Trihydroxyflavones with antioxidant and anti-inflammatory efficacy

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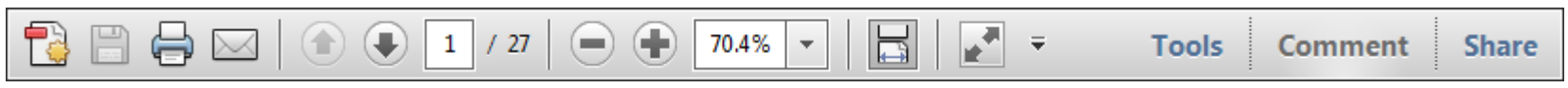

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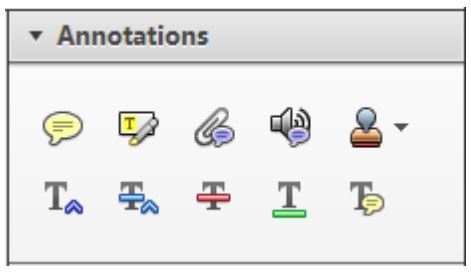

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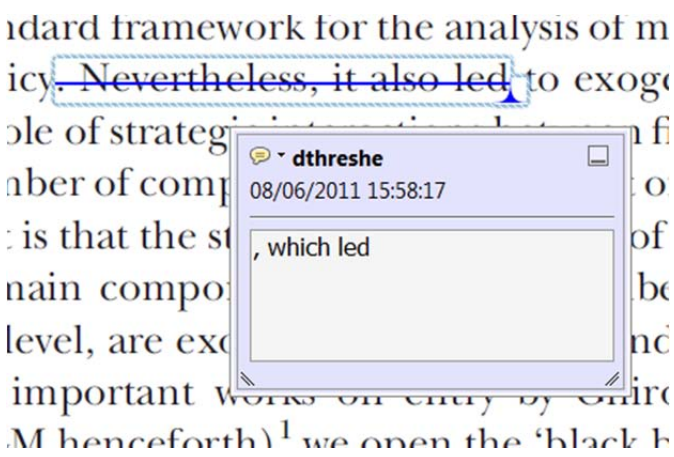

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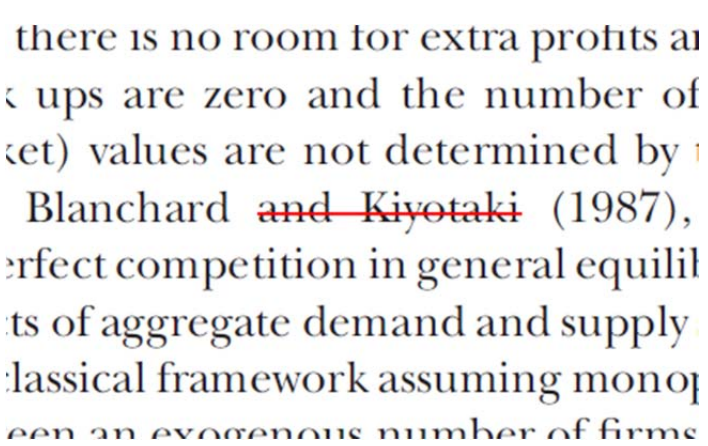

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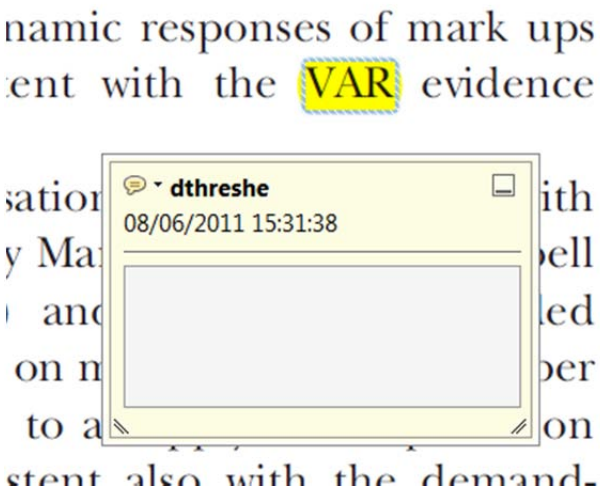

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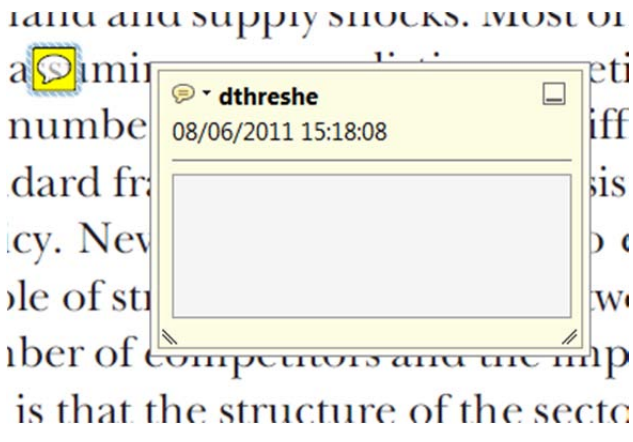

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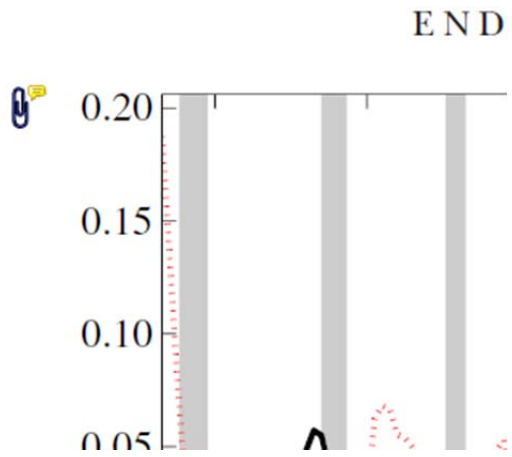

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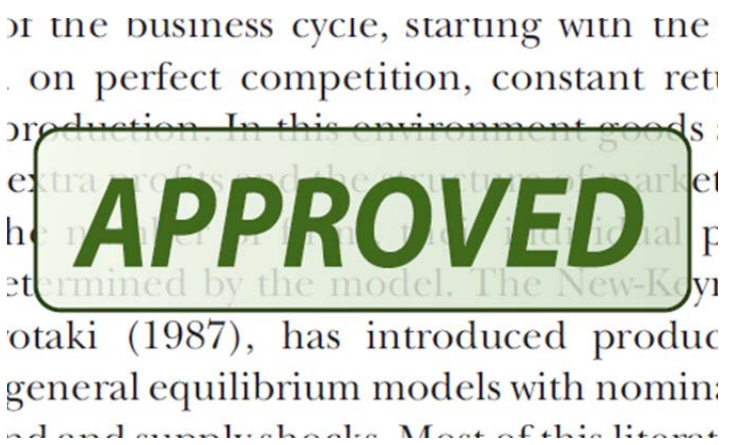

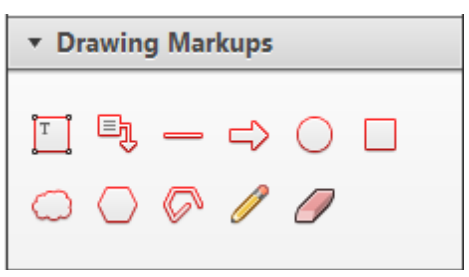

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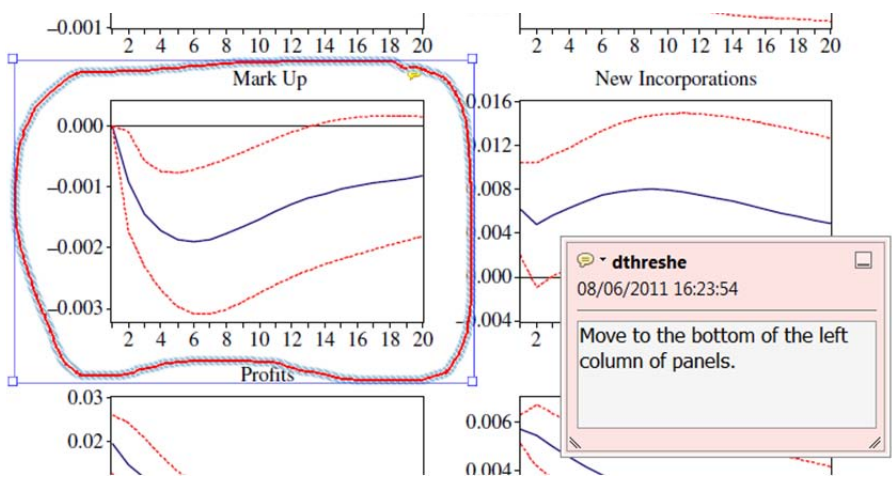

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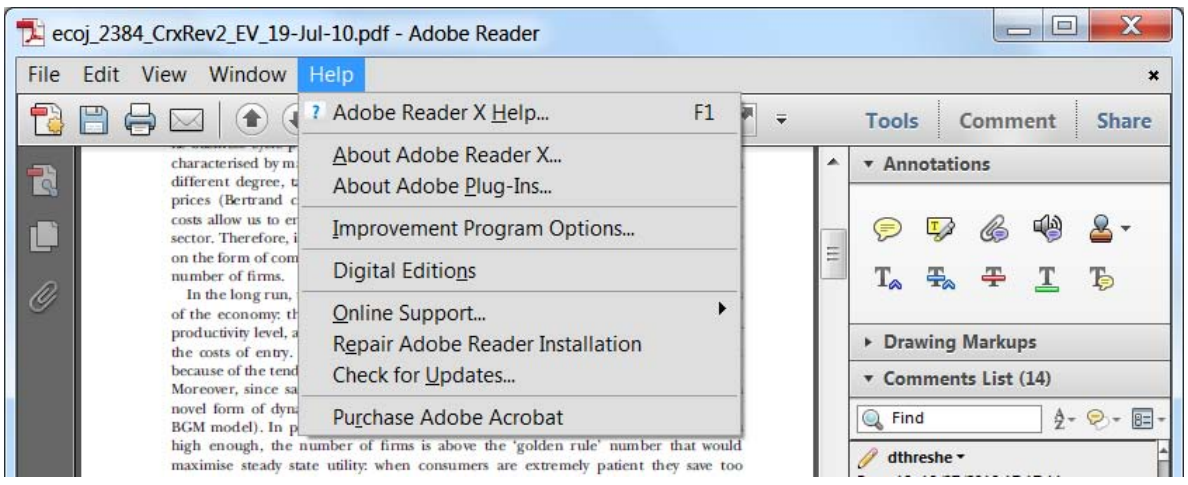

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Trihydroxyflavones with antioxidant and anti-inflammatory efficacy

*Ana Gomes[1,2] Diana Couto[1] Andreia Alves[1] Irene Dias[1] Marisa Freitas[1] Graça Porto[1] José Alberto Duarte[3] and Eduarda Fernandes[1]**

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Abstract.

The classical anti-inflammatory therapies are frequently ineffective and present numerous and severe side effects, especially in long term use, which requires the development of anti-inflammatory drugs with different scaffolds and mechanisms of action. Owing to the high antioxidant potential and anti-inflammatory activities already inferred for hydroxyflavones, we found it would be relevant to evaluate the anti-inflammatory potential of a series of trihydroxyflavones by testing their ability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells and cell-free systems and to inhibit the proinflammatory pathways mediated by the enzymes cyclooxygenase (COX) and 5-lipoxygenase (5-LOX), in which reactive species have a proven involvement. The tested trihydroxyflavones proved to be effective inhibitors of neutrophils' oxidative burst and were shown to scavenge different ROS and RNS in cell-free systems. The most active compound in the majority of the assays was 3,3′,4′-trihydroxyflavone, which was somehow expected due to the presence of the *ortho*-dihydroxy in the B-ring, an important structural feature in terms of free radical scavenging activity. Additionally, the studied compounds were able to inhibit the production of leukotriene B_4 by 5-LOX in activated neutrophils. 3,5,7-Trihydroxyflavone was able to inhibit both COX-1 and COX-2, which makes it a dual inhibitor of COX and 5-LOX pathways and, therefore, a promising candidate for a new therapeutic option in the treatment of inflammatory processes.

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Keywords: hydroxyflavones, oxidative burst, reactive oxygen species, reactive nitrogen species, cyclooxygenase-1, cyclooxygenase-2, 5-lipoxygenase

1. Introduction

Flavonoids have long been recognized to possess several biological activities and have been considered to produce beneficial effects in age associated diseases such as cardiovascular and neurodegenerative diseases and some forms of cancer [1]. The potential health-promoting properties of flavonoids have been attributed mainly to their antioxidant properties such as the capacity to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are overproduced in oxidative-stress related pathologies. One example of this overproduction is the one occurring in the inflammatory response, during phagocytosis, in cells such as neutrophils, monocytes, and macrophages. Despite being an important mechanism of host defense, the overproduction of ROS and RNS may provoke or exacerbate damage in inflammatory sites [2,3]. In fact, these reactive species are known to be involved in the pathogenesis of chronic inflammatory conditions such as rheumatoid arthritis (RA), systemic lupus erythematosus, and inflammatory bowel disease [4,5]. In addition, it has been suggested by several studies that the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs (NSAIDs) may be due, in part, to their ability to interfere with ROS- and RNS-mediated reactions [6–13]. All of this suggests the relevance of the antioxidant properties of flavonoids in the control of proinflammatory conditions.

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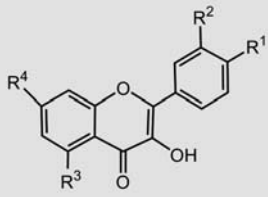
One of the most important mechanisms of action of the conventional anti-inflammatory therapies is the inhibition of cyclooxygenases (COXs). These enzymes, which exist in at least two isoforms (COX-1 and COX-2) are responsible for the biosynthesis of prostanoids from arachidonic acid in different mammalian cells. COX-1 is classically considered a constitutive enzyme responsible for the physiological production of prostaglandins and thromboxane A_2, while COX-2 is known to be highly expressed in cells involved in the inflammatory response to diverse stimuli and is considered as the isoform primarily responsible for the synthesis of the prostanoids implicated in pathological processes such as acute and chronic inflammatory states [14,15]. On the other hand, 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 COX-1 may be induced in sites of inflammation [16]. Leukotrienes (LTs) are produced through the 5-lipoxygenase (5-LOX) pathway of the arachidonic acid cascade. While LTC_4, LTD_4, and LTE_4, also known as Cys-leukotrienes, have shown to be essential mediators in asthma pathophysiology [17], LTB_4 is a potent chemoattractant mediator of inflammation. Although the major pathophysiological implication of LTs has been 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 [17–19] for reviews). However, to this date, drugs that specifically interfere with the 5-LOX pathway such as 5-LOX inhibitors and LT receptor antagonists are only being used in the therapeutics of asthma.

As it was pointed above, the classical anti-inflammatory medicines are based on the inhibition of the COX pathway and include NSAIDs and selective COX-2 inhibitors (COXIBs). While the use of NSAIDs is associated with several adverse effects such as gastric ulceration, renal failure and asthma, the safety of COXIBs has been recently questioned due to the apparent association of these drugs with an increased risk of adverse cardiovascular events [20–22]. It is known that the COX inhibition shunts the arachidonic acid metabolism toward the 5-LOX pathway enhancing the gastric mucosal damage due to the augmented production of LTB_4 [23,24] and inducing respiratory adverse reactions in predisposed patients as a result of the overgeneration of Cys-leukotrienes [23]. Thus, the pursuit for new therapeutic options with fewer side-effects than those of the presently available medicines is a matter of current interest. Knowing that flavonoids are promising molecules in this context, the aim of the present study was to test a series of flavone derivatives with the intent of finding new structures with anti-inflammatory potential. For this purpose, a group of trihydroxy-substituted flavones was tested, owing to the high antioxidant potential [25–27] and anti-inflammatory activities [28,29] already inferred for different flavones with this type of structure. Thus, the trihydroxy-substituted flavones depicted in Figure 1 were tested *in vitro* for their ability to scavenge ROS and RNS, as well as to prevent the oxidative burst and LTB_4 production by human neutrophils, and finally their potential to inhibit COX-1 and COX-2 activities.



	R1	R2	R3	R4
3,3',4'-Trihydroxyflavone	OH	OH		
3,5,7-Trihydroxyflavone (Galangin)			OH	OH
3,7,3'-Trihydroxyflavone		OH		OH
3,7,4'-Trihydroxyflavone	OH			OH

Fig. 1. Chemical structures of the tested trihydroxyflavones.

2. Material and methods

2.1. Reagents

All the chemicals and reagents were of analytical grade. Histopaque 1077, Histopaque 1119, dihydrorhodamine 123 (DHR), 30% hydrogen peroxide, ascorbic acid, sodium hypochlorite solution (with 4% available chlorine), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), β-nicotinamide adenine dinucleotide reduced (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), diethylenetriaminepentaacetic acid (DTPA), trypan blue, Hanks' balanced salt solution (HBSS), Dulbecco's phosphate buffered saline, nordihydroguaiaretic acid (NDGA), ~~DMSO~~, calcium ionophore A23187, arachidonic acid, indomethacin, lucigenin, luminol, phorbol myristate acetate (PMA), superoxide dismutase (SOD), amplex red, horseradish peroxidase (HRP) and 2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF) were obtained from Sigma-Aldrich (Steinheim, Germany). Histidine and tiron were obtained from Fluka Chemie GmbH. Quercetin was obtained from Aldrich. 4,5-Diaminofluorescein (DAF-2) was obtained from Calbiochem. Trihydroxyflavones were obtained from Indofine Chemical Company, Inc (Hillsborough, NJ). The "Leucotriene B4 Enzyme Immunoassay (EIA) Kit" and "COX Inhibitor Screening Assay" were obtained from Cayman Chemical, Ann Arbor, MI. Celecoxib was an offer from Pfizer.

2.2. Equipment

A multimode microplate reader (Synergy HT, BIO-TEK) was used in all the fluorescence, absorbance, and luminescence measurements.

2.3. ROS and RNS scavenging assays

2.3.1. Superoxide radical scavenging assay. The superoxide radical ($O_2^{\cdot-}$) was generated by the NADH/PMS/O_2 system and the $O_2^{\cdot-}$ scavenging activity was determined by monitoring the reduction of NBT to diformazan at 560 nm induced by this reactive specie, as previously described [30]. The production was controlled by superoxide dismutase (SOD), which inhibited NBT reduction in a concentration-dependent manner. The assay was performed at room temperature. The reaction mixtures in the sample wells contained the following reagents at the indicated final concentrations (in a final volume of 300 μL): NADH (166 μM), NBT (43 μM), the tested compounds at various concentrations (0.98–~~1000~~ μM),

dissolved in DMSO, and PMS (2.7 μM). NADH, NBT, and PMS were dissolved in 19 mM phosphate buffer, pH 7.4. Tiron was used as positive control. The scavenging effect of the tested compounds was expressed as the inhibition of $O_2^{\cdot-}$-induced reduction of NBT (% control). Each study corresponds to four experiments, performed in triplicate.

2.3.2. Hydrogen peroxide scavenging assay. The hydrogen peroxide (H_2O_2) scavenging activity was measured by monitoring the H_2O_2-induced oxidation of lucigenin, as previously described [30]. 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 (~~125~~–1000 μM), 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. Ascorbic acid was used as positive control. The effect of the tested compounds was expressed as the inhibition of H_2O_2-induced oxidation of lucigenin (% control). Each study corresponds to four experiments, performed in triplicate.

2.3.3. Hypochlorous acid scavenging assay. The hypochlorous acid (HOCl) was measured by using a previously described fluorescence methodology [30] based on the HOCl-induced oxidation of DHR to rhodamine 123. 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 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 reagents 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. Quercetin was used as positive control. The range of concentrations of the tested compounds was 0.98–~~1,000~~ μM (3,3′,4′-trihydroxyflavone and 3,5,7-trihydroxyflavone) and 31.2–~~1,000~~ μM (3,7,3′-trihydroxyflavone and 3,7,4′-trihydroxyflavone). The effect of the tested compounds was expressed as the inhibition of HOCl-induced oxidation of DHR (% control). Each study corresponds to four experiments, performed in triplicate.

2.3.4. Singlet oxygen scavenging assay. The singlet oxygen ($^1O_2$) scavenging activity was measured by monitoring the $^1O_2$-induced oxidation of nonfluorescent DHR to fluorescent rhodamine 123, as previously described [30]. $^1O_2$ was generated by 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 reagents 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, using the emission wavelength 528 ± 20 nm with excitation at 485 ± 20 nm. The fluorescence was measured after a 30-min incubation period. The range of concentrations of the tested compounds was 1.88–30.0 μM (3,3′,4′-trihydroxyflavone and 3,5,7-trihydroxyflavone) and 7.50-~~240~~ μM (3,7,3′-trihydroxyflavone and 3,7,4′-trihydroxyflavone). Quercetin was used as positive control. The effect of the tested compounds was expressed as the inhibition of $^1O_2$-induced oxidation of DHR (% control). Each study corresponds to four experiments, performed in triplicate.

2.3.5. Peroxynitrite scavenging assay. The peroxynitrite ($ONOO^-$) scavenging activity was measured by monitoring the $ONOO^-$-induced oxidation of nonfluorescent DHR to fluorescent rhodamine 123, as previously described [30]. $ONOO^-$ was synthesized by mixing an acidic solution (HCl 0.7 M) of H_2O_2 0.6 M with $NaNO_2$ 0.66 M in a Y junction. The reaction mixture was quenched with ice-cold NaOH 3 M. Residual H_2O_2 was removed by mixing with granular MnO_2 prewashed 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 (ε_{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 reagents 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. Quercetin was used as positive control. In a parallel set of experiments, the assays were performed in the presence of 25

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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 ($k_2 = 3-5.8 \times 10^4\ M^{-1}\ s^{-1}$) [31]. The range of concentrations of the tested compounds was 1.88–30.0 μM (3,3′,4′-trihydroxyflavone and 3,7,4′-trihydroxyflavone) and 3.91–125 μM (3,5,7-trihydroxyflavone and 3,7,3′-trihydroxyflavone), without bicarbonate and 1.88–30.0 μM (3,3′,4′-trihydroxyflavone and 3,7,4′-trihydroxyflavone), 0.98–125 μM (3,5,7-trihydroxyflavone) and 62.5–250 (3,7,3′-trihydroxyflavone), with bicarbonate. The effect of the tested compounds was expressed as the inhibition of $ONOO^-$–induced oxidation of DHR (% control). Each study corresponds to four experiments, performed in triplicate.

2.3.6. Nitric oxide scavenging assay. The nitric oxide ($^{\cdot}NO$) scavenging activity was measured by monitoring the $^{\cdot}NO$-induced oxidation of nonfluorescent DAF-2 to the fluorescent triazolofluorescein (DAF-2T), as previously described [30]. $^{\cdot}NO$ 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. The range of concentrations of the tested compounds was 0.03–1.00 μM (3,3′,4′-trihydroxyflavone), 0.12–30.0 μM (3,5,7-trihydroxyflavone), 15.0–1000 μM (3,7,3′-trihydroxyflavone) and 0.04–10.0 μM (3,7,4′-trihydroxyflavone). Quercetin was used as positive control. The effect of the tested compounds was expressed as the inhibition of $^{\cdot}NO$–induced oxidation of DAF-2 (% control). Each study corresponds to four experiments, performed in triplicate.

2.4. Isolation of human neutrophils by the density gradient centrifugation method

Venous blood was collected from healthy human volunteers by antecubital venipuncture, into vacuum tubes with K_3EDTA. The isolation of human neutrophils was performed by the density gradient centrifugation method as previously reported [32]. Isolated neutrophils were kept in ice until use. The neutrophils were from one volunteer per experiment. Cell viability and cell yield were evaluated by the trypan blue exclusion method, using a Neubauer chamber and an optic microscope with the 400x magnification. In the studies of human neutrophils' oxidative burst inhibition, Tris-G (25 mM Tris, 1.26 $CaCl_2.2H_2O$, 5.37 mM KCl, 0.81 mM $MgSO_4$, 140 mM NaCl, 0.55 mM D-glucose) was used as incubation medium.

2.5. Measurement of neutrophils' oxidative burst by the luminol amplified chemiluminescence assay

The chemiluminescent probe luminol has been thoroughly studied and used for monitoring reactive species production by neutrophils, namely $O_2^{\cdot-}$, H_2O_2, $HO^{\cdot}$, HOCl, $^{\cdot}NO$, and $ONOO^-$ [33]. The measurement of neutrophil's oxidative burst was undertaken by chemiluminescence, by monitoring the oxidation of luminol by neutrophil-generated reactive species, according to a previously described procedure [32]. Neutrophils (final suspension = 1 x 10^6 cells/mL) were preincubated with various concentrations of the tested compounds (0.16–10 μM for 3,3′,4′-trihydroxyflavone and 10–40 μM for the other three compounds) and luminol (500 μM) for 5 min at 37°C. After incubation, PMA (160 nM) was added. The reaction mixture was subjected to soft shaking at 37°C during the course of the assays. Kinetic readings were initiated immediately after cell stimulation and the values at the peak of the obtained curves were used for calculations. Quercetin was used as positive control. The effect of the tested compounds was expressed as the oxidation of luminol by neutrophil-generated reactive species (% control inhibition). Each study corresponds to three experiments, performed in duplicate.

2.6. Measurement of neutrophils' oxidative burst by the Amplex Red assay

Amplex red is a highly sensitive and chemically stable fluorescent probe for the extracellular detection of H_2O_2 [33]. Neutrophils (final suspension = 1 x 10^6 cells/mL) were preincubated with various concentrations of trihydroxyflavones (0.16–20 μM for 3,3′,4′-trihydroxyflavone and 1.25–40 μM for the other three compounds), amplex red (25 μM), and HRP (0.25 U/mL) for 5 min at 37°C. After incubation, PMA (160 nM) was added. The reaction mixture was subjected to soft shaking at 37°C during the course of the assays. The excitation and emission wavelengths used were 530 ± 20 and 590 ± 20 nm, respectively. Kinetic readings were initiated immediately after cell stimulation and the slope obtained was used to calculate the percentage of inhibition. Quercetin was used as positive control. The effect of the tested compounds was expressed as the oxidation of amplex red by neutrophil-generated H_2O_2 (% control inhibition). Each study corresponds to three experiments, performed in duplicate.

2.7. Measurement of neutrophils' oxidative burst by the APF assay

The fluorescent probe APF reacts with $HO^{\cdot}$, HOCl, and $ONOO^-$ [33]. Neutrophils (final suspension = 2 x 10^6 cells/mL) were preincubated with various concentrations of trihydroxyflavones (0.16–10 μM for 3,3′,4′-trihydroxyflavone and 3,5,7-trihydroxyflavone and 1.25–40 μM for 3,7,3′-trihydroxyflavone and 3,7,4′-trihydroxyflavone) and APF (2 μM) for 5 min at 37°C. After incubation, PMA (160 nM) was added. The reaction mixture was subjected to soft shaking at 37°C during the course of the assays. The excitation and emission wavelengths used were 485 ± 20 and 528 ± 20 nm,

respectively. Kinetic readings were initiated immediately after cell stimulation and the slope obtained was used to calculate the percentage of inhibition. Quercetin was used as positive control. The effect of the tested compounds was expressed as the oxidation of APF by neutrophil-generated reactive species (% control inhibition). Each study corresponds to three experiments, performed in duplicate.

2.8. Determination of LTB_4 production by human neutrophils

Human neutrophils were isolated from peripheral blood of healthy volunteers as described above. The cells were resuspended in HBSS (5×10^6 cells/mL) and placed in a 96-well microplate (140 μL/well) at 37° for 10 min to equilibrate. The tested compounds were then added to the reaction mixture and the plate was incubated at 37°C for another 10 min. Subsequently, the cells were activated with A23187 (5 μM) and arachidonic acid (10 μg/mL) for 8 min and the reactions were stopped by the addition of cold methanol. Samples were subsequently centrifuged at 13,000*g* for 5 min at 4°C and the supernatants were stored at −80°C until analysis. The amount of LTB_4 in the samples was measured using a commercial EIA kit from Cayman Chemical, according to the manufacturer's instructions. The 5-LOX inhibitor NDGA (10 μM) and quercetin (10 μM) were used as positive controls. Each study corresponds to three experiments, performed in duplicate.

2.9. COX-1 and COX-2 inhibition assays

The inhibition of COX-1 (ovine) and COX-2 (human recombinant) by the trihydroxyflavones was determined in a cell-free system by quantifying the levels of $PGF_{2\alpha}$, produced by the COX-dependent catalysis of arachidonic acid, using a commercial EIA kit from Cayman Chemical, according to the manufacturer's instructions. The COX inhibitors indomethacin (1 μM) and celecoxib (10 μM) were used as positive controls. Each study corresponds to three experiments, performed in duplicate.

3. Results

3.1. ROS and RNS scavenging activity

All the tested trihydroxyflavones revealed a concentration-dependent scavenging activity for $O_2^{\cdot-}$, except 3,5,7-trihydroxyflavone, which could not be tested due to its lack of solubility under the tested conditions. 3,3′,4′-Trihydroxyflavone was the most active and 3,7,3′-trihydroxyflavone and 3,7,4′-trihydroxyflavone revealed a similar activity (Table 1). Tiron, which was used as positive control, presented an IC_{50} of 241 ± 29 μM.

None of the tested trihydroxyflavones was able to scavenge H_2O_2 up to the concentration of 1 mM. The IC_{50} obtained for ascorbic acid was 1033 ± 157 μM.

All trihydroxyflavones were able to scavenge $^1O_2$ in a concentration-dependent manner, 3,3′,4′-trihydroxyflavone and 3,5,7-trihydroxyflavone being the most active ones, showing similar IC_{50} values between them, and 3,7,3′-trihydroxyflavone and 3,7,4′-trihydroxyflavone the least active ones, also with similar IC_{50} values (Table 1). The IC_{50} obtained for quercetin was 3.1 ± 0.4 μM.

Table 1
ROS scavenging effects (IC_{50}, mean ± SE) of the tested trihydroxyflavone

Tested compound	IC_{50} (μM) $O_2^{\cdot-}$	$^1O_2$	HOCl
3,3′,4′-Trihydroxyflavone	47 ± 4	14 ± 1	184 ± 6
3,5,7-Trihydroxyflavone[a]	–	17 ± 2	24 ± 5
3,7,3′-Trihydroxyflavone	140 ± 15	91 ± 11	293 ± 41
3,7,4′-Trihydroxyflavone	138 ± 3	86 ± 15	168 ± 19

~~Each study corresponds to four experiments, performed in triplicate.~~
[a] ~~Precipitated in the reaction media.~~

All of the tested compounds showed a concentration-dependent scavenging activity against HOCl. 3,5,7-Trihydroxyflavone was the most active and 3,7,3′-trihydroxyflavone the least active (Table 1). Quercetin presented an IC_{50} of 1.5 ± 0.2 μM.

All trihydroxyflavones were able to scavenge $ONOO^-$ either with or without $NaHCO_3$, each one showing a similar behavior in both situations, except 3,7,3′-trihydroxyflavone, which was much more active in the absence of $NaHCO_3$ than in its presence (Table 2). The IC_{50} values obtained for quercetin were 0.7 ± 0.1 μM (without bicarbonate) and 0.97 ± 0.04 μM (with bicarbonate). T2

Concerning the $^{\cdot}NO$ scavenging activity, 3,3′,4′-trihydroxyflavone, 3,5,7-trihydroxyflavone and 3,7,4′-trihydroxyflavone showed to be very active, with IC_{50} values in the nanomolar range, 3,3′,4′-trihydroxyflavone being the most active of the three. 3,7,3′-Trihydroxyflavone was also active but in much higher concentrations (Table 2). The IC_{50} obtained for quercetin was 0.39 ± 0.03 μM.

3.2. Inhibition of oxidative burst in human neutrophils

Luminol unspecifically detects $O_2^{\cdot-}$, H_2O_2, $HO^{\cdot}$, HOCl, $^{\cdot}NO$, and $ONOO^-$. Among the tested compounds, 3,3′,4′-trihydroxyflavone showed the strongest inhibition of luminol chemiluminescence, followed by 3,5,7-trihydroxyflavone, 3,7,4′-trihydroxyflavone and 3,7,3′-trihydroxyflavone (Table 3). The IC_{50} obtained for quercetin was 14 ± 3 μM. The results obtained with amplex red showed that 3,3′,4′-trihydroxyflavone and 3,7,4′-trihydroxyflavone were the compounds that inhibited H_2O_2 production by neutrophils more efficiently, followed by 3,7,3′-trihydroxyflavone and 3,5,7-trihydroxyflavone (Table 3). Quercetin presented an IC_{50} of 5.1 ± 0.5 μM. The results obtained with APF are in accordance with those obtained with luminol, 3,3′,4′-trihydroxyflavone being the strongest T3

Table 2
RNS scavenging effects (IC_{50}, mean ± SE) of the tested trihydroxyflavone. Each study corresponds to four experiments, performed in triplicate

	IC_{50} (μM)		
Tested compound	·NO	$ONOO^-$ without $NaHCO_3$	$ONOO^-$ with $NaHCO_3$
3,3′,4′-Trihydroxyflavone	47 ± 4	4.3 ± 0.3	5.1 ± 0.2
3,5,7-Trihydroxyflavone	–	10.5 ± 0.2	9.5 ± 0.9
3,7,3′-Trihydroxyflavone	140 ± 15	12 ± 1	643 ± 29
3,7,4′-Trihydroxyflavone	138 ± 3	6.6 ± 0.9	6.6 ± 0.7

inhibitor of APF oxidation, followed by 3,5,7-trihydroxyflavone. 3,7,4′-Trihydroxyflavone and 3,7,3′-trihydroxyflavone were less effective, showing approximate values of IC_{50} (Table 3). The IC_{50} obtained for quercetin was 3.1 ± 0.8 μM.

3.3. Inhibition of LTB_4 production by human neutrophils

All trihydroxyflavones were able to inhibit the LTB_4 production by human neutrophils in the tested concentrations (10 and 25 μM), with approximate levels of activity, except 3,7,3′-trihydroxyflavone which was slightly less active (Fig. 2). Quercetin (10 μM) showed a 96.9 ± 1.2 % effect and NDGA (10 μM) a 94.6 ± 2.8% effect.

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

From the tested trihydroxyflavones, only 3,5,7-trihydroxyflavone was able to inhibit both COX-1 and COX-2, in a concentration-dependent manner (12.5–100 μM), showing a similar level of inhibition to both enzymes in the different concentrations tested (Fig. 3). 3,7,3′-Trihydroxyflavone inhibited COX-1 in a concentration-dependent manner (25–200 μM) (Fig. 4). 3,3′,4′-Trihydroxyflavone, 3,7,4′-trihydroxyflavone and quercetin were unable to inhibit either COX-1 or COX-2 up to 200 μM. Indomethacin 1 μM inhibited COX-1 by 63 ± 5% and celecoxib 10 μM inhibited COX-2 by 82.3 ± 0.9%.

4. Discussion

The tested trihydroxyflavones proved to be effective inhibitors of neutrophils' oxidative burst, as shown by the results of luminol, amplex red, and APF assays. The chemiluminescent probe luminol has been thoroughly studied and used to monitor the production of reactive species by neutrophils, namely $O_2^{\cdot-}$, H_2O_2, $HO^{\cdot}$, HOCl, ·NO, and $ONOO^-$ [33]. The most active inhibitor of luminol's chemiluminescence was 3,3′,4′-trihydroxyflavone. This was expected due to the presence of the *ortho*-dihydroxy in the B-ring, which is an important structural feature for free radical scavenging activity [27,34]. APF is a fluorescence probe that reacts highly with reactive species such as HOCl, $ONOO^-$ and $HO^{\cdot}$ [35]. However, Setsukinai et al. [36] verified that after stimulating with PMA neutrophils loaded with APF and 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF), a fluorescence probe that only detects $ONOO^-$ and $HO^{\cdot}$, the fluorescence intensity of APF-loaded neutrophils greatly increased whereas that of HPF-loaded neutrophils did not change, showing that under these conditions APF allows a selective detection of HOCl. We were able to confirm that selectivity by using the myeloperoxidase inhibitor 4-aminobenzoyl hydrazide (ABAH). Myeloperoxidase is a heme protein present in azurophil granules of neutrophils that is released, upon cell activation, into the phagolysosome or into the extracellular space. This enzyme considerably contributes to the bactericidal capabilities of these cells via formation of HOCl from H_2O_2 and chloride ions [37,38]. The addition of ABAH to human

Table 3
Inhibitory effects of the tested trihydroxyflavone on the oxidation of luminol, amplex red and APF by reactive species generated by PMA-stimulated neutrophils

	IC_{50} (μM)		
Tested compound	Luminol	Amplex red	APF
3,3′,4′-Trihydroxyflavone	4.6 ± 0.6	6.1 ± 1.7	0.6 ± 0.1
3,5,7-Trihydroxyflavone	18.2 ± 1.5	21.9 ± 7.5	1.7 ± 0.6
3,7,3′-Trihydroxyflavone	27.5 ± 2.7	15.8 ± 0.7	11.3 ± 4.1
3,7,4′-Trihydroxyflavone	24.7 ± 3.8	6.9 ± 0.7	12.1 ± 3.4

Each study corresponds to three experiments, performed in duplicate.

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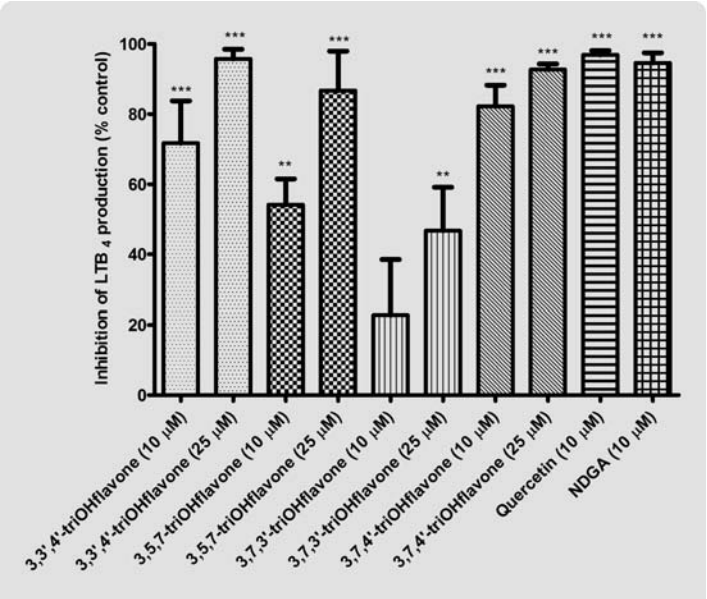

Fig. 2. Inhibition of LTB_4 production in human PMNL by the studied trihydroxyflavones determined by EIA. Each value represents mean ± SEM of three experiments, in duplicate. ***$P < 0.001$, **$P < 0.01$, compared to control.

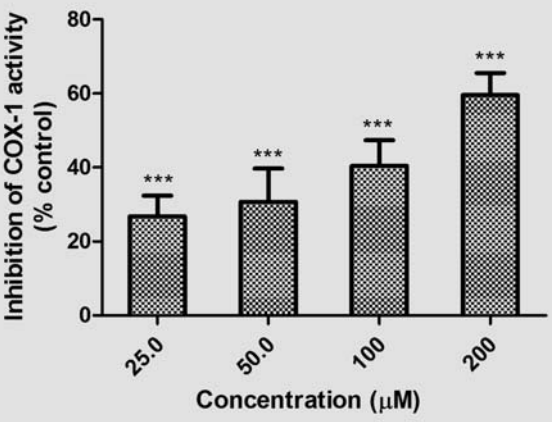

Fig. 4. Inhibition of COX-1 activity by 3,7,3′-trihydroxyflavone, determined by EIA. Each value represents mean ± SEM of three experiments, in triplicate. ***$P < 0.001$, compared control.

neutrophils which were subsequently stimulated with PMA decreased the APF-dependent fluorescence signal to the level of the control assay, ruling out the involvement of $HO^{\bullet}$ and RNS (data not shown). Taking this into account, it could be expected to obtain similar results, in terms of compounds' order of activity, in the APF assay and the HOCl scavenging assay. That ~~didn't~~ happen and the reason might be related to the different capacity of the compounds to penetrate the cell membrane since APF is able to reach the intracellular media [36]. Thus, a compound that penetrates the neutrophil cell membrane probably will achieve a better location to inhibit the fluorescence signal. This becomes evident when we compare the results of 3,3′,4′-trihydroxyflavone and quercetin. Although quercetin was shown to be a better scavenger of HOCl, this flavonol was a worse inhibitor of APF-dependent fluorescence, most probably due to the presence of two additional hydroxyl substituents in its molecule, making it less lipophilic than 3,3′,4′-trihydroxyflavone. The specificity of amplex red to detect H_2O_2 gives us information about the capacity of the test compounds to scavenge this reactive species or to inhibit its production. As it was shown by the results of the H_2O_2-dependent oxidation of lucigenin, the tested trihydroxyflavones were unable to scavenge H_2O_2, meaning that they act upstream by scavenging $O_2^{\cdot-}$, which was shown in the NBT reduction assay, and might as well inhibit the enzyme NADPH oxidase. In fact, 3,7,4′-trihydroxyflavone, which showed a $O_2^{\cdot-}$-scavenging activity similar to 3,7,3′-trihydroxyflavone, was able to inhibit the fluorescence caused by the oxidation of amplex red more efficiently than the latter. This comes in agreement with the structure-activity relationships of NADPH oxidase inhibition postulated by Steffen et al. [39] in which is referred the importance of a 4′-hydroxy substituent in the B-ring.

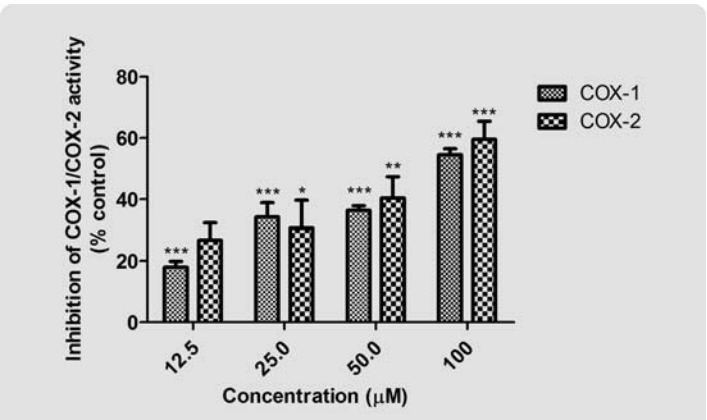

Fig. 3. Inhibition of COX-1 and COX-2 activity by 3,5,7-trihydroxyflavone, determined by EIA. Each value represents mean ± SEM of three experiments, in duplicate. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, compared with control.

The results from the scavenging assays are in agreement with previous findings [27,34] about the structural features that are essential for an effective free radical scavenging activity, such as the *ortho*-dihydroxy groups, like the one in the B-ring, present in 3,3′,4′-trihydroxyflavone, and the combination of 3- and 5-OH groups with the 4-oxo group, present in 3,5,7-trihydroxyflavone. The importance of 4′-hydroxy is also evident, particularly from the results of RNS scavenging assays in which 3,7,4′-trihydroxyflavone shows high activity. The 5,7-dihydroxylated A-ring assumes great importance to the HOCl-scavenging effect as it can be seen by the high effectiveness of 3,5,7-trihydroxyflavone. According to previous findings [40], this can be explained by the formation of a stable dichlorinated product modified at the

C6 and C8 sites of the A-ring, in which 5-hydroxy and 7-hydroxy function as activators of the chlorination reaction.

The results from the COX experiments showed that 3,5,7-trihydroxyflavone was able to inhibit the activity of both isoforms of the enzyme in a concentration-dependent manner. Previous studies also reported the capacity of this compound, also known as galangin, to inhibit COX activity in rat lungs and J774 macrophages and to inhibit COX-2 expression in J774A.1. macrophages [41–43]. On the other hand, the inhibition of COX-1 activity by 3,7,3′-trihydroxyflavone was reported here for the first time. Although 3,3′,4′-trihydroxyflavone and 3,7,4′-trihydroxyflavone were shown to be inactive in this study, their effectiveness as COX inhibitors ~~can't~~ be completely discarded. In fact, previous reports indicate COX inhibitors to be more potent in intact cells than against purified enzymes or enzymes originating from broken cells [44]. On the other hand, 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 (see [1] for review).

The inhibition of the 5-LOX pathway by the tested trihydroxyflavones was, to our knowledge, reported here for the first time. Although several mechanisms, other than a direct inhibition of 5-LOX, may explain the observed inhibition of LTB_4 production by the tested compounds, it is conceivable to presume that trihydroxyflavones, in conformity with other flavonoids and 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. 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 [45,46]. Moreover, in accordance to what has been previously stated for other flavonoids [47] it is expected that the tested trihydroxyflavones access the catalytic site of the enzyme, through a hydrophobic cavity, due to their likely planar structure conferred by the 3-hydroxy substituent, which forces the coplanarity of the B-ring with the rest of the molecule [48].

The capacity of 3,5,7-trihydroxyflavone to inhibit both COX and 5-LOX pathways plays in favor of its potential use as an effective and secure anti-inflammatory drug, when compared with the commonly used NSAIDs and COXIBs. According to Bertolini et al. [49], a dual acting anti-inflammatory drug should be advantageous for the following reasons: (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 proinflammatory and gastrointestinal damaging effects of leukotrienes. Supporting this theory, there are already some dual inhibitor agents who have proven their efficacy in the treatment of osteoarthritis, one of them consisting in a mixture of flavonoids [50–52].

In this study, a group of trihydroxyflavones was tested for their antioxidant and anti-inflammatory capacities, showing high ability to scavenge ROS and RNS and to inhibit the oxidative burst in human inflammatory cells. Also, due to its capacity to inhibit simultaneously two important pro-inflammatory pathways (COX and 5-LOX), 3,5,7-trihydroxyflavone appeared as a promising candidate for a new therapeutic option in the treatment of inflammatory pathologies.

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