{ox}}$ and the scavenging activity against ROS and RNS of the tested 2-SC[10]

Graph	Pearson *r*
	0.6527
	−0.9192**
	0.3782
	0.8591**
	0.7369*,a
	0.7345*,b

[a] With bicarbonate.
[b] Without bicarbonate.
* Significant at $p < 0.05$.
** Significant at $p < 0.01$.

4. Experimental

4.1. Materials

All the chemicals and reagents were of analytical grade. Luteolin, apigenin, 5-hydroxyflavone, 7-hydroxyflavone and aluminium oxide were obtained from Sigma–Aldrich. Chrysin was obtained from Aldrich. DMSO was obtained from Fluka. The other reagents were obtained from Merck.

4.2. Cyclic voltammetry

2-SC (**1A**–**1D**, **2A**–**2D** and **3A**–**3D**) were synthesised according to procedures described in the literature.[28]

Water purified by the Millipore Milli-Q system (conductivity < 0.1 μS cm^{-1}) was used in the preparation of solutions. Flavonoids and 2-SC were dissolved in DMSO and further diluted in a supporting electrolyte, consisting of a NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4 (ionic strength of 0.2), reaching a final concentration of 0.1 mM. To determine the effect of pH on the first oxidation peak potential, for compound **1B**, the pH was varied from 3.8 to 12.5. To cover the whole pH range, the following solutions were used as supporting electrolytes: $HCH_3COO/NaCH_3COO$, NaH_2PO_4/Na_2HPO_4, NH_4Cl/NH_3, KCl/NaOH.

Electrochemical measurements were carried out in an Autolab electrochemical system (Eco Chemie model PGSTAT 10), and data acquisition was accomplished through GPES software (Version 4.6). Voltammetric signals were recorded at room temperature. The working electrode was a glassy carbon electrode (3.0 mm diameter), a Ag/AgCl (KCl 3 M) electrode was used as reference and a carbon electrode was used as auxiliar. The glassy carbon

working electrode was polished with alumina 0.075 μm aqueous slurry before every experiment. Cyclic voltammograms were obtained by a single cycle performed at a scan rate of 100 mV s^{-1}. For the scan rate studies, the scan rate was varied from 10 to 200 mV s^{-1}. Voltammetric scans were carried out in the potential interval of −0.2 to +1.0 V versus Ag/AgCl.

4.3. Statistical analysis

Pearson's correlation test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

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II.3. ANTI-INFLAMMATORY POTENTIAL OF 2-STYRYLCHROMONES REGARDING THEIR INTERFERENCE WITH ARACHIDONIC ACID METABOLIC PATHWAYS

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Anti-inflammatory potential of 2-styrylchromones regarding their interference with arachidonic acid metabolic pathways

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ABSTRACT

Cyclooxygenases (COXs) are the key enzymes in the biosynthesis of prostanoids. COX-1 is a constitutive enzyme while the expression of COX-2 is highly stimulated in the event of inflammatory processes, leading to the production of large amounts of prostaglandins (PGs), in particular PGE_2 and PGI_2, which are pro-inflammatory mediators.

Lipoxygenases (LOXs) are enzymes that produce hydroxy acids and leukotrienes (LTs). 5-LOX metabolizes arachidonic acid to yield, among other products, LTB_4, a potent chemoattractant mediator of inflammation.

The aim of the present work was to evaluate the anti-inflammatory potential of 2-styrylchromones (2-SC), a chemical family of oxygen heterocyclic compounds, vinylogues of flavones (2-phenylchromones), by studying their COX-1 and COX-2 inhibitory capacity as well as their effects on the LTB_4 production by stimulated human polymorphonuclear leukocytes (PMNL).

Some of the tested 2-SC were able to inhibit both COX-1 activity and LTB_4 production which makes them dual inhibitors of the COX and 5-LOX pathways. The most effective compounds in this study were those having structural moieties with proved antioxidant activity (3′,4′-catechol and 4′-phenol substituted B-rings).

This type of compounds may exhibit anti-inflammatory activity with a wider spectrum than that of classical non-steroidal anti-inflammatory drugs (NSAIDs) by inhibiting 5-LOX product-mediated inflammatory reactions, towards which NSAIDs are ineffective.

1. Introduction

Arachidonic acid is the most abundant polyunsaturated fatty acid in the phospholipid bilayer of cell membranes. This second-messenger molecule is released by phospholipase A_2 in activated cells and further metabolized in different eicosanoids by the cyclooxygenase and lipoxygenase pathways (Fig. 1). Cyclooxygenases (COXs) are the key enzymes in the biosynthesis of prostanoids. In mammalian cells, COXs exist in at least two isoforms (COX-1 and COX-2). COX-1 is a constitutive enzyme, expressed in almost every cell type, responsible for the physiological production of prostaglandins (PGs) i.e., PGI_2, PGE_2, $PGF_{2\alpha}$, and PGD_2, in diverse organs and thromboxane A_2 in platelets. COX-2, on the other hand, is usually absent in most tissues (with the exception of kidney, parts of the brain, and gravid uterus where COX-2 is constitutive) but its expression can be readily induced by numerous stimuli such as growth factors, tumor promoters, or cytokines [1,2]. The earliest expression of COX-2 is detected in stromal cells, but it also can be found in multiple cells, like epithelial, endothelial, and stromal cells in several types of tumors [3]. In addition, COX-2 is highly expressed by cells that are involved in inflammation (e.g., neutrophils, macrophages, monocytes, mast cells, synoviocytes), emerging as the isoform primarily responsible for the synthesis of the prostanoids involved in pathological processes, such as acute and chronic inflammatory states. These inflammation-related cell types can be stimulated by lipopolysaccharide, phorbol esters, cytokines, or growth factors, producing large amounts of PGs, in particular PGE_2 and PGI_2, which are pro-inflammatory mediators that increase vascular permeability and promote edema at the sites of inflammation. Furthermore, these

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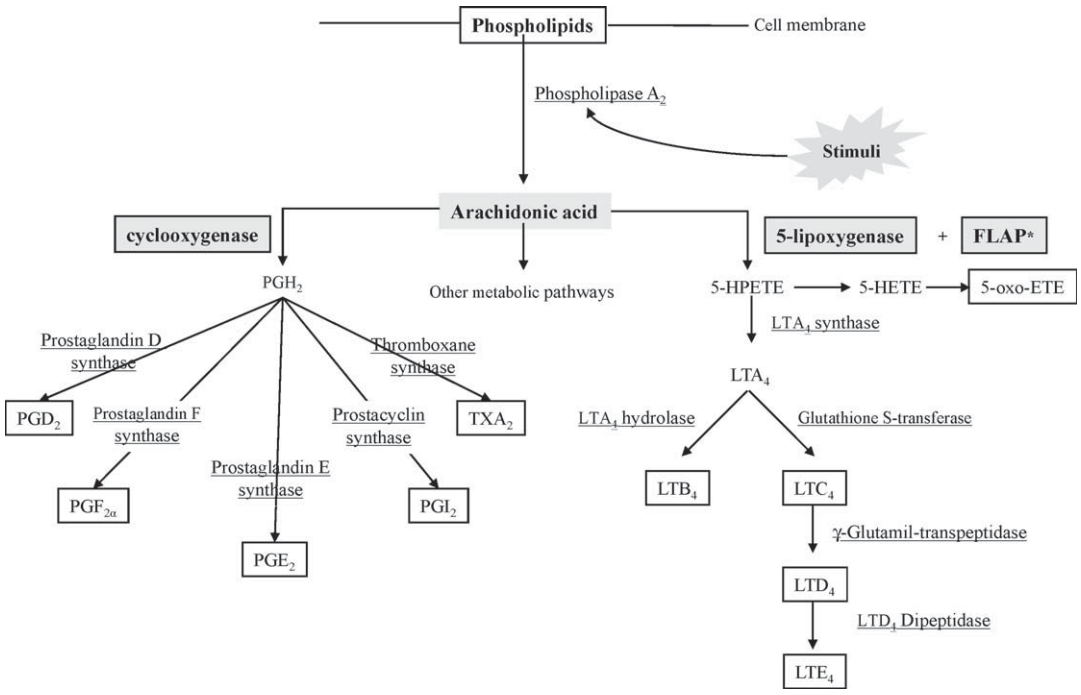


Fig. 1. Metabolism of arachidonic acid by the cyclooxygenase and 5-lipoxygenase pathways. (*) 5-Lipoxygenase-activating protein.

PGs are involved in the nociception of the inflammatory pain [4]. Thus, COX-2 isoform has been associated with acute and chronic inflammatory disorders [5] as well as in the carcinogenesis and tumor progression [3].

Lipoxygenases (LOXs) are enzymes that produce hydroxy acids and leukotrienes (LTs). From the LOXs existent in the mammalian tissues, 5-LOX, which is mainly found in cells of myeloid origin, i.e., polymorphonuclear leukocytes (PMNL), mast cells, macrophages, is the most implicated in inflammatory and allergical disorders [6]. 5-LOX metabolizes arachidonic acid to yield 5(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), which is further metabolized to the bioactive 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) by PMNL 5-hydroxyeicosanoid dehydrogenase [7], and various LTs (LTA_4-LTE_4) [8]. 5-oxo-ETE and LTB_4, produced by PMNL, are potent chemoattractant mediators of inflammation. LTB_4 stimulates neutrophil chemotaxis, enhances neutrophil-endothelial interactions, and stimulates neutrophil activation, leading to degranulation and the release of mediators, enzymes, and superoxide radicals [9], while 5-oxo-ETE is much more active than LTB_4 as an eosinophil chemoattractant [10]. LTC_4, LTD_4 and LTE_4, also known as Cys-leukotrienes, have shown to be essential mediators in asthma pathophysiology [11].

Although the major pathophysiological implication of LTs was considered to be the bronchial asthma, these eicosanoids contribute to the pathogenesis of other human inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, Crohn's disease, and psoriasis (see [11–13] for reviews). Recently, the 5-LOX pathway has also been associated with atherosclerosis, osteoporosis and certain types of cancer like prostate cancer [12].

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used remedy in inflammatory disorders. However, they cause several adverse effects, the most important being gastric injury up to gastric ulceration, renal failure and asthma [14]. On the other hand, the COX-2 selective drugs, generically known as COXIBs, have recently been a cause of controversy due to the enhanced cardiovascular risk they carry [13]. Thus, alternative therapeutic solutions, with similar anti-inflammatory potency but with fewer side effects, are needed, especially for the control of chronic inflammatory diseases, which implicate longer therapies.

2-Styrylchromones (2-SC) are a chemical family of oxygen heterocyclic compounds, vinylogues of flavones (2-phenylchromones), whose occurrence in nature has been reported [15,16]. Natural and synthetic 2-styrylchromones have been tested in different chemical and biological systems, showing activities with potential therapeutic applications [17–24]. The anti-inflammatory potential of 2-styrylchromones, concerning their interference with the arachidonic acid metabolic pathways, has not been explored so far. However, this may represent a promising field of research considering the potent inhibition of eicosanoids production by certain flavonoids containing structural similarities with 2-SC (see [25] for review). Therefore, the purpose of this work was to evaluate the inhibition of COX-1 and COX-2 activities, in a cell-free system, as well as the inhibition of LTB_4 production, in human PMNL, by a group of 2-SC (Fig. 2).

2. Material and methods

2.1. Reagents

All the chemicals and reagents were of analytical grade. Hanks' balanced salt solution (HBSS), Dulbecco's phosphate buffered saline, DMSO, nordihydroguaiaretic acid (NDGA), calcium ionophore (A23187), indomethacin, iron(II) chloride, iron(III) chloride, ascorbic acid, and arachidonic acid were obtained from Sigma–Aldrich (Steinheim, Germany). The "Leucotriene B_4 Enzyme Immunoassay (EIA) Kit" and "COX Inhibitor Screening Assay" were obtained from Cayman Chemical Co., Ann Arbor, MI, USA. Celecoxib was an offer from Pfizer. Potassium ferricyanide [$K_3Fe(CN)_6$] and trichloroacetic acid were obtained from Merck (Darmstadt, Germany).

2-Styrylchromones 1A–1D, 2A–2D and 3A–3D were synthesised by the three step Baker–Venkataraman method, starting from the *O*-protected 2′-hydroxyacetophenones and cinnamoyl chlorides [26]. The first step involves the *O*-acylation of appropriate 2′-hydroxyacetophenones with cinnamoyl chloride derivatives to give 2′-cinnamoyloxyacetophenones. These intermediates were converted into 5-aryl-3-hydroxy-1-(2-hydroxyaryl)-2,4-pentadien-1-ones by the base-catalysed Baker–Venkataraman rearran-

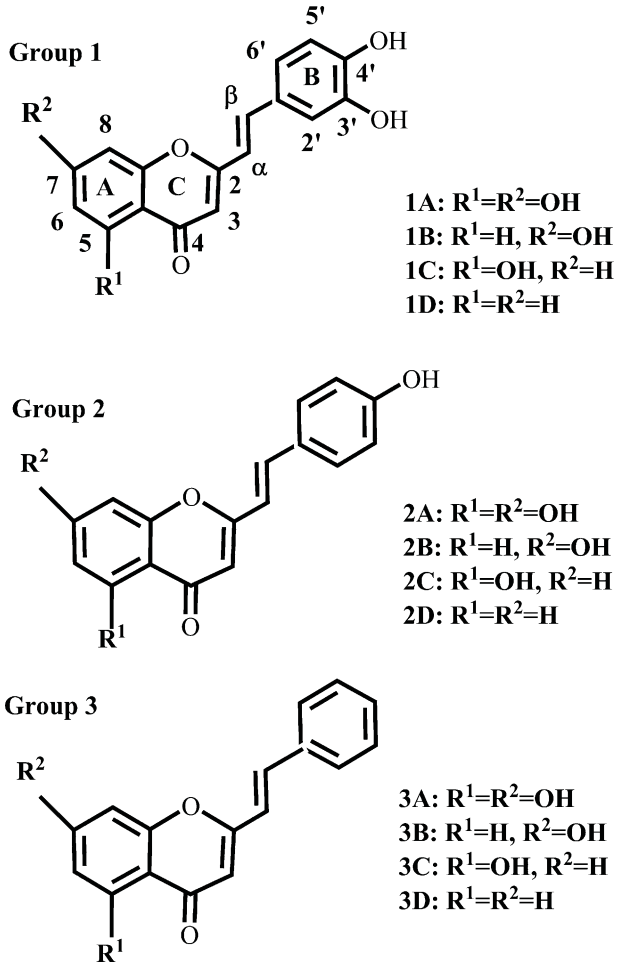


Fig. 2. Chemical structures of the tested 2-SC.

gement [27–29]. The cyclodehydration of these β-diketones with a mixture of DMSO and a catalytic amount of iodine or *p*-toluenesulfonic acid gives polybenzyloxy-2-SC, which were debenzylated by treatment with hydrogen bromide in acetic acid at reflux, to give the expected hydroxy-2-SC 1A–1D, 2A–2D and 3A–3D.

2.2. Equipment

A microplate reader (Synergy HT, BIO-TEK), was used to perform the spectrophotometric readings in all the assays.

2.3. Determination of LTB_4 production by human PMNL

Human PMNL were isolated from peripheral blood of healthy volunteers as previously described [30]. Neutrophil suspensions (5×10^6 cells/mL) in HBSS were pre-incubated at 37 °C for 10 min with the 2-SC (25 and 10 μM) or with the lipoxygenase inhibitor, NDGA (1 μM). The cells were subsequently incubated with A23187 (5 μM) and arachidonic acid (10 μg/mL) for 8 min. The reactions were stopped by the addition of cold methanol. Samples were subsequently centrifuged at 13,000 × *g* for 1 min, and the supernatants were stored at −70 °C until analysis. The amount of LTB_4 in the samples was measured using the above mentioned commercial EIA kit, according to the manufacturer's instructions. The results were expressed as the percent inhibition of control LTB_4 production. At least four determinations were done for each experiment.

2.4. COX-1 and COX-2 inhibition assays

The inhibition of COX-1 (ovine) and COX-2 (human recombinant) by 2-SC was determined in a cell-free system by quantifying the levels of $PGF_{2\alpha}$, produced by catalysis of arachidonic acid, using the above mentioned specific EIA kit according to the manufacturer's instructions. The COX inhibitors indomethacin and celecoxib were used as positive controls. The results were expressed as the percent inhibition of control COX-1 or COX-2 activity. At least four determinations were done for each experiment.

2.5. Determination of the reducing power

The reductive potential of 2-SC was determined according to a previously described method [31]. Test compounds, in 1 mL of a ultrapure water/DMSO 3:1 solution, were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1% w/v). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10% w/v) was added to the mixture, which was then centrifuged for 10 min at 3000 *g*. The upper layer of the solution (2.5 mL) was mixed with ultrapure water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1% w/v), and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as standard compound.

2.6. Determination of metal chelating activity

The metal chelating activity was assessed by monitoring the changes in UV–vis spectrum of 2-SC after addition of Fe(II) [32]. Titration experiments were performed by sequential additions of 2 μL of $FeCl_2$ solution (300 μM stock solution) to 300 μL of a 10 μM (final concentration) 2-SC solution. The titrations were performed in phosphate buffer 20 mM, pH 7.2.

2.7. Cytotoxicity

The effect of the 2-SC on the cell viability was assessed by the trypan blue exclusion method and by the release of LDH (as a measure of cell membrane integrity). The LDH activity was determined by following the rate of oxidation of NADH at 340 nm.

2.8. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multicomparison test using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California, USA, http://www.graphpad.com. When comparing with control group, values of *P* less than 0.05 were considered significant.

Pearson correlation tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California, USA, http://www.graphpad.com.

3. Results

3.1. Inhibition of LTB_4 production by human PMNL

All the tested 2-SC showed to have inhibitory effects on the LTB_4 production by human PMNL. 2-SC from group 1 were more effective inhibitors of LTB_4 production than the correspondent compounds from the other groups. Compounds 1A, 1C, and 1D, at the concentration of 25 μM, were able to inhibit the LTB_4 production with a ≈90% effect, while the compound 1B showed a 66.0 ± 12.4% effect at the same concentration (Fig. 3). These

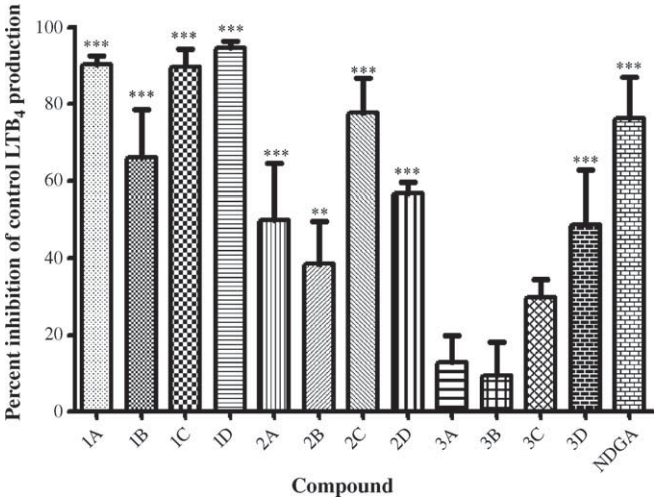


Fig. 3. Inhibition of human PMNL production of LTB_4 by 2-SC (25 μM) and NDGA (1 μM) determined by EIA. Each value represents mean ± SEM of at least four experiments performed in duplicate. (***) $P < 0.001$, (**) $P < 0.01$, (*) $P < 0.05$, significantly different from control.

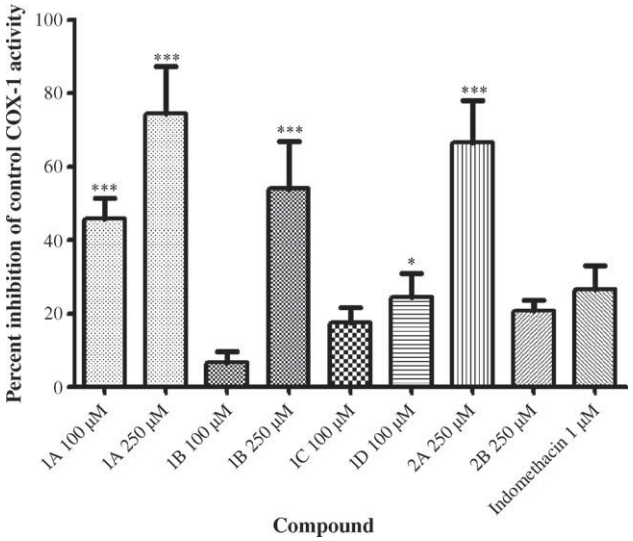


Fig. 4. Inhibition of COX-1 activity by 2-SC, determined by EIA. Each value represents mean ± SEM of at least 4 experiments performed in duplicate. (***) $P < 0.001$, (*) $P < 0.05$, significantly different from control.

compounds were still very effective at the lowest tested concentration (10 μM), 1C being the strongest inhibitor (80.9 ± 10.1% effect, $P < 0.001$) and 1B the weakest inhibitor (38.5 ± 11.8% effect, $P < 0.01$). 1A showed a 63.9 ± 9.2% effect ($P < 0.001$) and 1D showed a 62.3 ± 7.9% effect ($P < 0.001$). The compounds from group 2 were more effective inhibitors than the correspondent compounds from group 3. 2C was the most effective compound from group 2, reaching a 78.0 ± 9.0% effect at the concentration of 25 μM, while 3D was the most effective compound from group 3, showing a 48.9 ± 10.3% effect at the same concentration (Fig. 3). These compounds were still significantly effective at the concentration of 10 μM, where 2C showed an effect of 50.6 ± 11.9% ($P < 0.001$) and 3D showed an effect of 42.2 ± 9.6% ($P < 0.01$). The compounds 1B, 2B, and 3B were the weakest inhibitors in the respective groups. The 5-LOX inhibitor NDGA (1 μM) reached a 76.1 ± 11.0% inhibitory effect (Fig. 3).

3.2. Inhibition of COX-1 and COX-2 in a cell-free system

All 2-SC from group 1 were able to inhibit COX-1. 1A significantly inhibited the enzyme's activity at the concentrations of 100 μM (46.0 ± 5.6%) and 250 μM (74.4 ± 12.8%). 1B inhibition was only significant at 250 μM (54.2 ± 12.6%). Due to solubility issues, compounds 1C and 1D could only be tested at a maximum concentration of 100 μM, with 1D showing a significant inhibition (24.6 ± 6.4%). From group 2, only 2A and 2B where shown to inhibit COX-1, when tested at the concentration of 250 μM, 2A being a significant inhibitor (66.5 ± 11.4%) (Fig. 4). No other 2-SC was able to inhibit COX-1. None of the studied 2-SC inhibited COX-2 at the tested concentrations (100 μM and 250 μM). Indomethacin (1 μM) inhibited COX-1 (26.6 ± 6.6%) and COX-2 (92.6 ± 2.1%) and the selective COX-2 inhibitor celecoxib (10 μM) could only inhibit this isoenzyme (68.2 ± 3.0%).

3.3. Reducing power

From the tested 2-SC, compounds 1A–1D and 2B were able to reduce, significantly, ferric ion. Compounds from group 1 were shown to be more efficient reducers than ascorbic acid (Fig. 5).

3.4. Metal chelating activity

The UV–vis absorption spectrum of 2-SC 1A–1D showed a common absorption peak at 380 nm which presented bathochromic shift upon addition of growing amounts of Fe(II), increasing gradually until the 400 nm (Fig. 6).

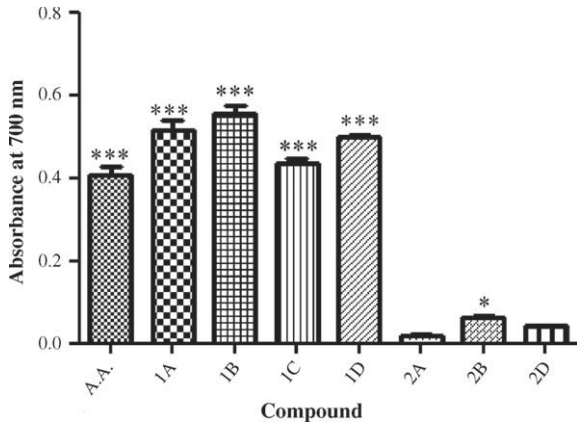


Fig. 5. Reducing activity of 2-SC and ascorbic acid (AA). All the compounds were tested at the final concentration of 25 μM. Each value represents mean ± SEM of triplicate measurements. (***) $P < 0.001$, (*) $P < 0.05$, significantly different from control.

No relevant changes were observed in the UV–vis spectrum of 2-SC from groups 2 and 3 upon addition of Fe(II).

3.5. Cytotoxicity

None of the 2-SC caused an increased in the release of LDH compared to the control. The cell viability, assessed by the trypan blue exclusion method, was above 95% for all the compounds.

3.6. Correlations between the inhibition of LTB_4 production and scavenging activity

Significant correlations were found between the inhibition of LTB_4 production, described in Section 3.1, and the scavenging activity for singlet oxygen ($^1O_2$), and peroxynitrite anion ($ONOO^-$) (these assays were performed in a previous work [24]).

4. Discussion

In this work, 2-SC were tested for their anti-inflammatory potential, through their capacity to interfere with the arachidonic

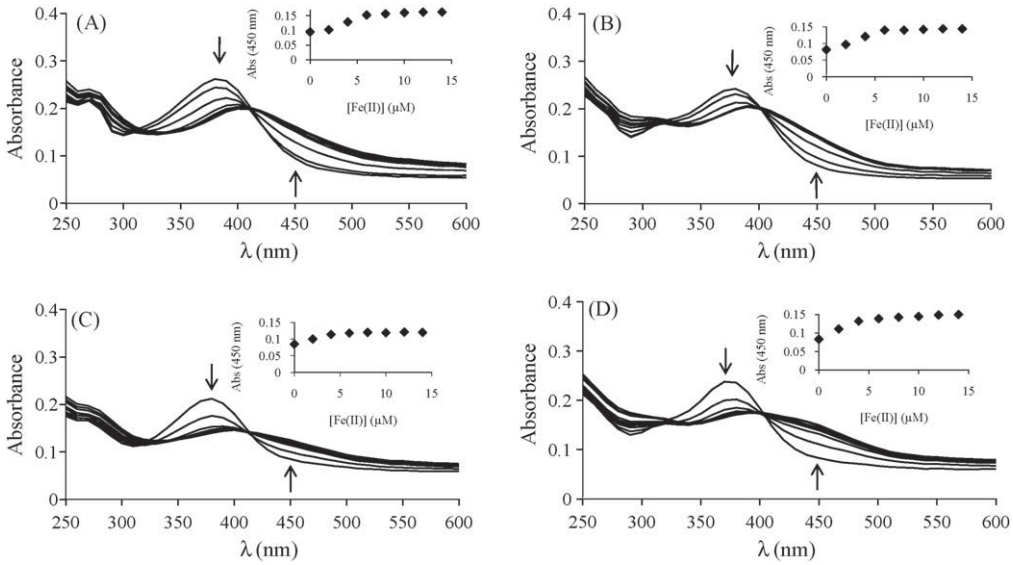


Fig. 6. UV–vis absorption spectrum of 10 μM of compounds (A) 1A, (B) 1B, (C) 1C, and (D) 1D in the presence of 0, 2, 4, 6, 8, 10, 12, 14, and 16 μM of Fe(II), in 20 mM phosphate buffer, pH 7.2. The direction of the arrows indicates crescent amounts of Fe(II). Insets: titration curves.

acid metabolic pathways, in particular, the COX-1 and COX-2 pathways, studied in a cell-free system, and 5-LOX pathway, studied in a cellular system using human PMNL. All the studied compounds were shown to inhibit the LTB_4 production by human leukocytes, especially those with a catechol substituted B-ring. This biological effect of 2-SC is shown for the first time, in the present study. As previously mentioned, 2-SC are vinylogues of flavones. Accordingly, Yoshimoto et al. [33] had previously found that the flavone structure represents a pharmacophore for 5-LOX inhibitory activity. In a recent theoretical study [34], a correlation was found between the lipoxygenase inhibitory activity of flavonoid molecules and their planar character, which probably facilitates the access, through a hydrophobic cavity, to the catalytic site of the enzyme. Considering this rationale, the planar character of 2-SC [16,29,35], conferred by the styryl link between the B-ring and the C-ring, may contribute to their effectiveness as 5-LOX inhibitors.

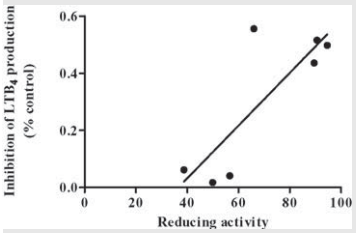
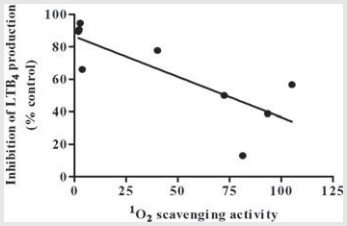
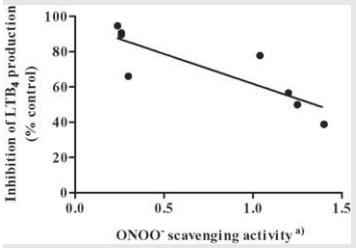
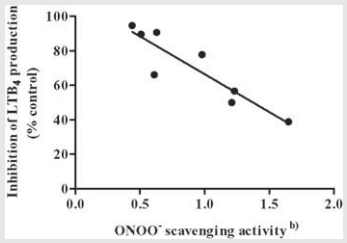
Several mechanisms, other than a direct inhibition of 5-LOX, can explain the observed inhibition of LTB_4 production by 2-SC. These include the inhibition of phospholipase A_2, and thus of the arachidonic acid release, the inhibition of LTA_4 hydrolase, or the blockade of 5-lipoxygenase activating protein. Nevertheless, it is conceivable that 2-SC, in conformity with flavonoids and other phenolic compounds, act as redox 5-LOX inhibitors. Catalytically active 5-LOX requires the conversion of Fe(II) to Fe(III), conferred by certain lipid hydroperoxides (LOOH). Most 5-LOX inhibitors act at the catalytic domain by reducing or chelating the active-site iron or by scavenging radical intermediates in the redox cycle of the iron [6,25]. Accordingly, the reduction of ferric ion is a possible mechanism of inhibition of LTB_4 production by 2-SC, since the most effective compounds (1A–1D) also behaved as strong reducers (Fig. 3 *vs.* Fig. 5). Indeed, significant correlation was found between the inhibition of LTB_4 production and the reducing power of the tested compounds (Table 1). On the other hand, the spectral changes of those compounds upon addition of Fe(II) indicate the formation of iron complexes. The similar changes in all the compounds indicate that the Fe(II)-binding site is between the *ortho*-hydroxyl groups in the B-ring, considered to be one "iron binding motif" in flavonoid molecules [32,36]. Furthermore, the Fe(II) titration curves reveal the formation of 1:2 Fe(II):compound complexes (Fig. 6). Besides indicating a possible mechanism of inhibition of LTB_4 production, the metal chelating capacity of the 2-SC from group 1, shown here for the first time, is also a very important indicator of their antioxidant activity. Finally, the capacity of some 2-SC to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS), demonstrated in a previous study [24], is also likely to contribute to their LTB_4 production inhibitory effect. Noteworthy, significant correlations were found between the inhibition of LTB_4 production observed in the present study and the scavenging activity for singlet oxygen ($^1O_2$), and peroxynitrite anion ($ONOO^-$) reported before (Table 1).

2-SC 1A, 1B, 1D and 2A where shown to significantly inhibit COX-1. This is also the first time that such biological activity of 2-SC is disclosed. On the other hand, none of the tested compounds was able to inhibit COX-2. Still, it is conceivable that this kind of effect might be seen at higher concentrations in accordance to what has been previously observed with a structural-similar compound (7,3′,4′-trihydroxyflavone) [37]. However, due to solubility issues, the studied 2-SC could not be tested at higher concentrations. On the other hand, several flavonoids have previously shown to inhibit COX-2 expression (see [38] for review). The flavonoid's structural features considered by others [39] as relevant for this kind of activity are shared by some of the tested 2-SC, specifically compounds 1A and 2A. Thus, 2-SC, particularly the two referred compounds, may still be COX-2 inhibitors *in vivo* despite the negative results obtained in the present study.

The mechanism through which 2-SC inhibit COX-1 is likely to consist in the scavenging of the radical intermediates involved in COX enzyme catalysis, especially the phenoxy radical formed on a tyrosine residue, according to what has been previously suggested for other compounds with antioxidant moieties [40]. This mechanism is consistent with the fact that the only effective 2-SC were those which have shown higher ROS and RNS scavenging effects in our previous study [24].

The capacity of some of the tested 2-SC to inhibit both COX-1 and LOX pathways plays in favour of their potential use as effective and secure anti-inflammatory drugs, when compared to the commonly used NSAIDs and COXIBs. It is well known that NSAIDs may cause several adverse effects such as gastric ulceration, renal failure and asthma. On the other hand, the safety of selective COXIBs has been recently questioned due to the apparent association of these drugs with an increased risk of cardiovascular events. The cardiovascular adverse effects are probably due to a reduction on the levels of PGI_2, an important anti-thrombotic

Table 1
Pearson correlations between the inhibition of LTB_4 production and the reducing activity and between the LTB_4 production and the scavenging activity against $^1O_2$ (IC_{30}) and $ONOO^-$ (with and without bicarbonate) (IC_{50}) [24] of the tested 2-SC.

Graph	Pearson (r)
	0.8254*
	−0.7951*
	−0.8482**
	−0.9056**

(a) Without bicarbonate; (b) with bicarbonate.
* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

eicosanoid, in concomitance to an excess of thromboxane A_2, which has the opposite effect [41–43]. Considering gastric effects, COX-2 selective inhibitors can delay the repair of existing gastrointestinal damage caused by ulcers, most likely by reducing the synthesis of PGs that are thought to play an important role in the healing process [44]. In fact, the inhibition of COX pathway has to be interpreted with careful because of the diversity of COX-derived mediators with different properties. Indeed, the PGE_2, which has been mostly considered a pro-inflammatory mediator, has, on the other hand, given proves of involvement in anti-inflammatory and pro-resolving responses [45–47]. Furthermore, the COX inhibition shunts the arachidonic acid metabolism toward the 5-LOX pathway enhancing the gastric mucosal damages due to the augmented production of LTB_4 [44,48] and inducing adverse reactions in patients with asthma as a result of the cys-LTs overgeneration [44].

According to Bertolini et al. [14], dual acting anti-inflammatory drugs may represent a breakthrough in the treatment of rheumatic diseases, in view of several important arguments: (i) the same molecule (i.e., one drug alone) inhibits both COXs and 5-LOX; (ii) the inhibition of both COX isoforms ensures a high anti-inflammatory efficacy and the concurrent preservation of the cardiovascular protective effects; (iii) the simultaneous inhibition of 5-LOX prevents pro-inflammatory and gastrointestinal damaging effects of leukotrienes. From our point of view, the use of dual inhibitors of the COX/5-LOX pathways seems advantageous to the treatment of other inflammatory disorders beyond rheumatic diseases. In fact, this type of compounds may exhibit anti-inflammatory activity with a wider spectrum than that of classical NSAIDs by inhibiting 5-LOX product-mediated inflammatory reactions towards which NSAIDs are ineffective [44].

Importantly, it has been demonstrated that the antioxidant effect of many NSAIDs may contribute to their therapeutic effectiveness [30,49–51]. Thus, the strong antioxidant effects of 2-SC will certainly contribute to their anti-inflammatory efficacy *in vivo*.

In conclusion, in the present study, several of the tested 2-SC were shown, for the first time, to prevent LTB_4 production by human leukocytes and to inhibit COX-1 activity. These effects, together with the well known antioxidant properties of these compounds may become a new therapeutic option in the treatment of inflammatory processes.

Acknowledgements

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II.4. INHIBITION OF NF-KB ACTIVATION AND CYTOKINES PRODUCTION IN THP-1 MONOCYTES BY 2-STYRYLCHROMONES

Manuscript submitted for publication

Inhibition of NF-κB activation and cytokines production in THP-1 monocytes by 2-styrylchromones

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Abstract

Nuclear factor kappa B (NF-κB) is one of the most important transcription factors whose modulation triggers a cascade of signaling events, namely the expression of many cytokines, enzymes, chemokines, and adhesion molecules, some of which being potential key targets for intervention in the treatment of inflammatory conditions.

The 2-styrylchromones (2-SC) designation represents a well recognized group of natural and synthetic chromones, vinylogues of flavones (2-phenylchromones). Several 2-SC were recently tested for their anti-inflammatory potential, regarding the arachidonic acid metabolic cascade, showing some motivating results. In addition, several flavones with structural similarities to 2-SC have shown NF-kB inhibitory properties. Hence, the aim of the present work was to continue the investigation on the interference of 2-SC in inflammatory pathways. Herein we report their effects on LPS-induced NF-κB activation and consequent production of proinflammatory cytokines/chemokine, using a human monocytic cell line (THP-1). From the twelve 2-SC tested, three of them were able to significantly inhibit the NF-κB activation and to reduce the production of the proinflammatory cytokines/chemokine. The compound 3',4',5-trihydroxy-2-styrylchromone stood up as the most active in both assays, being a promising candidate for an anti-inflammatory drug.

Keywords: Nuclear factor-κB; Proinflammatory cytokines; 2-Stryrylchromones; THP-1 monocytes; Anti-inflammatory

Introduction

Nuclear factor kappa B (NF-κB) is one of the most important transcription factors whose modulation triggers a cascade of signaling events, some of which are potential key targets for intervention in the treatment of inflammatory conditions. In resting cells, NF-κB, which is composed mainly of two proteins, p50 and p65, is present within the cytoplasm in an inactive state, bound to its inhibitory protein, IκB. However, a number of proinflammatory stimuli (cytokines such as tumour necrosis factor (TNF)-α or interleukin (IL)-1, oxidative stress, infectious agents) can activate NF-κB in different cell types. These inflammatory stimulations can initiate an intracellular signaling cascade leading to IκBα phosphorylation, by IκB kinase (IKK) complex, with its subsequent dissociation from NF-κB and degradation by the proteasome. Once liberated from its inhibitory protein, NF-κB translocates to the nucleus, where it orchestrates the transcription of a number of proinflammatory genes by binding to κB motifs found in the promoter or enhancer region. This activates the expression of many mediators of the inflammation including, cytokines, enzymes, chemokines, and adhesion molecules [1].

Lipopolysaccharide (LPS) is a surface component of gram-negative bacteria released upon host infection. Its interaction with Toll-like receptor 4 (TLR4) located in cell membrane, initiates a signal transduction pathway, which leads, among other events, to the activation of NF-κB [2]. In monocytes, LPS-dependent activation of NF-κB complexes induces a rapid but transient expression of a defined set of genes including TNF-α, IL-1β, IL-6 and IL-8 [3]. These proinflammatory mediators have shown to play important roles in the pathogenesis of chronic inflammatory and autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, or multiple sclerosis [4-7].

The 2-styrylchromones (2-SC) designation represents a well recognized group of natural and synthetic chromones, vinylogues of flavones (2-phenylchromones). As it occurs for flavones, several studies performed with 2-SC have disclosed interesting activities with potential therapeutic applications, possibly in the treatment of cancers [8-12], allergies [13], viral infections [14, 15], gout [16], and oxidative stress related damage [17]. In fact the antioxidant properties of 2-SC have been shown in cellular [17] and non cellular systems [18, 19]. In a recent study, performed by our group, a number of 2-SC was shown to have anti-inflammatory potential by inhibiting both the production of leukotriene B_4 in human neutrophils and the activity of the enzyme cyclooxygenase-1 in a non-cellular system [20]. Those promising results lead us to continue the investigation on the interference of 2-SC (Figure1) in inflammatory pathways. Herein we report their effects on LPS-induced NF-κB activation and production of TNF-α, IL-1β, IL-6 and IL-8 due to the above mentioned relevance of the NF-κB pathway in the inflammatory process and also to the fact that several flavonoids with structural similarities to 2-SC have shown interesting results in this

field (see [21, 22] for reviews). The present studies were performed with the THP-1 human monocytic leukemia cell line. These cells were chosen because they display many characteristics similar to human monocytes and have been used previously in the study of the inflammatory process [3, 23-26].

Materials and methods

Reagents

THP-1 human monocytic cell line, RPMI 1640 medium, penicillin-streptomycin, L-glutamine, fetal bovine serum (FBS), igepal, dithiothreitol, phenylmethanesulfonyl fluoride, aprotinin, leupeptin, pepsatin, and LPS from *Escherichia Coli* O26:B6 were obtained from Sigma-Aldrich (St Louis, MO). All the reagents used were of analytical grade.

2-Styrylchromones **1A**-**1D**, **2A**-**2D** and **3A**-**3D** were synthesised by the three step Baker-Venkataraman method, starting from the *O*-protected 2'-hydroxyacetophenones and cinnamoyl chlorides [27]. The first step involves the *O*-acylation of the appropriate 2'-hydroxyacetophenones with cinnamoyl chloride derivatives to give 2'-cinnamoyloxyacetophenones. These intermediates were converted into 5-aryl-3-hydroxy-1-(2-hydroxyaryl)-2,4-pentadien-1-ones by the base-catalysed Baker-Venkataraman rearrangement [28-30]. The cyclodehydration of these β-diketones with a mixture of DMSO and a catalytic amount of iodine or *p*-toluenesulfonic acid gives polybenzyloxy-2-SC, which were debenzylated by treatment with hydrogen bromide in acetic acid at reflux, to give the expected hydroxy-2-SC **1A**-**1D**, **2A**-**2D** and **3A**-**3D**.

Equipment

A microplate reader (Synergy HT, BIO-TEK), was used to perform the chemiluminescence and absorbance readings.

Cell culture

THP-1 human monocytic cells were maintained in RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mmol/L) and 10% FBS in a 5% CO_2 humid atmosphere at 37°C.

NF-κB activation

THP-1 cells ($1x10^6$ cells/mL) were cultivated in 6-well plates and incubated with the test compounds or vehicle (DMSO) for 90 min and subsequently stimulated with LPS (0.1 µg/mL) for 30 min. The nuclear extracts were then obtained according to a previously described method [3], with minor modifications. Cells were collected on ice, centrifuged (870 x g) at 4ºC, for 5 min, and ressuspended in 1 mL of cell lysis buffer [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 0.1 mM EGTA] supplemented with 2% igepal, 1 mM dithiothreitol and 0.25 mM phenylmethanesulfonyl fluoride. The samples were then incubated on ice for 15 min and centrifuged (13,000 x g) at 4ºC, for 5 min. The supernatant was removed and nuclear pellets were ressuspended in 100 µL of nuclear lysis buffer solution [20 mM Hepes (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, and 20% glycerol] supplemented with 1 mM dithiothreitol, 0.25 mM phenylmethanesulfonyl, and 5 µg/mL of each of the following protease inhibitors: aprotinin, leupeptin, and pepsatin. The mixture was kept on ice for 30 min, under intermittent agitation, and further centrifuged (13,000 x g) at 4ºC, for 10 min. The supernatant was collected and stored at -80 ºC. The protein content was measured using the DC Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, California, USA).

The activated NF-κB contained in the nuclear extracts was measured by immunoassay, using a commercially available ELISA kit (Trans AM NF-κB p50 chemi) from Active Motif (Carlsbad, CA), according to the manufacturer's instructions. Briefly, NF-κB was captured by binding to a consensus oligonucleotide (5′-GGGACTTTCC-3′) immobilized on a 96-well plate. The p50 subunit of NF-kB was determined by a chemiluminescent reaction using a specific primary antibody and a secondary horseradish peroxidase-conjugated antibody. The results were expressed as the reduction of p50-dependent chemiluminescence signal relatively to control (%).

Cytokines/chemokine production

THP-1 cells ($1x10^6$ cells/mL) were cultivated in 6-well plates and incubated with the test compounds or vehicle (DMSO) for 90 min and subsequently stimulated with LPS (0.1 µg/mL) for 14 h. The cultures were then collected into microcentrifuge tubes and centrifuged at 1,000 x g for 10 min. The supernatant was assayed for secreted cytokines (TNF-α, IL-1β, and IL-6), and chemokine (IL-8) using a commercially available ELISA Kit from SABiosciences (Frederick, MDbe), according to the manufacturer's instructions. The detection was made by reading the absorbance at 450 nm. The results were expressed as the reduction of cytokines/chemokine-dependent absorption at 450 nm relatively to control (%).

Cytotoxicity

The effect of the 2-SC on the cell viability was assessed by the lactate dehydrogenase (LDH) leakage (as a measure of cell membrane integrity). The LDH activity was determined by following the rate of oxidation of NADH at 340 nm [31].

Statistical analysis

The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multicomparison test using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California, USA, http://www.graphpad.com. When comparing with control group, values of *P* less than 0.05 were considered significant.

Results

NF-κB activation

Compounds **1C**, **2C**, and **2D** (50 μM) were able to significantly reduce the NF-κB activation induced by stimulation with LPS, compound 1C being the most effective. Therefore, this compound was tested at smaller concentrations exhibiting a concentration dependent effect, which was still significant at 25 μM (Figure 2).

Cytokines/chemokine production

The three compounds that inhibited the NF-κB activation induced by incubation with LPS were tested at the 50 μM concentration for their capacity to reduce proinflammatory cytokines/chemokine production. Thereby, compound **1C** totally inhibited the production of IL-6 and significantly reduced the production of IL-1β, TNF-α, and IL-8. Compound **2D** was able to significantly reduce the production of IL-1β, TNF-α, and IL-6 while **2C** could only significantly inhibit TNF-α and IL-6 production (Figure 3).

Cytotoxicity

None of the tested 2-SC under the concentration of 50 μM was cytotoxic to THP-1 cells after a 90 min incubation period, as verified by the LDH leakage assay. Compounds **1C**, **2C**, and **2D** were also further incubated for 16 h with the cells showing no cytotoxic effect (data not shown).

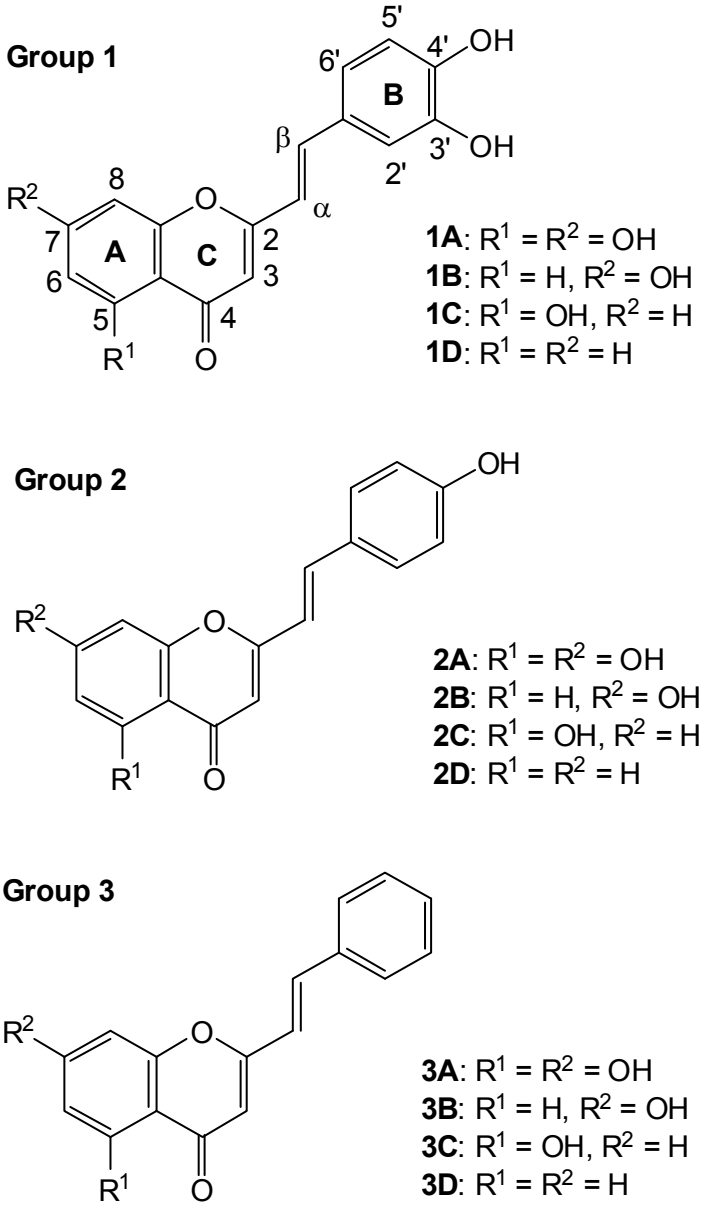

Figure 1 - Chemical structures of the tested 2-SC.

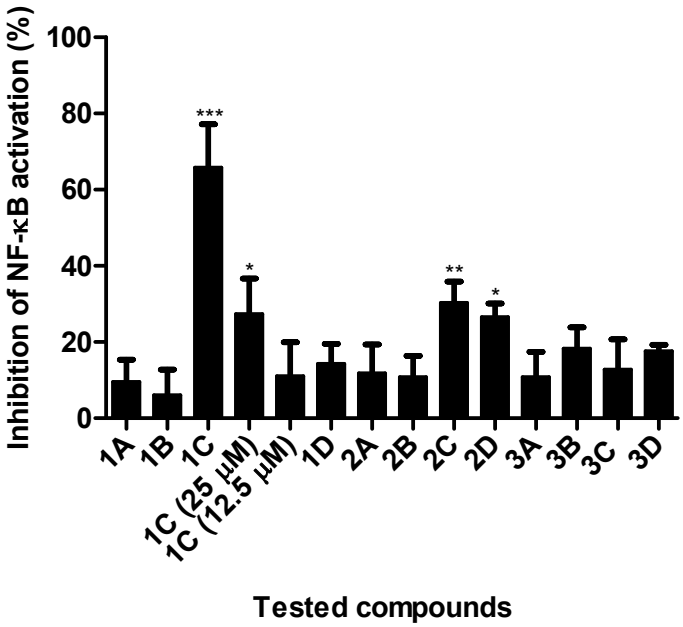

Figure 2 - Inhibition of LPS-induced NF-kB activation in THP-1 cells by 2-SC (**1A**-**1D**, **2A**-**2D**, **3A**-**3D**). Each bar represents the percentage of reduction of p-50-dependent chemiluminescence signal relatively to control. The compounds were tested at the concentration of 50 μM, except where otherwise indicated. The control represents LPS-stimulated cells with DMSO. Each value represents mean±SEM of four experiments. ***P<0.001, **P<0.01, *P<0.05, significantly different from control.

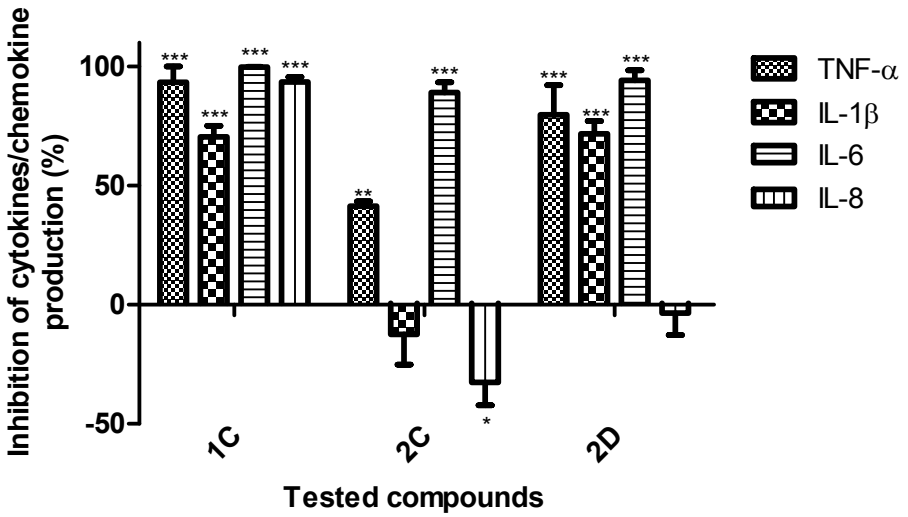

Figure 3 - Inhibition of LPS-induced production of TNF-α, IL-1β, IL-6, and IL-8 in THP-1 cells by 2-SC (**1C**, **2C**, and **2D**). Each bar represents the percentage of reduction of the respective cytokine/chemokine-dependent absorption at 450 nm relatively to control. The compounds were tested at the concentration of 50 μM. The control represents LPS-stimulated cells with DMSO. Each value represents mean±SEM of three experiments. ***P<0.001, **P<0.01, *P<0.05, significantly different from control.

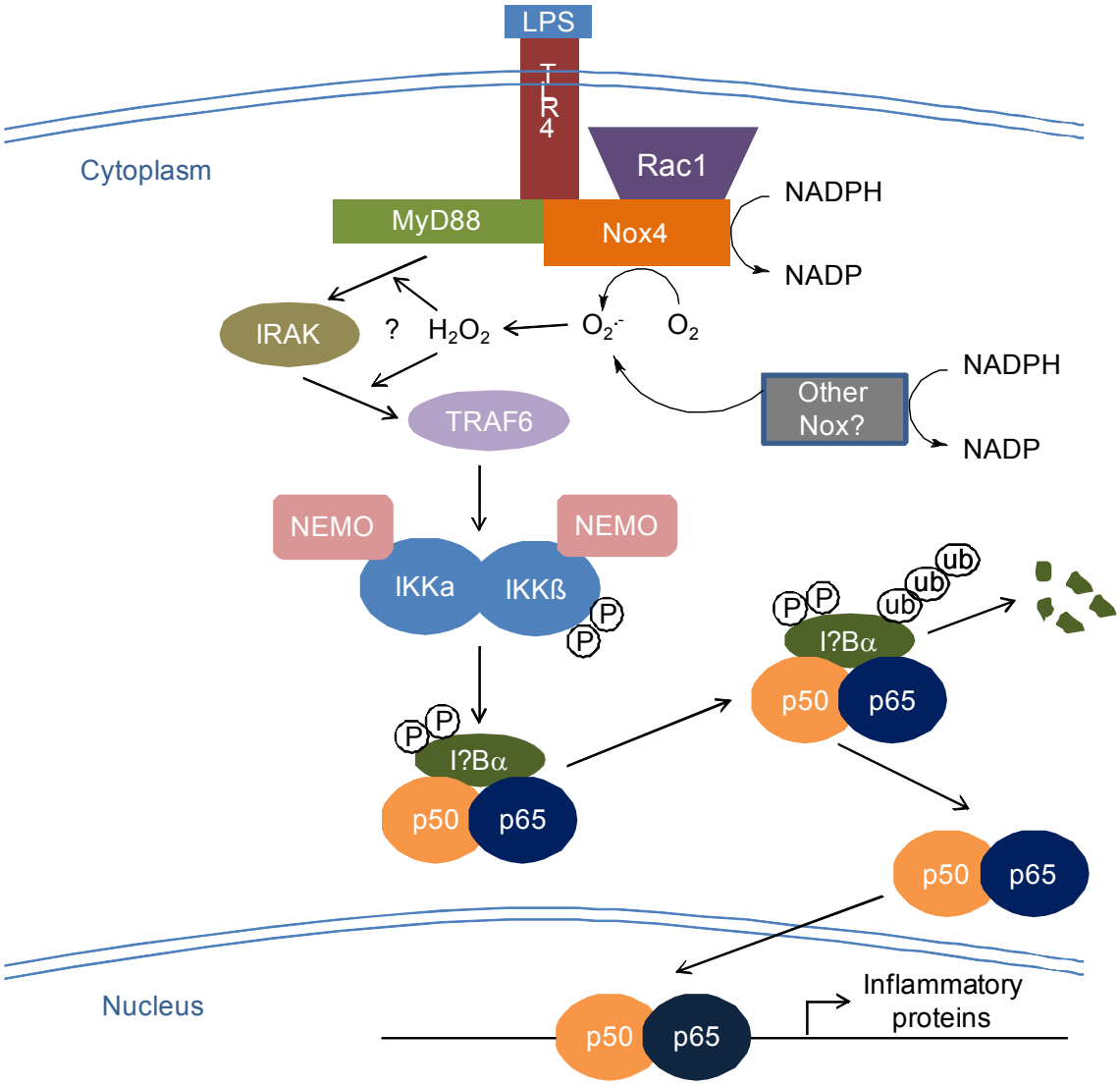

Figure 4 – LPS-induced NF-kB activation. The interaction of TLR4 with Nox4 and the subsequent production of ROS are key steps in the NF-kB activation. The possible role of another Nox enzyme is indicated. The NF-kB activation mediated by TLR4 follows the classical pathway: IKK-dependent IkBα phosphorylation (P) on Ser32 and Ser36, which induces ubiquitination (ub) and degradation of the inhibitory protein by the proteasome, thus allowing NF-kB to migrate into the nucleus and transactivate inflammatory genes. NEMO, NF-kB essential modulator (also called IKKγ). Adapted from [32].

Discussion

The involvement of NF-κB proteins in the development of common autoimmune and chronic inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, type 1 diabetes mellitus, thyroid autoimmune diseases, systemic lupus erythematosus as well as inflammatory bowel disease and psoriasis has been demonstrated by *in vitro* and *in vivo* data. Moreover, accepted therapeutic options such as glucocorticoids, cyclosporine, tacrolimus, non-steroidal anti-inflammatory drugs (NSAIDs), and sulfasalazine were shown to modulate the NF-κB pathway by several mechanisms. In addition, novel therapeutic strategies that specifically inhibit key elements of this pathway are being developed, showing some interesting results in animal models of the above mentioned diseases (reviewed in [33]).

The modulation of NF-κB activation by hydroxylated 2-SC was reported here for the first time. Some of the studied compounds had previously shown high scavenging activity against reactive oxygen and nitrogen species (ROS and RNS) [19]. Recent studies indicate that ROS production after IL-1β and LPS stimulation is a key messenger for subsequent NF-κB activation [32]. The mechanisms of NF-κB activation depend on the activator and on the cell type. Herein we focused on the LPS-induced NF-κB activation in phagocytic cells. LPS is recognized by TLR4, a member of the TLR family that is involved in innate immunity and inflammation response. Upon binding of LPS to TLR4, the cytoplasmic region of the receptor recruits MyD88 (myeloid differentiation primary response gene 88), which links TLR4 to IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor-6 (TRAF6) that mediates NF-kB activation. On the other hand, the activation of Rac1 and the subsequent production of ROS are key steps involved in NF-kB activation and TNF secretion in macrophages challenged with LPS. LPS-induced ROS generation and NF-kB activation was shown to be mediated by direct interaction of TLR4 with NADPH oxidase 4 (Nox4), a protein related to the NADPH oxidase 2 (Nox2) present in phagocytic cells, although another Nox enzyme might be involved (Figure 4) [32].

While the mechanism of NF-κB inhibition has been considered by some to be related to the antioxidant properties of the compounds [34, 35], the relevance of these properties to the activity of flavonoids was questioned by Comalada *et al*. [36] based on the observation that a good antioxidant like kaempferol was a worse NF-κB inhibitor than apigenin, which is a poor antioxidant. A similar situation occurred with 2-SC, i.e., compounds like **1A** and **1B**, which previously showed high ROS scavenging activity [19], could not inhibit the LPS-induced NF-κB DNA-binding, while compounds **2C** and **2D**, which are much less active scavengers, were effective in the present study. However, the lack of activity of good ROS scavengers such as **1A** and **1B** might be related to their poorer hydrophobicity, compared with the active compounds **1C**, **2C** and **2D**, which can difficult their entry into cells. This

rational comes in line with results from a previous study [18], in which compounds **1A**-**1D** were tested for their inhibitory effect on Cu^{2+}-induced oxidation of isolated human serum low density lipoproteins (LDL), an *in vitro* model of lipid peroxidation. In that work, compounds **1C** and **1D** were much more effective than **1A** and **1B**. The unexpected differences observed in the antioxidant effectiveness were suggested to be due to the different partitioning of 2-SC into LDL as a function of the number of hydroxyl groups in the molecule.

Meanwhile, diverse mechanisms have been suggested for the NF-κB inhibitory activity of flavonoids with structural similarities to 2-SC. Luteolin was shown to inhibit LPS-induced NF-κB activation by inhibition of IKK activity [21]. In addition the attenuation of IKK phosphorylation by this flavonoid was observed in LPS-stimulated macrophages [37]. In fact, IKK seems to be a target shared by some flavonoids as shown by the several studies demonstrating the inhibitory effect of compounds from this group on the activity of that kinase complex [38-41]. Noticeably, the function of conventionally used anti-inflammatory agents such as sulindac and salicylates has been re-evaluated and shown to be due, at least partially, to the interference with the IKK–NF-κB system [42]. Nonetheless, the NF-κB inhibition by flavonoids, flavones in particular, may be explained by other mechanisms. Thereby, apigenin was found to inactivate NF-κB by suppressing the LPS-induced phosphorylation of the p65 subunit at Ser536, a step subjacent to the interaction of p65 with other components of the basal transcriptional machinery, without affecting IκBα degradation or NF-κB DNA-binding [41]. On the other hand, nobiletin was shown to suppress NF-κB DNA-binding activity without affecting LPS-induced phosphorylation and degradation of IκBα protein, and nuclear translocation of NF-κB [43]. According to the authors nobiletin itself is unlikely to react directly with NF-κB given that direct addition of this flavone to nuclear extracts did not inhibit NF-κB DNA-binding. This suggests that a metabolite of nobiletin is responsible for the effect or that a nobiletin-induced product drives the inhibition of the interaction between NF-κB and DNA.

The proinflammatory cytokines TNF-α, IL-1β, and IL-6 and the chemokine IL-8 are known to be implicated in chronic inflammatory and autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease [4-7]. Furthermore, the clinical efficacy of drugs that target TNF-α, IL-1β, and IL-6 (e.g. monoclonal antibodies, receptor antagonists) has been well demonstrated in rheumatoid arthritis and Crohn's disease [4, 44-46]. The results obtained in this work show that **1C** is capable of reducing the production of TNF-α, IL-1β, IL-6, and IL-8, most likely by the previous inhibition of NF-κB activation. **2D**, however, was unable to reduce IL-8 production. The same happened with **2C**, which could not reduce IL-1β production as well. The fact that these two compounds are less potent inhibitors of NF-κB activation than **1C** may be an explanation for the different results. Furthermore, it cannot be forgotten that the production of the studied

proinflammatory mediators in monocytes is also regulated by the activator protein-1 (AP-1) transcription factor complex [47-49].

In this study, new inhibitors of NF-κB activity were found. Of all compounds tested **1C** showed superior activity in inhibiting this transcription factor and was also superior in reducing the production of all proinflammatory cytokines/chemokine tested. Thus, compound **1C** is a promising candidate for anti-inflammatory drug and should be tested in further preclinical studies, particularly those with animal models and those aimed to explain in more detail the mechanism of action of this molecule.

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CHAPTER III

DISCUSSION AND CONCLUSIONS

III.1. INTEGRATED DISCUSSION OF THE PERFORMED STUDIES

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 inflammatory and autoimmune diseases such as rheumatoid arthritis (RA) and Crohn's disease and when it is linked to an allergic response like asthma and anaphylactic shock [1]. An uncontrolled inflammatory response can manifest itself in several ways. Taking as example RA, probably the most studied chronic autoimmune disease, with an incidence of about 1% in the western population, the inflammation occurs essentially in the joints, with a pathophysiology characterised by hyper-proliferation of the synovial tissue and infiltration of blood derived cells, resulting in invasion and progressive erosion of cartilage and bone, which impairs range of movements and leads to deformity [2]. On the other hand, Crohn's disease is a type of inflammatory bowel disease which shows a transmural pattern of inflammation, meaning that the inflammation may span the entire depth of the intestinal wall. The activation of T cells and macrophages generates cytokines including interferon (IFN)-γ and tumour necrosis factor (TNF)-α, leading to release and activation of matrix metalloproteinases that digest the stromal tissue. Cytokine actions also change epithelial barrier function and lead to recruitment of neutrophils from the circulation which amplify tissue damage and migrate transepithelially, further increasing permeability and uptake of bacteria from the lumen [3]. This inflammatory state leads to a variety of symptoms including abdominal pain and diarrhea (which may be bloody), with several extraintestinal associated symptoms [4]. Due to the permanent affliction, disability, and, many times, premature death of the millions of patients suffering from these diseases, chronic inflammation is associated with severe socio-economic problems. Unfortunately, the available treatments aren't always sufficiently effective and many times present numerous and severe side effects especially in long-term use. Therefore, the pursuit for alternative therapeutic approaches in inflammation becomes imperative.

The interest for 2-SC properties began in the late 1970s with the evaluation of their antiallergic activity by Doria *et al*. [5]. Since then, several compounds from this group, with different patterns of substitution, were shown to possess various biological activities such as antitumor, antiviral, and antioxidant. However, the anti-inflammatory potential of 2-styrylchromone derivatives had not been explored so far. This seemed to us a promising field of investigation considering the good results obtained by other groups with structurally similar compounds i.e. flavonoids (reviewed in [6]). So a question emerged: what would be a good starting point to evaluate the anti-inflammatory potential of 2-SC? Our group's

extensive experience on reactive oxygen species (ROS) and reactive nitrogen species (RNS) detection techniques along with the knowledge that these species are overproduced in sites of inflammation led, as a first approach, to the study of the scavenging activity against ROS and RNS by a series of 2-SC.

The overproduction of ROS and RNS at inflammation sites has been well recognized [7-10]. In fact, it has been suggested by several studies that the anti-inflammatory activity of non-steroidal anti-inflammatory drugs (NSAIDs) may be due, in part, to their ability to interfere with ROS- and RNS-mediated reactions [11-18]. Furthermore, these reactive species are known to be involved in the pathogenesis of chronic inflammatory conditions such as RA, systemic lupus erythematosus, and inflammatory bowel disease [19, 20]. The results from the scavenging assays performed in the scope of this dissertation show that, generally, the 3',4'-dihydroxy derivatives (**1A-1D**) were the most effective compounds meaning that the catecholic B-ring is important to the molecule's effectiveness. By comparing the scavenging effects of 2-SC with structurally similar flavones, only differing from the former by the absence of the vinyl link between C-ring and B-ring (e.g. **1A** vs. luteolin), it became clear that this structural feature favors the reactivity of the molecule (Section II.1).

Our second study was performed with the aim of understand the relationship between the scavenging activities of 2-SC and their electrochemical behavior (Section II.2). Generally, the compounds that generated peaks with the lowest oxidation potentials (Ep_{ox}) had been the most effective scavengers of ROS/RNS. It became clear, in this study, which parts of the molecule were more reactive from the redox point of view. The 3',4'-dihydroxy substituent in the B-ring originated a peak with the lowest oxidation potential, followed by the peak originated by the 4'-hydroxy group. The 5-hydroxy and/or 7-hydroxy groups originated the peaks with the highest Ep_{ox}. These results confirmed our suspicions about the relevance of each substituent to the scavenging effect, supporting the theory that the catecholic B-ring confers the highest reactivity to the molecule. The superior reactivity of 2-SC *vs.* structurally similar flavones was also observed in this study and is probably due to the improved molecule's electron-donating capacity caused by the vinyl link, which is supposed to contribute to the stabilization of the aryloxyl radical formed in redox reactions by facilitating the electron delocalization from the B-ring. Finally this work demonstrated that the oxidation potential can be used as a generic indicator of the radical scavenging ability.

Our evaluation of the anti-inflammatory potential of 2-SC got on by studying the interference of these compounds in the pathways of the arachidonic acid metabolic cascade most relevant to inflammation (Section II.3). Since the disclosure, in 1971, that cyclooxygenase (COX) was the molecular target of NSAIDs [21, 22] and the first purifications [23, 24] and cloning [25, 26] of COX-1 (then simply called COX), the inhibition of this enzyme has been the most common approach for the development of anti-

inflammatory drugs. The discovery, in 1991, of an inducible COX isoform (now called COX-2) [27], which is highly expressed by cells that are involved in inflammation (e.g., neutrophils, macrophages, monocytes, mast cells, synoviocytes), led to the extensive search for selective inhibitors of COX-2 with the hope of finding new NSAIDs without the well known gastrointestinal side effects. These side effects have been attributed to the inhibition of the constitutive isoform (COX-1) and its consequent inhibition of the production of prostaglandins (PGs) with important physiological functions such as gastric cytoprotection. Indeed, the introduction in the market of the first COX-2 selective inhibitors (celecoxib and rofecoxib) was a success since these drugs accomplished the aims for which they had been proposed: clinical efficacy and gastric tolerability [28, 29]. However, the safety of selective COX-2 inhibitors (generically known as COXIBs) was questioned few years later due the increased risk of cardiovascular side effects, leading to the voluntary withdrawal of Vioxx® (rofecoxib) in 2004 by its producing company [30] and to the recommendation of restrictions in the use of COXIBs by the European Medicines Agency (EMEA) in 2005 [31]. Furthermore, there are increasing evidences that, contrarily to the previous beliefs, COX-2 plays a physiological role in several body functions such as gastric tissue repair, bone repair, and kidney homeostasis and that, on the other hand, COX-1 may be induced in sites of inflammation [32]. While the benefits of COX's (-1 or -2) inhibition are being questioned, the interest for the 5-lipoxigenase (LOX) pathway of the arachidonic acid cascade is growing. Leukotrienes (LTs), which are produced through this enzymatic pathway, are known to be involved in the pathogenesis of human inflammatory diseases such as asthma, RA, inflammatory bowel disease, and psoriasis [33-35]. However, to this date, drugs that interfere with the 5-LOX pathway such as 5-LOX inhibitors (zileuton) and LT receptor antagonists (montelukast, zafirlukast, pranlukast) are only being used in the therapeutics of asthma. Thus, the search for new therapeutic accomplishments based on the inhibition of this pathway is a field worthy of further research. The results described in Section II.3. produced very promising results, especially in what concerns to the inhibition of LTB_4 production by stimulated human polymorphonuclear leukocytes (PMNL). In accordance to the results of the scavenging assays, the most effective compounds in the LTB_4 assay were those carrying the catecholic B-ring (**1A-1D**). This fact, along with the iron reducing capacity of these compounds shown in this study, supported our hypothesis, based on the knowledge about flavonoids and other phenolic compounds, that 2-SC act as 5-LOX redox inhibitors, although this theory could not be totally proved. Nonetheless, the compounds **1A-1D** may inhibit 5-LOX by iron chelation as well, since they proved to form iron complexes in the presence of Fe(II). The results obtained in the COX-1/-2 inhibition assays showed a selective inhibition of COX-1 by compounds **1A**, **1B**, **1D**, and **2A**. It may be expected that these results origin some skepticism about the gastrointestinal safety of the referred 2-SC. However, one should not forget the importance of the concomitant

inhibition of 5-LOX pathway exhibited by the same compounds. It was previously suggested that the COX inhibition by NSAIDs shunts the arachidonic acid metabolism toward the 5-LOX pathway, enhancing the gastric mucosal damage due to the augmented production of LTB_4 [36, 37]. Additionally, the increased production of cys-LTs (LTC_4, LTD_4, LTE_4), known to be essential mediators in asthma pathophysiology, induces respiratory adverse reactions in predisposed patients [37]. In this scenario, the dual inhibition of COX and 5-LOX pathways should confer protection from the most common side effects of COX-1/-2 inhibitors while maintaining the well recognized therapeutic properties resultant from the inhibition of these isoenzymes, which could not be obtained, so far, with selective 5-LOX inhibitors.

The pathophysiology of common autoimmune diseases such as RA, multiple sclerosis, type 1 diabetes mellitus, thyroid autoimmune diseases, systemic lupus erythematosus as well as inflammatory bowel disease and psoriasis is known to involve the activation of nuclear factor kappa B (NF-κB). In turn, different inhibitors of key elements of the NF-κB pathway were shown to reduce or suppress inflammatory signs in animal models of some of those diseases (reviewed in [38]). Therefore, the inhibition of the NF-κB pathway is a promising therapeutic strategy for chronic inflammatory diseases. Thus, the initial aim of the fourth experimental work included is this dissertation was to evaluate the capacity of 2-SC to inhibit the LPS-induced NF-κB activation in a human monocytic cell line (THP-1) (Section II.4). In addition, the finding of three effective inhibitors (**1C**, **2C**, and **2D**) took us further in the investigation, specifically through the study of the influence of these compounds in the production of proinflammatory cytokines/chemokine [TNF-α, interleukin (IL)-1β, IL-6, and IL-8] in LPS-stimulated THP-1 cells, knowing the important role of NF-κB in their expression. We found out that all the three compounds were able to reduce the production of TNF-α and IL-6, while compounds **1C** and **2D** also reduced the production of IL-1β, and **1C** additionally reduced the production of IL-8. The results from this work achieved our best expectations since these biological properties, found for the first time in 2-SC, are in sight of the most recent therapeutic approaches for chronic inflammatory/autoimmune diseases. Actually, the development of inhibitors of the NF-κB pathway, in particular selective IKK inhibitors, has recently been a research strategy common to several pharmaceutical companies (reviewed in [39]). However, the clinical efficacy of this class of compounds is still to be proven. There are several other approaches to inhibit the NF-κB pathway other than IKK inhibition: regulation of NF-κB protein expression and binding to DNA; interference with the formation of the IKK complex; blockade of IKK-β activation process; proteasome inhibition; inhibition NF-κB nuclear translocation; and inhibition of NF-κB transcriptional activity [39]. So far, the only compound showing clinical efficacy was a proteasome inhibitor, bortezomib, when tested for the treatment of multiple myeloma. In fact, besides its role in inflammation, persistent NF-κB

activation has been suggested to contribute to cancer [40]. Studies with animal models show that IKKβ-dependent NF-κB activation is involved in the development of cancers secondarily to chronic inflammations, which may entail different mechanisms. On the one hand, NF-κB contributes to tumour promotion by inhibiting apoptosis with the consequent increase of cancer cell survival and resistance to drug and radiation therapies [41, 42]. On the other hand, NF-κB is required for expression of proinflammatory factors (e.g. COX-2) which are believed to contribute to tumour growth [41]. Thereby, the usefulness of drugs that inhibit the NF-κB pathway seems to be far broader that initially thought. It has to be remembered, however, the important physiological roles of the NF-κB pathway in the survival, maturation, and activation of lymphocytes, in their resistance to apoptosis, and in the generation of robust antibody responses [43]. Thus, special attention should be given to the risks that may arise from the therapeutic solutions based on NF-κB modulation such as immune suppression.

While the knowledge about the clinical efficacy and safety of inhibitors of NF-κB activation is still very limited, drugs that target a specific inflammatory cytokine, also known as biological agents, are being used for more than one decade in the therapeutics of RA and other autoimmune diseases. The first approved biological agents for the treatment of RA were inhibitors of TNF. There are now three available agents in this class: the monoclonal antibodies infliximab and adalimumab and the soluble TNF receptor fusion protein etanercept. Although TNF inhibitors are very effective at improving signs and symptoms and at slowing or preventing structural damage in patients with RA, about 30% of patients treated with these agents failed to achieve a 20% improvement in the American College of Rheumatology criteria [44]. Besides the TNF inhibitors, other biological agents are being used in the treatment of RA. One of them is the IL-1 receptor antagonist (IL-1Ra) anakinra. Although anakinra improves the clinical response to methotrexate, the most commonly used agent in the treatment of RA, controlled trials and clinical practice suggest that this drug is less effective than anti-TNF agents [45]. Recently, a humanized monoclonal antibody against IL-6 receptor antagonist, tocilizumab, was approved in EU to treat patients suffering from RA with insufficient response to methotrexate, or other disease modifying antirheumatic drugs, and TNF inhibitors [46, 47]. Even though biological agents have shown higher efficacy than the classical therapeutic options, these drugs are still used with some reluctance due the uncertainty about long-term safety and to their high cost. Furthermore, the fact that this kind of drugs is only available for intravenous or subcutaneous administration might be an inconvenient for therapeutic adhesion.

The experimental studies performed in the scope of this dissertation allowed to establish important structure-activity relationships about the tested 2-SC. Additionally, they enabled to find out, starting from twelve compounds, one that was among the most effective in practically all of the experiments performed. Thereby, compound **1C** was able to

scavenge all of the tested ROS and RNS, the latter with IC_{50s} lower than 1 μM (Figure 1). Then, it was shown to be capable of inhibiting the production of LTB_4, in stimulated human PMNL, for more than 80% at the concentration of 10 μM and approximately 90% at 25 μM. Although the capacity of 1C to inhibit COX-1 was not statistically significant at 100 μM, the results suggest that it probably would at a higher concentration (Figure 2). Finally, 1C was shown to inhibit the LPS-induced activation of NF-κB and production of proinflammatory cytokines/chemokine in THP-1 cells at the concentration of 50 μM (Figure 3). Thus, we believe that **1C** is worthy to be tested in further pre-clinical experiments and clinical trials as a potential anti-inflammatory or even anti-carcinogenic drug. We hope that this dissertation has contributed to the worldwide research of alternative anti-inflammatory drugs.

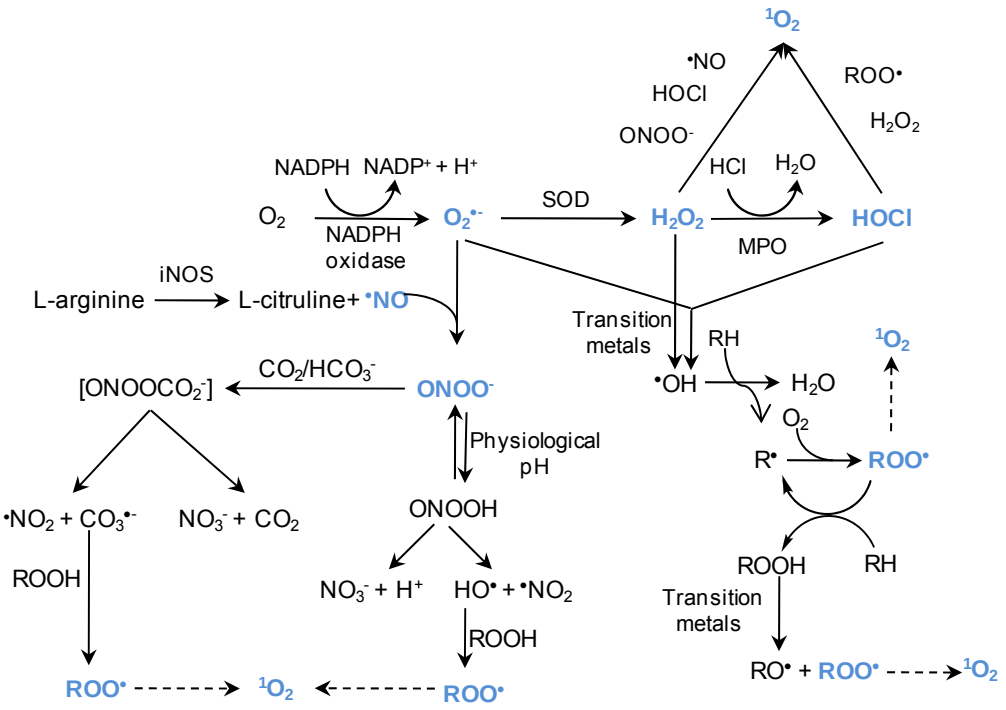

Figure 1 – ROS and RNS produced in the event of inflammatory processes (those which were shown to be scavenged by **1C** are detached in blue). For details about the generation of ROS and RNS during inflammatory processes please consult Section II.1.. $O_2^{\bullet-}$, superoxide radical; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; HO•, hydroxyl radical; HOCl, hypochlorous acid; $^1O_2$, singlet oxygen; MPO, myeloperoxidase; R•, carbon radical; RO•, alkoxyl radical; ROO•, peroxyl radical; ROOH, hydroperoxide; iNOS, inducible nitric oxide synthases; •NO, nitric oxide; ONOO$^-$, peroxinitrite; •NO$_2$, nitrogen dioxide radical; ONOOCO$_2^-$, nitrosoperoxycarbonate anion; $CO_3^{\bullet-}$, carbonate radical. Adapted from [48].

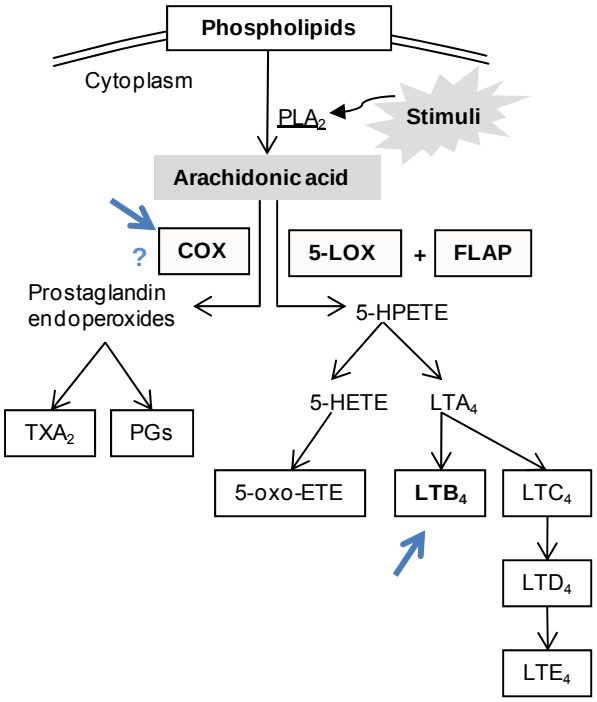

Figure 2 - Arachidonic acid metabolism by the COX and 5-LOX pathways (the blue arrows point to the molecules affected by compound **1C**). For details about the arachidonic acid metabolic pathway please consult Section II.3.. PLA_2, phospholipase A_2; FLAP, 5-lipoxygenase-activating protein; 5-HPETE, 5(*S*)-hydroperoxy-6,8,11,14-eicosa-tetraenoic acid; 5-HETE, 5(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-oxo-HETE, 5-oxo-6,8,11,14-eicosatetraenoic acid.

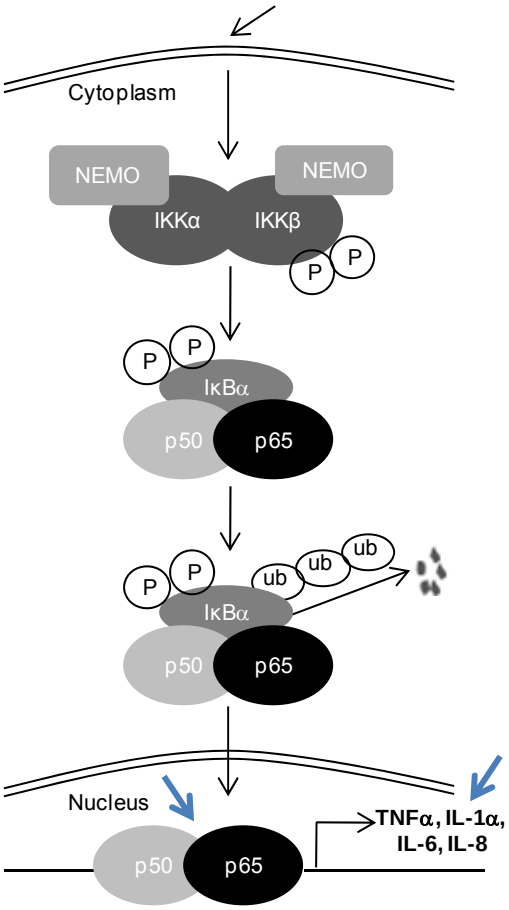

Figure 3 - Classical pathway of NF-κB activation (the blue arrows point to the molecules affected by compound **1C**). For details about the NF-κB activation please consult Section II.4.. NEMO, NF-kB essential modulator (also called IKKγ); IKK, IκB kinase; p, phosphate; ub, ubiquitin. Adapted from [49].

III.2. CONCLUSIONS

- From the studied 2-SC, those bearing a catecholic B-ring, i.e., the 3',4'-dihydroxy derivatives were, generally, the most efficient scavengers of reactive species, being able to scavenge all the ROS ($O_2^{\bullet -}$, H_2O_2, HOCl, $^1O_2$, $ROO^{\bullet}$) and RNS ($^{\bullet}NO$, $ONOO^-$);

- The ROS and RNS scavenging activity of the studied 2-SC depends on the hydroxylation pattern of the molecule, especially in the B-ring;

- The oxidation potential of 2-SC, measured by cyclic voltammetry, can be used as a generic indicator of the radical scavenging ability;

- Compounds **1A**, **1B**, **1D**, and **2A** were shown to be dual inhibitors of the COX and 5-LOX pathways. These compounds have structural moieties with proved antioxidant activity, such as 3',4'-dihydroxy and 4'-hydroxy substituents;

- Compounds **1C**, **2C**, and **2D** were able to inhibit the activation of NF-κB in the THP-1 human monocytic cell line, **1C** being the most effective;

- Compound **1C** totally inhibited the production of IL-6 and reduced the production of TNF-α, IL-1β, and IL-8 in THP-1 cells;

- From the twelve 2-SC tested, compound **1C** was the most effective in practically all of the experiments performed.

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