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Synthesis of chlorinated flavonoids with anti-inflammatory and pro-apoptotic activities in human neutrophils

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ABSTRACT

Neutrophils are considered the central cells of acute inflammation. Flavonoids have been suggested as therapeutic agents to avoid damages induced by inflammatory processes. It is well known the reactivity of flavonoids with hypochlorous acid produced by neutrophils, to form stable mono and dichlorinated products. In this study, we synthesized novel chlorinated flavonoids and investigated their effect in neutrophils' oxidative burst and in its lifespan, in comparison with the parent non-chlorinated flavonoids. The obtained results demonstrate that chlorinated flavonoids were more efficient than their parent compounds in modulating neutrophils' oxidative burst in phorbol myristate acetate-activated neutrophils. Some of the tested flavonoids drive neutrophil apoptosis in a caspase 3-dependent fashion. The present data showed that 8-chloro-3′,4′,5,7-tetrahydroxyflavone (**4a**) constitute an alternative anti-inflammatory therapy, due to the proven ability to suppress mechanisms engaged at the onset and progression of inflammation.

1. Introduction

Inflammation is an orchestrated biological process, induced by microbial infection or tissue injury, characterized by redness, oedema, fever, pain, and loss of function [1]. Inflammation is increasingly found to be involved in the development of several chronic diseases such as arteriosclerosis, obesity, diabetes, neurodegenerative diseases and even cancer. Among them, cardiovascular diseases and cancer are main causes of mortality in many countries [1]. A major trigger of inflammation is the recognition of microbes by specific receptors of the innate immune system, which play a crucial role in the induction of early signals, initiating and establishing the inflammatory setting [2,3]. Neutrophils are considered to be the central cells of acute inflammation [4]. Accordingly, in the event of an inflammatory process, it is observed an increase in the number, lifespan, mobility, tissue influx ability, and phagocytic capacity of neutrophils [5,6].

One of the most important mechanisms used by neutrophils to protect the organism against the invader is the production of an array of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [5]. However, the sustained overproduction of reactive species or the impairment of antioxidant defenses, may result in a pro-oxidant status of the cells known as oxidative stress, leading to detrimental effects to the host, namely alterations on the normal function of lipids, proteins or DNA [4,5,7,8]. In addition, an abnormal, ineffective or absent resolution of inflammation leads to tissue irreversible damages. Despite the lifespan of neutrophils of only a few hours, under physiological conditions, in inflammatory environment their survival is promoted by delaying apoptosis, a form of cell death. Therefore, novel strategies of anti-inflammatory therapy that manipulate neutrophils activity namely the production of reactive species or their lifespan will be useful in both acute and chronic inflammation [9]. In this respect, the antioxidant and anti-inflammatory properties of flavonoids present in a wide variety of plants, may represent valid alternatives in the treatment of chronic inflammatory processes. Flavonoids occur mainly in fruit, vegetables, nuts, seeds, flowers, and bark and are generally present in plants as glycosides [10,11]. They are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, isoflavones, flavonols, flavanonols, flavanols and flavanones. It was already reported the ability of flavonoids to modulate

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neutrophils' oxidative burst [12]. However, despite the known reactivity of flavonoids with HOCl to form stable mono and dichlorinated products [13], there are no reports in literature about the effect of chlorinated metabolites of flavonoids on human neutrophils oxidative burst. Taking into account the lack of information about flavonoids ability to alter neutrophils' apoptosis, it is also of paramount importance to investigate their effect on neutrophils lifespan. As such, it is our purpose to understand if flavonoids metabolites may have biological and chemical properties distinct from their parent compounds. Thus, in this study, we synthesized novel chlorinated flavonoids and investigated their effect in neutrophils' oxidative burst and in its lifespan, in comparison with the parent non-chlorinated flavonoids.

Table 1 shows the chemical structure of the studied flavonoids.

2. Materials and methods

2.1. Materials

The following reagents were purchased from Sigma–Aldrich Co. LLC (St. Louis, USA): phorbol-12-myristate-13-acetate (PMA), *N*-acetyl-3,7-dihydroxyphenoxazine (amplex red), peroxidase from horseradish (HRP), histopaque 1077, histopaque 1119, Dulbecco's Phosphate Buffer saline, without calcium chloride and magnesium chloride (PBS), trypan blue solution 0.4%, dimethylsulfoxide (DMSO), trizma, superoxide dismutase (SOD), catalase, D-(+)-glucose, RPMI 1640 medium, fetal bovine serum, L-glutamine, penicillin, streptomycin, luteolin (**6**), and quercetin (**7**). 4-Aminobenzoyl hydrazide (ABAH) was purchased from Calbiochem (San Diego, CA, USA). 2-[6-(4′-Amino)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (APF) was purchased from Invitrogen, Life Technologies Ltd (Paisley, UK). Luminol was purchased from Fluka Chemie GmbH (Steinheim, Germany). Hemacolor® was obtained from Merck (Darmstadt, Germany). Annexin-V-FLUOS Staining Kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Z-DEVD-FMK was obtained from BD Pharmingen™. All chemicals and solvents used in the synthesis procedures were obtained from commercial sources and used as received or dried by standard procedures.

2.2. General measurements

Melting points were measured in a Reichert Thermovar apparatus fitted with a microscope and are uncorrected. NMR spectra were recorded on a Bruker Avance 300 spectrometer (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C), in $CDCl_3$ as solvent if not stated otherwise. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz; the internal standard was TMS. Unequivocal ^{13}C assignments were made with the aid of 2D gHSQC and gHMBC (delays for one-bond and long-range $J_{C/H}$ couplings were optimised for 145 and 7 Hz, respectively) experiments. Positive-ion ESI mass spectra were acquired with a Q-TOF 2 instrument [dilution of 1 μL of the sample in chloroform solution (ca. 10^{-5} M) in 200 μL of 0.1% trifluoroacetic acid/methanol solution]. Nitrogen was used as nebuliser gas and argon as collision gas. The needle voltage was set at 3000 V, with the ion source at 80 °C and the desolvation temperature at 150 °C. The cone voltage was 30 V. High-resolution mass spectra analyses were performed on a Bruker MicrOTOF spectrometer (University of Vigo). Elemental analyses were obtained with a LECO 630-200-200 CHNS analyser (University of Aveiro). Preparative thin-layer chromatography was performed with Merck silica gel (60 DGF_{254}). Column chromatography was performed with Merck silica gel (60, 70–230 mesh). All chemicals and solvents used were obtained from commercial sources and used as received or dried by standard procedures.

2.3. Synthesis of 2′-hydroxyacetophenones ***1a–d***

2.3.1. Synthesis of 2′-hydroxy-4′,6′-dimethoxyacetophenone (***1a***)

2′-Hydroxy-4′,6′-dimethoxyacetophenone (**1a**) was prepared according to a procedure previously described in the literature [14].

2.3.2. Synthesis of chloro-2′-hydroxyacetophenones ***1b–d***

To a stirred solution of 2′-hydroxy-4′,6′-dimethoxyacetophenone (**1a**) (150 mg, 0.76 mmol) in THF (30 mL) it was added *N*-chlorosuccinimide (NCS) (0.11 g, 0.84 mmol, for **1b**,**d** or 0.21 g, 1.61 mmol, for **1c**). The solution was refluxed, under nitrogen atmosphere, for 24 h. The reaction mixture was filtered, evaporated to dryness and purified by preparative thin-layer chromatography using a (1:1) mixture of light petroleum:dichloromethane (for **1b**,**d**) or dichloromethane (for **1c**) as eluent. The obtained oily residues were precipitated in ethanol:water giving chloro-2′-hydroxy-4′,6′-dimethoxyacetophenones **1b–d** in moderate yields: **1b** (62 mg, 35%) and **1d** (70 mg, 39%) as light yellow solids and **1c** (100 mg, 49%) as a white solid.

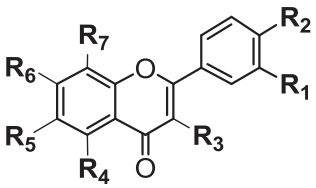
2.3.3. 3′-Chloro-2′-hydroxy-4′,6′-dimethoxyacetophenone (***1b***)

Mp 191–192 °C. ^{1}H NMR: δ 2.62 (s, 3H, 2-C*H*$_3$), 3.93 (s, 3H, 4′-OC*H*$_3$), 3.96 (s, 3H, 6′-OC*H*$_3$), 6.00 (s, 1H, H-5′), 14.45 (s, 1H, 2′-O*H*) ppm. ^{13}C NMR: δ 33.1 (2-*C*H$_3$), 55.7 (4′-OCH$_3$), 56.2 (6′-OCH$_3$), 86.5 (C-5′), 101.8 (C-3′), 106.2 (C-1′), 161.0 (C-6′), 161.6 (C-4′), 161.7 (C-2′), 203.4 (C=O) ppm. MS (ESI$^+$) m/z (rel. int.): 231 ($[C_{10}H_{11}^{35}ClO_4+H]^+$, 67), 233 ($[C_{10}H_{11}^{37}ClO_4+H]^+$, 24), 253 ($[C_{10}H_{11}^{35}ClO_4+Na]^+$, 92), 255 ($[C_{10}H_{11}^{37}ClO_4+Na]^+$, 27). Anal. Calcd for $C_{10}H_{11}ClO_4$ (230.64): C 52.07, H 4.81; found: C 52.13, H 4.79%.

2.3.4. 3′,5-Dichloro-2′-hydroxy-4′,6′-dimethoxyacetophenone (***1c***)

Mp 103–104 °C. ^{1}H NMR: δ 2.76 (s, 3H, 2-C*H*$_3$), 3.95 (s, 3H, 6′-OC*H*$_3$), 3.98 (s, 3H, 4′-OC*H*$_3$), 13.69 (s, 1H, 2′-O*H*) ppm. ^{13}C NMR:

Table 1
Chemical structure of the studied flavonoids.

Compound		R_1	R_2	R_3	R_4	R_5	R_6	R_7
4a		OH	OH	H	OH	H	OH	**Cl**
4b		OH	OH	H	OH	**Cl**	OH	**Cl**
4c		OH	OH	**Cl**	OH	H	OH	H
4d		OH	OH	**Cl**	OH	H	OH	**Cl**
5 Flavone		H	H	H	H	H	H	H
6 Luteolin		OH	OH	H	OH	H	OH	H
7 Quercetin		OH	OH	OH	OH	H	OH	H

δ 31.9 (2-*C*H$_3$), 61.0 (4′-O*C*H$_3$), 61.8 (6′-O*C*H$_3$), 112.9 (C-5′), 113.7 (C-3′), 114.1 (C-1′), 157.6 (C-6′), 158.9 (C-4′), 159.3 (C-2′), 204.0 (C=O) ppm. MS (ESI$^+$) *m/z* (rel. int.): 265 ([$C_{10}H_{10}^{35}Cl^{35}ClO_4$+H]$^+$, 25), 267 ([$C_{10}H_{10}^{35}Cl^{37}ClO_4$+H]$^+$, 19), 269 ([$C_{10}H_{10}^{37}Cl^{37}ClO_4$+H]$^+$, 5), 287 ([$C_{10}H_{10}^{35}Cl^{35}ClO_4$+Na]$^+$, 31), 289 ([$C_{10}H_{10}^{35}Cl^{37}ClO_4$+Na]$^+$, 25), 291 ([$C_{10}H_{10}^{37}Cl^{37}ClO_4$+Na]$^+$, 6). Anal. Calcd for $C_{10}H_{10}Cl_2O_4$ (265.09): C 45.31, H 3.80; found: C 44.88, H 4.07%.

2.3.5. 5′-Chloro-2′-hydroxy-4′,6′-dimethoxyacetophenone (**1d**)

Mp 89–90 °C. ^{1}H NMR: δ 2.70 (s, 3H, 2-C*H*$_3$), 3.91 (s, 3H, 6′-OC*H*$_3$), 3.92 (s, 3H, 4′-OC*H*$_3$), 6.30 (s, 1H, H-3′), 13.50 (s, 1H, 2′-O*H*) ppm. ^{13}C NMR: δ 31.2 (2-*C*H$_3$), 56.4 (4′-O*C*H$_3$), 61.3 (6′-O*C*H$_3$), 96.9 (C-3′), 107.8 (C-5′), 109.5 (C-1′), 159.0 (C-6′), 161.2 (C-4′), 164.4 (C-2′), 203.0 (C=O) ppm. MS (ESI$^+$) *m/z* (rel. int.): 231 ([$C_{10}H_{11}^{35}ClO_4$+H]$^+$, 14), 233 ([$C_{10}H_{11}^{37}ClO_4$+H]$^+$, 4). Anal. Calcd for $C_{10}H_{11}ClO_4$ (230.64): C 52.07, H 4.81; found: C 51.79, H 4.76%.

2.4. Synthesis of 2′-hydroxychalcones **2a**–**c**

2′-Hydroxychalcones **2a**–**c** were prepared accordingly to a procedure we previously described for 2′-hydroxychalcones with other substituents [12] in good yields: **2a** 87% (canary yellow solid); **2b**, 74% (dark yellow solid), **2c**, 73% (cottony orange solid).

2.4.1. 2′-Hydroxy-3,4,4′,6′-tetramethoxychalcone (**2a**)

Mp 154–155 °C. ^{1}H NMR: δ 3.84 (s, 3H, 4′-OC*H*$_3$), 3.91 (s, 3H, 6′-OC*H*$_3$), 3.93 (s, 3H, 4-OC*H*$_3$), 3.94 (s, 3H, 3-OC*H*$_3$), 5.97 (d, *J* 2.4 Hz, 1H, H-5′), 6.11 (d, *J* 2.4 Hz, 1H, H-3′), 6.90 (d, *J* 8.3 Hz, 1H, H-5), 7.13 (d, *J* 1.9 Hz, 1H, H-2), 7.22 (dd, *J* 8.3 and 1.9 Hz, 1H, H-6), 7.75 (d, *J* 15.5 Hz, 1H, H-β), 7.81 (d, *J* 15.5 Hz, 1H, H-α), 14.21 (s, 1H, 2′-O*H*) ppm. ^{13}C NMR (lit. [15]): δ 55.7 (4′-O*C*H$_3$), 55.8 (6′-O*C*H$_3$), 55.8 (3-O*C*H$_3$), 56.0 (4-O*C*H$_3$), 91.2 (C-5′), 93.8 (C-3′), 106.3 (C-1′), 110.4 (C-2), 111.1 (C-5), 122.6 (C-6), 125.4 (C-α), 128.6 (C-1), 142.6 (C-β), 149.1 (C-3), 151.0 (C-4), 162.4 (C-6′), 166.0 (C-4′), 168.4 (C-2′), 192.4 (C=O) ppm. MS (ESI$^+$) *m/z* (rel. int.): 345 ([M+H]$^+$, 100), 367 ([M+Na]$^+$, 23). Anal. Calcd for $C_{19}H_{20}O_6$ (344.36): C 66.27, H 5.85; found: C 66.24, H 5.92%.

2.4.2. 3′-Chloro-2′-hydroxy-3,4,4′,6′-tetramethoxychalcone (**2b**)

Mp 189–190 °C. ^{1}H NMR: δ 3.94 (s, 3H, 4-OC*H*$_3$), 3.95 (s, 3H, 3-OC*H*$_3$), 3.98 (2× s, 6H, 4′-OC*H*$_3$ and 6′-OC*H*$_3$), 6.04 (s, 1H, H-5′), 6.90 (d, *J* 8.3 Hz, 1H, H-5), 7.11 (d, *J* 1.9 Hz, 1H, H-2), 7.22 (dd, *J* 8.3 and 1.9 Hz, 1H, H-6), 7.73 (d, *J* 15.4 Hz, 1H, H-β), 7.80 (d, *J* 15.4 Hz, 1H, H-α), 14.84 (s, 1H, 2′-O*H*) ppm. ^{13}C NMR: δ 55.8, 56.0 and 56.2 (3-OCH$_3$, 4-OCH$_3$, 4′-O*C*H$_3$ and 4′-O*C*H$_3$), 86.9 (C-5′), 102.3 (C-1′), 106.7 (C-3′), 110.4 (C-2), 111.1 (C-5), 122.9 (C-6), 124.8 (C-α), 128.2 (C-1), 143.7 (C-β), 149.1 (C-3), 151.3 (C-4), 160.8 (C-6′), 161.2 (C-4′), 162.2 (C-2′), 192.6 (C=O) ppm. MS (ESI$^+$) *m/z* (rel. int.): 401 ([$C_{19}H_{19}^{35}ClO_6$+Na]$^+$, 64), 403 ([$C_{19}H_{19}^{37}ClO_6$+Na]$^+$, 23), 779 ([$C_{38}H_{38}^{35}Cl^{35}ClO_{12}$+Na]$^+$, 50), 781 ([$C_{38}H_{38}^{35}Cl^{37}ClO_{12}$+Na]$^+$, 37), 783 ([$C_{38}H_{38}^{37}Cl^{37}ClO_{12}$+Na]$^+$, 9). Anal. Calcd for $C_{19}H_{19}ClO_6.{}^1/_3CH_3CH_2OH$ (394.16): C 59.93, H 5.37; found: C 59.77, H 5.01%. HRMS (EI), *m/z*. $C_{19}H_{19}^{35}ClO_6$: calcd 378.0870 [M]$^+$, found: 378.0862; $C_{19}H_{19}^{37}ClO_6$: calcd 380.0841 [M]$^+$, found: 380.0838.

2.4.3. 3′,5′-Dichloro-2′-hydroxy-3,4,4′,6′-tetramethoxychalcone (**2c**)

Mp 153–154 °C. ^{1}H NMR: δ 3.84 (s, 3H, 6′-OC*H*$_3$), 3.95 (s, 3H, 4-OC*H*$_3$), 3.96 (s, 3H, 3-OC*H*$_3$), 4.00 (s, 3H, 4′-OC*H*$_3$), 6.92 (d, *J* 8.4 Hz, 1H, H-5), 7.16 (d, *J* 2.0 Hz, 1H, H-2), 7.28 (dd, *J* 8.4 and 2.0 Hz, 1H, H-6), 7.80 (d, *J* 15.5 Hz, 1H, H-α), 7.94 (d, *J* 15.5 Hz, 1H, H-β), 13.74 (s, 1H, 2′-O*H*) ppm. ^{13}C NMR: δ 56.0 (3-OCH$_3$ and 4-OCH$_3$), 51.0 (4′-O*C*H$_3$), 62.7 (6′-OCH$_3$), 110.2 (C-3′), 111.1 (C-5), 113.3 and 113.9 (C-1′ and C-3′), 114.4 (C-5′), 122.7 (C-α), 123.8 (C-6), 127.6 (C-1), 146.3 (C-β), 149.3 (C-3), 152.0 (C-4), 157.0 (C-6′), 158.3 (C-4′), 159.4 (C-2′), 192.9 (C=O) ppm. MS (ESI$^+$) *m/z* (rel. int.): 413 ([$C_{19}H_{18}^{35}Cl^{35}ClO_6$+H]$^+$, 39), 415 ([$C_{19}H_{18}^{35}Cl^{37}ClO_6$+H]$^+$, 32), 417 ([$C_{19}H_{18}^{37}Cl^{37}ClO_6$+H]$^+$, 6), 435 ([$C_{19}H_{18}^{35}Cl^{35}ClO_6$+Na]$^+$, 28), 437 ([$C_{19}H_{18}^{35}Cl^{37}ClO_6$+Na]$^+$, 18), 439 ([$C_{19}H_{18}^{37}Cl^{37}ClO_6$+Na]$^+$, 4). Anal. Calcd for $C_{19}H_{18}Cl_2O_6$ (413.25): C 55.22, H 4.39; found: C 55.22, H 4.37%.

2.5. Synthesis of 3′,4′,5,7-tetramethoxyflavones **3a**,**c**

3′,4′,5,7-Tetramethoxyflavones **3a**,**c** were prepared accordingly to a procedure we previously described for a similar substituted methoxyflavone (3′,5,7-trimethoxyflavone) [12]. These flavones were obtained in good yields from the corresponding 3,4,4′,6′-tetramethoxychalcones **2a**,**c**: **3a** 91% (white solid); **3c**, 64% (white solid).

2.5.1. 3′,4′,5,7-Tetramethoxyflavone (**3a**)

Mp 193–194 °C (lit. [16] 193 °C). NMR: δ 3.92 (s, 3H, 7-OC*H*$_3$), 3.96 (2× s, 6H, 4′-OC*H*$_3$ and 5′-OC*H*$_3$), 3.98 (s, 3H, 3′-OC*H*$_3$), 6.38 (d, *J* 2.3 Hz, 1H, H-6), 6.56 (d, *J* 2.3 Hz, 1H, H-8), 6.61 (s, 1H, H-3), 6.96 (d, *J* 8.5 Hz, 1H, H-5′), 7.32 (d, *J* 2.1 Hz, 1H, H-2′), 7.51 (dd, *J* 8.5 and 2.1 Hz, 1H, H-6′) ppm. ^{13}C NMR: δ 55.7 (7-O*C*H$_3$), 56.0 and 56.4 (4-O*C*H$_3$, 5-O*C*H$_3$, 3′-O*C*H$_3$), 92.8 (C-8), 96.1 (C-6), 107.9 (C-3), 108.5 (C-2′), 109.1 (C-10), 111.0 (C-5′), 119.5 (C-6′), 124.0 (C-1′), 149.2 (C-3′), 152.0 (C-4′), 160.0 (C-9), 160.6 (C-2), 160.8 (C-5), 164.0 (C-7), 177.6 (C-4) ppm. MS (ESI+) *m/z* (rel. int.): 343 ([M+H]$^+$, 100), 365 ([M+Na]$^+$, 14), 381 ([M+K]$^+$, 7), 707 ([2M+Na]$^+$, 85). Anal. Calcd for $C_{19}H_{18}O_6$ (342.34): C 66.66, H 5.30; found: C 66.47, H 5.35%.

2.5.2. 6,8-Dichloro-3′,4′,5,7-tetramethoxyflavone (**3c**)

Mp 212–213 °C. NMR: δ 3.98 (s, 3H, 4′-OC*H*$_3$), 3.99 (s, 3H, 3′-OC*H*$_3$), 4.00 (s, 3H, 5-OC*H*$_3$), 4.04 (s, 3H, 7-OC*H*$_3$), 6.69 (s, 1H, H-3), 7.01 (d, *J* 8.5 Hz, 1H, H-5′), 7.46 (d, *J* 2.1 Hz, 1H, H-2′), 7.61 (dd, *J* 8.5 and 2.1 Hz, 1H, H-6′) ppm. ^{13}C NMR: δ 56.0 (3′-O*C*H$_3$), 56.1 (4′-O*C*H$_3$), 61.3 (7-O*C*H$_3$), 62.1 (5-O*C*H$_3$), 107.1 (C-3), 108.5 (C-2′), 111.2 (C-5′), 114.1 (C-8), 116.5 (C-10), 120.0 (C-6′), 121.4 (C-6), 123.0 (C-1′), 149.3 (C-3′), 152.1 (C-9), 152.3 (C-4′), 154.9 (C-5), 156.8 (C-7), 161.3 (C-2), 175.9 (C-4) ppm. MS (ESI$^+$) *m/z* (rel. int.): 411 ([$C_{19}H_{16}^{35}Cl^{35}ClO_6$+H]$^+$, 40), 413 ([$C_{19}H_{16}^{35}Cl^{37}ClO_6$+H]$^+$, 27), 415 ([$C_{19}H_{16}^{37}Cl^{37}ClO_6$+H]$^+$, 5), 433 ([$C_{19}H_{16}^{35}Cl^{35}ClO_6$+Na]$^+$, 22), 435 ([$C_{19}H_{16}^{35}Cl^{37}ClO_6$+Na]$^+$, 15), 437 ([$C_{19}H_{16}^{37}Cl^{37}ClO_6$+Na]$^+$, 3). Anal. Calcd for $C_{19}H_{16}Cl_2O_6.{}^1/_2CH_3CH_2OH$ (434.27): C 55.31, H 4.41; found: C 54.92, H 4.07%. HRMS (EI), *m/z*: $C_{19}H_{16}^{35}Cl^{35}ClO_6$: calcd 410.0324 [M]$^+$, found: 410.0325; $C_{19}H_{16}^{35}Cl^{37}ClO_6$: calcd 412.0294 [M]$^+$, found: 412.0307.

2.6. Synthesis of 8-chloro-3′,4′,5,7-tetramethoxyflavone (**3b**)

2.6.1. Method I

8-Chloro-3′,4′,5,7-tetramethoxyflavone (**3b**) was prepared in moderate yield from 3′-chloro-3,4,4′,6′-tetramethoxychalcone (**2b**), as described for flavones **3a**,**c**: **3b** 54% (white solid).

2.6.2. Method II

To a stirred solution of 3′,4′,5,7-tetramethoxyflavone (**3a**) (98 mg, 0.26 mmol) in a (1:1) mixture of methanol:chloroform (6 mL), at room temperature, it was added NaClO (10%, 0.53 mL) and pH was adjusted to 6.2 with diluted hydrochloric acid. The referred volume of NaClO was added four times with intervals of 2 h. Then, the reaction mixture was stirred, under nitrogen atmosphere, overnight. The reaction mixture was extracted with chloroform (100 mL), dried over anhydrous sodium sulphate and evaporated to dryness. The obtained residue was purified by preparative thin-layer chromatography using (1:1) mixture of chloroform:ethyl acetate as eluent and recrystallized in ethanol affording 8-chloro-3′,4′,5,7-tetramethoxyflavone (**3b**) in moderate yield (34 mg, 32%).

*2.6.3. 8-Chloro-3′,4′,5,7-tetramethoxyflavone (**3b**)*

Mp 248–249 °C. NMR: δ 3.96 (s, 3H, 4′-OCH_3), 3.98 (s, 3H, 3′-OCH_3), 4.02 (s, 3H, 7-OCH_3), 4.05 (s, 3H, 5-OCH_3), 6.46 (s, 1H, H-6), 6.64 (s, 1H, H-3), 6.98 (d, *J* 8.5 Hz, 1H, H-5′), 7.45 (d, *J* 2.1 Hz, 1H, H-2′), 7.61 (dd, *J* 8.5 and 2.1 Hz, 1H, H-6′) ppm. ^{13}C NMR: δ 55.9 (3′-OCH_3), 56.0 (4′-OCH_3), 56.5 (5-OCH_3 and 7-OCH_3), 91.9 (C-6), 102.0 (C-8), 107.0 (C-3), 108.5 (C-2′), 109.4 (C-10), 111.1 (C-5′), 119.7 (C-6′), 123.4 (C-1′), 149.1 (C-3′), 152.8 (C-4′), 154.2 (C-9), 159.1 (C-5), 159.3 (C-7), 160.5 (C-2), 177.3 (C-4) ppm. MS (ESI$^+$) *m/z* (rel. int.): 377 ([$C_{19}H_{17}^{35}ClO_6$+H]$^+$, 100), 379 ([$C_{19}H_{17}^{37}ClO_6$+H]$^+$, 13), 399 ([$C_{19}H_{17}^{35}ClO_6$+Na]$^+$, 9), 401 ([$C_{19}H_{17}^{37}ClO_6$+Na]$^+$, 1), 775 ([$C_{38}H_{34}^{35}Cl^{35}ClO_{12}$+Na]$^+$, 16), 777 ([$C_{38}H_{34}^{35}Cl^{37}ClO_{12}$+Na]$^+$, 8), 779 ([$C_{38}H_{34}^{37}Cl^{37}ClO_{12}$+Na]$^+$, 2). Anal. Calcd for $C_{19}H_{17}ClO_6$ (376.79): C 60.57, H 4.55; found: C 60.92, H 4.41%.

2.7. Synthesis of chloro-3′,4′,5,7-tetramethoxyflavones **3d**,**e**

To a stirred solution of 3′,4′,5,7-tetramethoxyflavone (**3a**) (102 mg, 0.30 mmol) in THF (40 mL) NCS was added (48 mg, 0.36 mmol, for **3d** or 0.88 g, 0.66 mmol, for **3e**). The solution was refluxed, under nitrogen atmosphere for 24 h. The reaction mixture was filtered, evaporated to dryness and then purified by preparative thin-layer chromatography using a (3:1) mixture of chloroform:ethyl acetate, and then recrystallized in ethanol (for **1b**,**c**) or directly recrystallized in ethanol (for **3d**). Mono- and dichloro-3′,4′,5,7-tetramethoxyflavones **3d**,**e** were obtained in moderate yields: **3d**, 49 mg, 44% (light yellow solid) and **3e**, 61 mg, 50% (white solid).

*2.7.1. 3-Chloro-3′,4′,5,7-tetramethoxyflavone (**3d**)*

Mp 181–182 °C. NMR: δ 3.90 (s, 3H, 7-OCH_3), 3.96 (s, 3H, 5-OCH_3), 3.97 (2× s, 6H, 3′-OCH_3 and 4′-OCH_3), 6.39 (d, *J* 2.3 Hz, 1H, H-6), 6.49 (d, *J* 2.3 Hz, 1H, H-8), 6.99 (d, *J* 8.5 Hz, 1H, H-5′), 7.45 (d, *J* 2.1 Hz, 1H, H-2′), 7.55 (dd, *J* 8.5 and 2.1 Hz, 1H, H-6′) ppm. ^{13}C NMR: δ 55.8 (7-OCH_3), 56.0 (4′-OCH_3), 56.1 (3′-OCH_3), 56.4 (5-OCH_3), 92.3 (C-8), 96.4 (C-6), 107.7 (C-10), 110.5 (C-5′), 112.0 (C-2′), 118.1 (C-3), 122.8 (C-6′), 123.7 (C-1′), 148.5 (C-3′), 151.2 (C-4′), 157.6 (C-2), 158.9 (C-9), 160.9 (C-5), 164.3 (C-7), 171.3 (C-4) ppm. MS (ESI$^+$) *m/z* (rel. int.): 377 ([$C_{19}H_{17}^{35}ClO_6$+H]$^+$, 18), 379 ([$C_{19}H_{17}^{37}ClO_6$+H]$^+$, 4), 399 ([$C_{19}H_{17}^{35}ClO_6$+Na]$^+$, 25), 401 ([$C_{19}H_{17}^{37}ClO_6$+Na]$^+$, 7), 775 ([$C_{38}H_{34}^{35}Cl^{35}ClO_{12}$+Na]$^+$, 100), 777 ([$C_{38}H_{34}^{35}Cl^{37}ClO_{12}$+Na]$^+$, 57), 779 ([$C_{38}H_{34}^{37}Cl^{37}ClO_{12}$+Na]$^+$, 7). Anal. Calcd for $C_{19}H_{17}ClO_6$ (376.79): C 60.28, H 5.58; found: C 60.57, H 4.55%.

*2.7.2. 3,8-Dichloro-3′,4′,5,7-tetramethoxyflavone (**3e**)*

Mp 258–259 °C. NMR: δ 3.97 (s, 3H, 3′-OCH_3), 3.98 (s, 3H, 4′-OCH_3), 4.02 (s, 3H, 7-OCH_3), 4.04 (s, 3H, 5-OCH_3), 6.47 (s, 1H, H-6), 7.01 (d, *J* 8.6 Hz, 1H, H-5′), 7.67 (d, *J* 2.1 Hz, 1H, H-2′), 7.79 (dd, *J* 8.6 and 2.1 Hz, 1H, H-6′) ppm. ^{13}C NMR: δ 56.0 (3′-OCH_3 and 4′-OCH_3), 56.6 (5′-OCH_3 and 7-OCH_3), 92.3 (C-6), 101.5 (C-8), 107.9 (C-10), 110.5 (C-5′), 112.1 (C-2′), 117.6 (C-3), 120.6 (C-1′), 123.3 (C-6′), 148.5 (C-3′), 151.5 (C-4′), 153.4 (C-9), 157.3 (C-2), 159.4 (C-5 and C-7), 171.2 (C-4) ppm. MS (ESI$^+$) *m/z* (rel. int.): 411 ([$C_{38}H_{32}^{35}Cl^{35}ClO_{12}$+H]$^+$, 100), 413 ([$C_{38}H_{32}^{35}Cl^{37}ClO_{12}$+H]$^+$, 45), 415 ([$C_{38}H_{32}^{37}Cl^{37}ClO_{12}$+H]$^+$, 3), 433 ([$C_{38}H_{32}^{35}Cl^{35}ClO_{12}$+Na]$^+$, 18), 435 ([$C_{38}H_{324}^{35}Cl^{37}ClO_{12}$+Na]$^+$, 8), 437 ([$C_{38}H_{32}^{37}Cl^{37}ClO_{12}$+Na]$^+$, 2). Anal. Calcd for $C_{18}H_{18}O_5$.½ H_2O (429.24): C 54.30, H 4.08; found: C 54.74, H 4.17%.

2.8. Synthesis of chloro-3′,4′,5,7-tetrahydroxyflavones **4a**–**d**

Mono and dichloro-3′,4′,5,7-tetrahydroxyflavones **4a**–**d** were prepared accordingly to a procedure we previously described for 3′,5,7-trihydroxyflavone [12]. The reaction time was 9 days for **4a**,**c**,**d** and 15 days for **4b**. All the obtained compounds were precipitated in a (2:8) mixture of acetone:hexane giving yellowish powders in good yields: **4a**, 85%; **4b**, 42%; **4c**, 86%; **4d**, 81%.

*2.8.1. 8-Chloro-3′,4′,5,7-tetrahydroxyflavone (**4a**)*

$Mp_{dec.}$ 329–330 °C. ^{1}H NMR (DMSO-d_6): δ 6.39 (s, 1H, H-6), 6.78 (s, 1H, H-3), 6.91 (d, *J* 7.9 Hz, 1H, H-5′), 7.45-7.48 (m, 1H, H-6′), 7.48 (brs, 1H, H-2′), 9.61 (s, 1H, 3′-O*H*), 10.07 (s, 1H, 4′-O*H*), 11.80 (brs, 1H, 7-O*H*), 12.90 (s, 1H, 5-O*H*) ppm. ^{13}C NMR (DMSO-d_6): δ 97.7 (C-8), 99.1 (C-6), 103.1 (C-3), 104.5 (C-10), 113.6 (C-5′), 116.3 (C-2′), 119.3 (C-6′), 121.4 (C-1′), 146.0 (C-3′), 150.2 (C-4′), 152.6 (C-9), 159.5 (C-5), 156.0 (C-7), 164.0 (C-2), 181.8 (C-4) ppm. MS (ESI$^+$) *m/z* (rel. int.): 321 ([$C_{15}H_9^{35}ClO_6$+H]$^+$, 36), 323 ([$C_{15}H_9^{37}ClO_6$+H]$^+$, 6). Anal. Calcd for $C_{15}H_9ClO_6$ (320.68): C 56.18, H 2.83; found: C 55.74, H 2.91%.

*2.8.2. 6,8-Dichloro-3′,4′,5,7-tetrahydroxyflavone (**4b**)*

$Mp_{dec.}$ 290–291 °C. ^{1}H NMR (DMSO-d_6): δ 6.80 (s, 1H, H-3), 6.90 (d, *J* 8.4 Hz, 1H, H-5′), 7.46 (dd, *J* 8.4 and 1.9 Hz, 1H, H-6′), 7.48 (d, *J* 1.9 Hz, 1H, H-2′), 9.55 (s, 1H, 4′-O*H*), 10.02 (s, 1H, 3′-O*H*), 13.71 (s, 1H, 5-O*H*) ppm. ^{13}C NMR (DMSO-d_6): δ 99.3 (C-8), 102.7 (C-3), 102.9 (C-10), 104.5 (C-6), 113.5 (C-2′), 116.2 (C-5′), 119.2 (C-6′), 121.3 (C-1′), 145.9 (C-3′), 150.1 (C-4′), 150.7 (C-9), 155.1 (C-5), 157.9 (C-7), 163.7 (C-2), 180.9 (C-4) ppm. MS (ESI$^+$) *m/z* (rel. int.): 355 ([$C_{15}H_8^{35}Cl^{35}ClO_6$+H]$^+$, 6), 357 ([$C_{15}H_8^{35}Cl^{37}ClO_6$+H]$^+$, 4), 359 ([$C_{15}H_8^{37}Cl^{37}ClO_6$+H]$^+$, 1). HRMS (ESI$^+$) *m/z*: $C_{15}H_8^{35}Cl^{35}ClO_6$: calcd 354.9771 [M+H]$^+$, found: 354.9769; $C_{15}H_8^{35}Cl^{37}ClO_6$: calcd 356.9741 [M+H]$^+$, found: 356.9740; $C_{15}H_8^{35}Cl^{37}ClO_6$: calcd 358.9712 [M+H]$^+$, found: 358.9711.

*2.8.3. 3-Chloro-3′,4′,5,7-tetrahydroxyflavone (**4c**)*

Mp_{dec} 318–319 °C. ^{1}H NMR (CD_3OD): δ 6.27 (d, *J* 2.0 Hz, 1H, H-6), 6.40 (d, *J* 2.0 Hz, 1H, H-8), 6.93 (d, *J* 8.4 Hz, 1H, H-5′), 7.39 (dd, *J* 8.4 and 2.0 Hz, 1H, H-6′), 7.44 (d, *J* 2.0 Hz, 1H, H-2′). ^{13}C NMR (CD_3OD): δ 94.9 (C-8), 100.4 (C-6), 104.4 (C-10), 114.6 (C-3), 116.1 (C-5′), 117.3 (C-2′), 123.3 (C-6′), 123.4 (C-1′), 146.2 (C-3′), 150.2 (C-4′), 158.6 (C-9), 162.7 (C-5), 162.8 (C-2), 166.4 (C-7), 178.2 (C-4) ppm. MS (ESI$^+$) *m/z* (rel. int.): 321 ([$C_{15}H_9^{35}ClO_6$+H]$^+$, 9), 323 ([$C_{15}H_9^{37}ClO_6$+H]$^+$, 2), 343 ([$C_{15}H_9^{35}ClO_6$+Na]$^+$, 8), 345 ([$C_{15}H_9^{37}ClO_6$+Na]$^+$, 2). Anal. Calcd for $C_{15}H_9ClO_6$.½ H_2O (329.69): C 54.65, H 3.06; found: C 54.69, H 2.93%. HRMS (EI), *m/z*: $C_{15}H_9^{35}ClO_6$: calcd 320.0088 [M]$^+$, found: 320.0099; $C_{15}H_9^{37}ClO_6$: calcd 322.0058 [M]$^+$, found: 322.0063.

*2.8.4. 3,8-Dichloro-3′,4′,5,7-tetrahydroxyflavone (**4d**)*

Mp_{dec} 292–293 °C. ^{1}H NMR (CD_3OD): δ 6.40 (s, 1H, H-6), 6.95 (d, *J* 8.4 Hz, 1H, H-5′), 7.54 (dd, *J* 8.4 and 2.2 Hz, 1H, H-6′), 7.60 (d, *J* 2.2 Hz, 1H, H-2′). ^{13}C NMR (CD_3OD): δ 99.4 (C-8), 100.4 (C-6), 104.9 (C-10), 114.8 (C-3), 116.1 (C-5′), 117.5 (C-2′), 123.1 (C-1′), 123.7 (C-6′), 146.3 (C-3′), 150.6 (C-4′), 153.6 (C-9), 160.6 (C-5), 161.9 (C-7), 162.4 (C-2), 178.1 (C-4) ppm. MS (ESI$^+$) *m/z* (rel. int.): 355 ([$C_{15}H_8^{35}Cl^{35}ClO_6$+H]$^+$, 8), 357 ([$C_{15}H_8^{35}Cl^{37}ClO_6$+H]$^+$, 6), 359 ([$C_{15}H_8^{37}Cl^{37}ClO_6$+H]$^+$, 2). Anal. Calcd for $C_{15}H_8Cl_2O_6$.3/2H_2O (382.16): C 47.14, H 2.90; found: C 47.02, H 3.08%. HRMS (ESI$^+$)$^{m/z}$: $C_{15}H_8^{35}Cl^{35}ClO_6$: calcd 354.9771 [M+H]$^+$, found: 354.9769; $C_{15}H_8^{35}Cl^{37}ClO_6$: calcd 356.9741 [M+H]$^+$, found: 356.9740; $C_{15}H_8^{35}Cl^{37}ClO_6$: calcd 358.9712 [M+H]$^+$, found: 357.9710.

2.9. Cell viability

Cell viability was determined by the trypan blue exclusion assay. Neutrophils were incubated with all the tested flavonoids (at the maximum concentration tested, 50 μM) for 4 h at 37 °C. 20 μL of neutrophils' suspension were added to an equal volume of trypan blue solution 0.4% in a microtube and gently mixed. After 2 min on ice, neutrophil number and viability (viable cells excluding trypan blue) were counted. Assays were performed in triplicate.

2.10. Measurement of human neutrophils' oxidative burst

Neutrophils were isolated from blood donated by healthy human volunteers to whom the design and execution of the experiment were thoroughly explained, and informed consent was obtained. Essentially, the study was based on the stimulation of human neutrophils with PMA and subsequent measurement of the neutrophil-ROS-generating-capacity. For this purpose, the chemiluminescence method using luminol as probe, and fluorescence methods, using amplex red and APF as probes, were adapted to a microplate reader (Synergy HT, BIO-TEK), as detailed below. Each study corresponds at least to 6 individual experiments and performed in triplicate in each experiment.

2.10.1. Isolation of neutrophils

Following informed consent, healthy human volunteers venous blood was collected 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 [17]. Tris-glucose (25 mM Tris, 1.26 mM $CaCl_2.2H_2O$, 5.37 mM KCl, 0.81 mM $MgSO_4$, 140 mM NaCl, and 5.55 mM D-Glucose) was the incubation media used in the evaluation of neutrophils' oxidative burst, as previously recommended [18]. In assessment of neutrophils' apoptosis the incubation media used was RPMI 1640 [(pH = 7.4) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin].

2.10.2. Evaluation of neutrophils' oxidative burst

2.10.2.1. Oxidation of luminol. The chemiluminescent probe luminol has been thoroughly studied and used for monitoring the production of reactive species by neutrophils, namely the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), hypochlorous acid (HOCl), nitric oxide ($^{\bullet}NO$) and peroxynitrite anion ($ONOO^-$) [5]. The measurement of neutrophils' oxidative burst was undertaken by chemiluminescence, by monitoring ROS-induced oxidation of luminol, according to a previously described procedure [19]. The reaction mixtures contained neutrophils (2×10^6 cells/mL) and the following reagents at the indicated final concentrations (in a final volume of 250 μL): tested flavonoids at various concentrations (0–50 μM), luminol (500 μM) and PMA (160 nM). Cells were pre-incubated with luminol and the tested flavonoids for 5 min before the addition of PMA and the measurements were carried out at 37 °C, subjected to continuous soft shaking. Kinetic readings were initiated immediately after cell stimulation. Measurements were taken at the peak of the curve. This peak was observed at around 10 min. Effects are expressed as the inhibition percentage of luminol oxidation. ABAH, SOD and catalase were used as positive controls.

2.10.2.2. Oxidation of amplex red. Amplex red is a highly sensitive and chemically stable fluorescent probe for the extracellular detection of H_2O_2 [5]. The measurement of neutrophils' oxidative burst was undertaken by fluorescence, by monitoring H_2O_2-induced oxidation of amplex red, according to a previously described procedure [19], with modifications. The reaction mixtures contained neutrophils (2×10^6 cells/mL) and the following reagents at the indicated final concentrations (in a final volume of 250 μL): tested flavonoids at various concentrations (0–50 μM), amplex red (25 μM), HRP (0.25 U/mL) and PMA (160 nM). Cells were pre-incubated with amplex red, HRP and the tested flavonoids for 5 min before the addition of PMA and the measurements were carried out at 37 °C, subjected to continuous soft shaking. Kinetic readings were initiated immediately after cell stimulation. The excitation and emission wavelengths used were 563 and 587 nm, respectively. Obtained values correspond to the slope measured between 5 and 10 min. Effects are expressed as the percentage inhibition of amplex red oxidation. Catalase was used as positive control.

2.10.2.3. Oxidation of APF. APF is a non-fluorescent derivative of fluorescein, which is oxidized by HOCl, $ONOO^-$ and $HO^{\bullet}$, in a concentration dependent manner. Its selectivity was tested using the myeloperoxidase (MPO) inhibitor, ABAH. The addition of ABAH to human neutrophils, stimulated with PMA, decreased the APF-dependent fluorescence signal to the level of the control assay, ruling out the involvement of $HO^{\bullet}$ and $ONOO^-$ [5]. The measurement of neutrophils' oxidative burst was undertaken by fluorescence, by monitoring HOCl-induced oxidation of APF, according to a previously described procedure [19], with modifications. The reaction mixtures contained neutrophils (2×10^6 cells/mL) and the following reagents at the indicated final concentrations (in a final volume of 250 μL): tested flavonoids at various concentrations (0–50 μM), PMA (160 nM) and APF (5 μM). Cells were pre-incubated with PMA and the tested flavonoids for 5 min before the addition of APF and the measurements were carried out at 37 °C, subjected to continuous soft shaking. Kinetic readings were initiated immediately after the addition of APF. The excitation and emission wavelengths used were 500 and 520 nm, respectively. Obtained values correspond to the slope measured between 5 and 15 min. Effects are expressed as the percentage inhibition of APF oxidation. ABAH was used as positive control.

2.11. Assessment of neutrophils' apoptosis

2.11.1. Cytomorphological alterations

Neutrophils (1×10^6 cells/mL) were incubated with the tested flavonoids for 4 h at 37 °C. At the end of the incubation, neutrophils were cytocentrifuged, stained with Hemacolor®, and counted under light microscopy (100×) to determine the proportion of cells showing characteristic apoptotic morphology. At least 400 cells were counted per slide.

2.11.2. Annexin-V binding assay

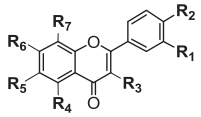
Apoptotic neutrophils were analysed by flow cytometry after simultaneous staining with annexin-V labelled with fluorescein and propidium iodide according to [19]. Neutrophils (1×10^6 cells/mL) were incubated with the tested flavonoids for 4 h at 37 °C. The commercial annexin-V-FLUOS Staining Kit (Roche Diagnostics GmbH, Mannheim Germany) was used according to the manufacturer's instructions.

Fluorescence signals for each sample were collected using an Accuri C6 flow. To restrict the analysis to neutrophils only, a polygon gate was set according to their light scattering properties (in a forward vs. side scatter plot) excluding cell debris and other blood cells. Fluorescence signals for at least 10,000 cells were collected in logarithmic mode and the data were analysed using C Flow (Accuri) software. The green fluorescence due to Annexin-V conjugated with FITC was followed in channel 1 (FL1) and plotted as a histogram of FL1 staining. Fluorescence due to the propidium iodide incorporation was followed in channel 3 (FL3).

2.11.3. Caspase 3-activity

The contribution of caspase 3 activity in flavonoids-induced apoptosis was evaluated by the use of a synthetic peptide inhibitor that irreversibly inhibits caspase 3 and related protease/caspase activity, blocking apoptosis. Neutrophils (1×10^6 cells/mL) were incubated with or without caspase 3 inhibitor, Z-DEVD-FMK (10 μM) for 30 min at 37 °C. After incubation, the tested flavonoids (50 μM) were added and incubated for 4 h. Apoptotic neutrophils

Table 2
Chemical structure and inhibition of PMA-induced neutrophils' oxidative burst by the studied flavonoids, assessed by the luminol, amplex red and APF (IC_{50} μM, mean ± SEM).

Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Luminol IC_{50} (μM)	Amplex red	APF
4a 	OH	OH	H	OH	H	OH	**Cl**	2.6 ± 0.5	1.6 ± 0.1	2.6 ± 0.4
4b	OH	OH	H	OH	**Cl**	OH	**Cl**	3.4 ± 0.7	6.2 ± 1.0	7.2 ± 0.6
4c	OH	OH	**Cl**	OH	H	OH	H	2.0 ± 0.4	1.4 ± 0.1	0.4 ± 0.1
4d	OH	OH	**Cl**	OH	H	OH	**Cl**	4.6 ± 0.8	1.7 ± 0.1	1.8 ± 0.4
5 Flavone	H	H	H	H	H	H	H	LA	LA	LA
6 Luteolin	OH	OH	H	OH	H	OH	H	5.2 ± 0.9	1.7 ± 0.1	2.2 ± 0.5
7 Quercetin	OH	OH	OH	OH	H	OH	H	2.8 ± 0.4	2.7 ± 0.2	1.8 ± 0.3

LA-less than 40% of inhibition neutrophils' oxidative burst, up to the highest tested concentration (50 μM).

were analysed by flow cytometry after simultaneous staining with annexin-V labelled with fluorescein and propidium iodide as reported in 2.11.2.

3. Results

3.1. Cell viability

Neutrophils' viability was assessed by the trypan blue assay after exposure to the tested flavonoids. Cell viability was maintained over 98%, after 4 h of exposure to the different flavonoids, at the maximum concentration tested, 50 μM.

3.2. Oxidation of luminol by neutrophils – generated ROS

As shown in Table 2, among the tested flavonoids, flavone **5** was noticeable less effective than the others tested flavonoids. Besides, it was not possible to achieve the IC_{50} value up to the highest tested concentration (50 μM). Flavonoid **4c** was the most active presenting an IC_{50} = 2.0 ± 0.4 μM, which was 2.6 times lesser than the IC_{50} found for luteolin (**6**) (IC_{50} = 5.2 ± 0.9 μM). The order of potencies found for the tested flavonoids was: **4c** (IC_{50} = 2.0 ± 0.4 μM) > **4a** > **7** > **4b** > **4d** > **6** (IC_{50} = 5.2 ± 0.9 μM). ABAH and catalase, which were used as positive controls, presented an IC_{50} of 43.6 ± 8.8 μM and 2597 ± 684 U/mL, respectively. SOD, which was also used as a positive control, presented a 49.3 ± 5.1% effect for the concentration of 1000 U/mL.

3.3. Oxidation of amplex red by neutrophils – generated H_2O_2

Table 2 shows the results of the amplex red oxidation. Once again it was not possible to achieve the IC_{50} value for flavone **5**, up to the highest tested concentration (50 μM). Flavonoid **4c** was the one that had the best activity (IC_{50} = 1.4 ± 0.1 μM), followed by flavonoid **4a**, luteolin (**6**), flavonoid **4d** and quercetin (**7**). The flavonoid **4b**, showed the highest IC_{50} value (6.2 ± 1.0 μM). Catalase, which was used as a positive control, presented an IC_{50} of 387 ± 7 U/mL.

3.4. Oxidation of APF by neutrophils – generated HOCl

In what concerns APF assays, one more time it was not possible to found the IC_{50} value for flavone **5**, up to the highest tested concentration (50 μM). Flavonoid **4b** appeared to be the less potent neutrophils' oxidative burst modulator presenting the highest IC_{50} value (7.2 ± 0.6 μM).

Regarding the results of the tested flavonoids, the order of potencies found was: **4c** (IC_{50} = 0.4 ± 0.1) > quercetin (**7**) = **4d** > luteolin (**6**) > **4a** > **4b** (IC_{50} = 7.2 ± 0.6 μM). ABAH, which was used as a positive control, presented an IC_{50} of 30.6 ± 6.1 μM.

3.5. Assessment of neutrophils' apoptosis

To investigate the ability of flavonoids to induce apoptosis, human neutrophils were incubated with increasing concentrations of the tested flavonoids for 4 h before apoptosis assessment by flow cytometry and morphological examination. In Fig. 1 are represented the flavonoids that induced a statistically significant difference in neutrophils' apoptosis between the control (without flavonoid) and the tested concentrations of the flavonoids. Flavone **4c**,**d** and **5** had no effect on human neutrophils' apoptosis up to the highest concentration (50 μM) tested. As shown in Fig. 1 luteolin (**6**) and flavone **4a** were the flavonoids that induced the greatest increase in annexin-V positive cells. At 50 μM flavone **4a** and luteolin (**6**) induced an intensification of neutrophils' apoptosis of 150 ± 26% and 115 ± 19%, respectively, when compared with the control (***$p < 0.001$). Similarly, cellular morphology revealed the appearance of cells with characteristic changes of apoptosis (such as nuclear condensation and cellular shrinkage) when in contact with the flavonoids quercetin (**7**), flavones **4a**,**b** and luteolin (**6**), the representative images are exposed in Fig. 2.

3.6. Evaluation of caspase 3 contribution to the induction of human neutrophils' apoptosis by flavonoids

Fig. 3 clearly show that the presence of Z-DEVD-FMK, a specific inhibitor of caspase 3, dramatically inhibited the ability of the

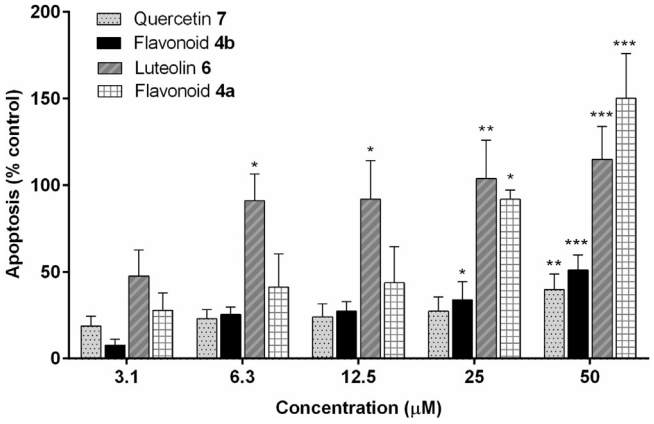


Fig. 1. Effect of flavonoids (3.1–50 μM) on human neutrophils' apoptosis assessed by flow cytometric analysis of annexin-V binding assay. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with the control assay (without flavonoid). The values are given as the mean ± SEM ($n \geq 6$).

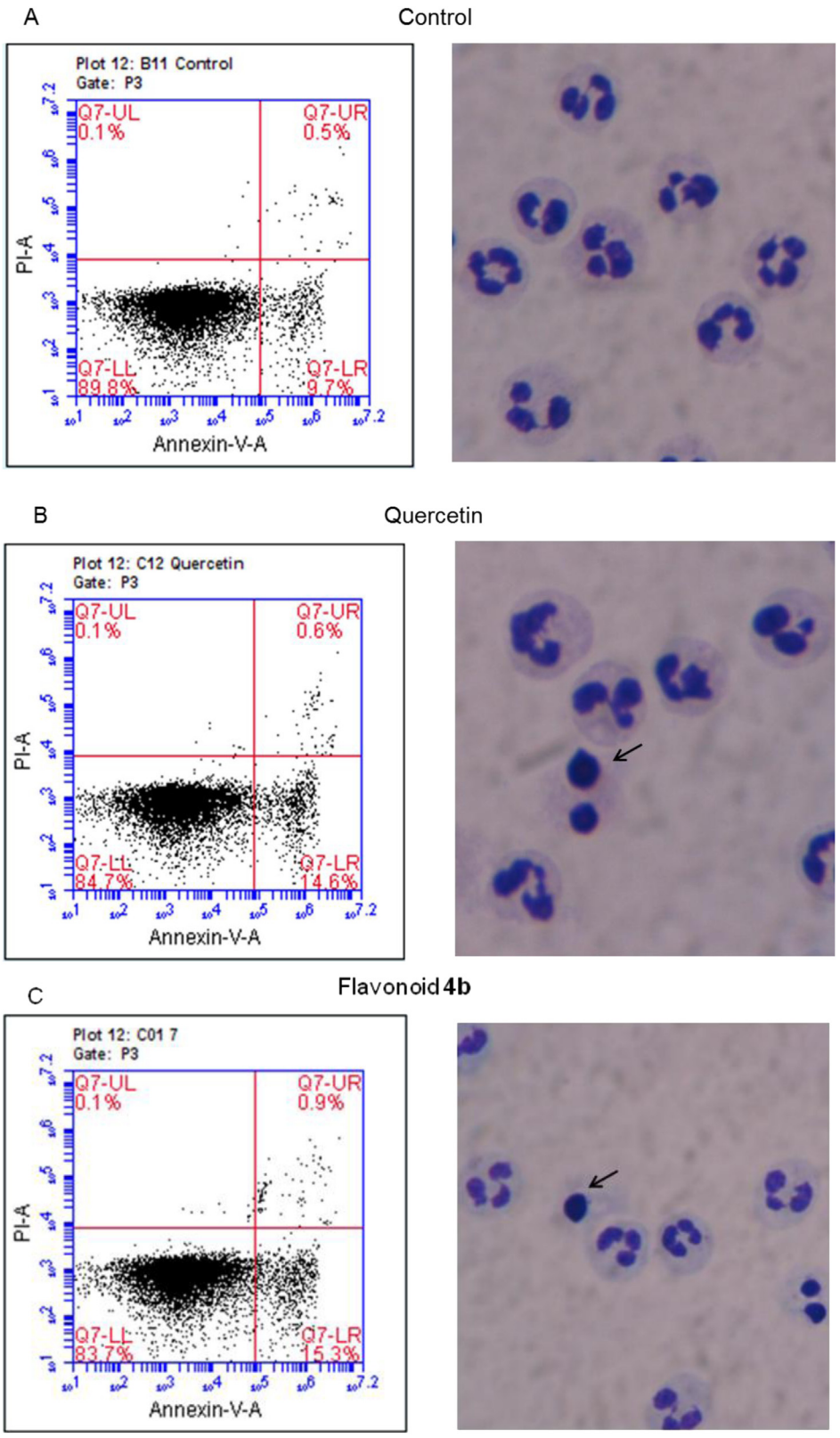


Fig. 2. Representative flow cytometry plots of annexin-V [(annexin-V; *x* axis)/PI (*y* axis)] binding and representative cytocentrifuge preparations after staining with Hemacolor are shown at 4 h for control (without flavonoid) (*A*), 50 μM of quercetin (**7**) (B), 50 μM of flavone **4b** (C), 50 μM of luteolin (**6**) (D), 50 μM of flavone **4a** (E). Arrowheads indicate an example of apoptotic neutrophil.

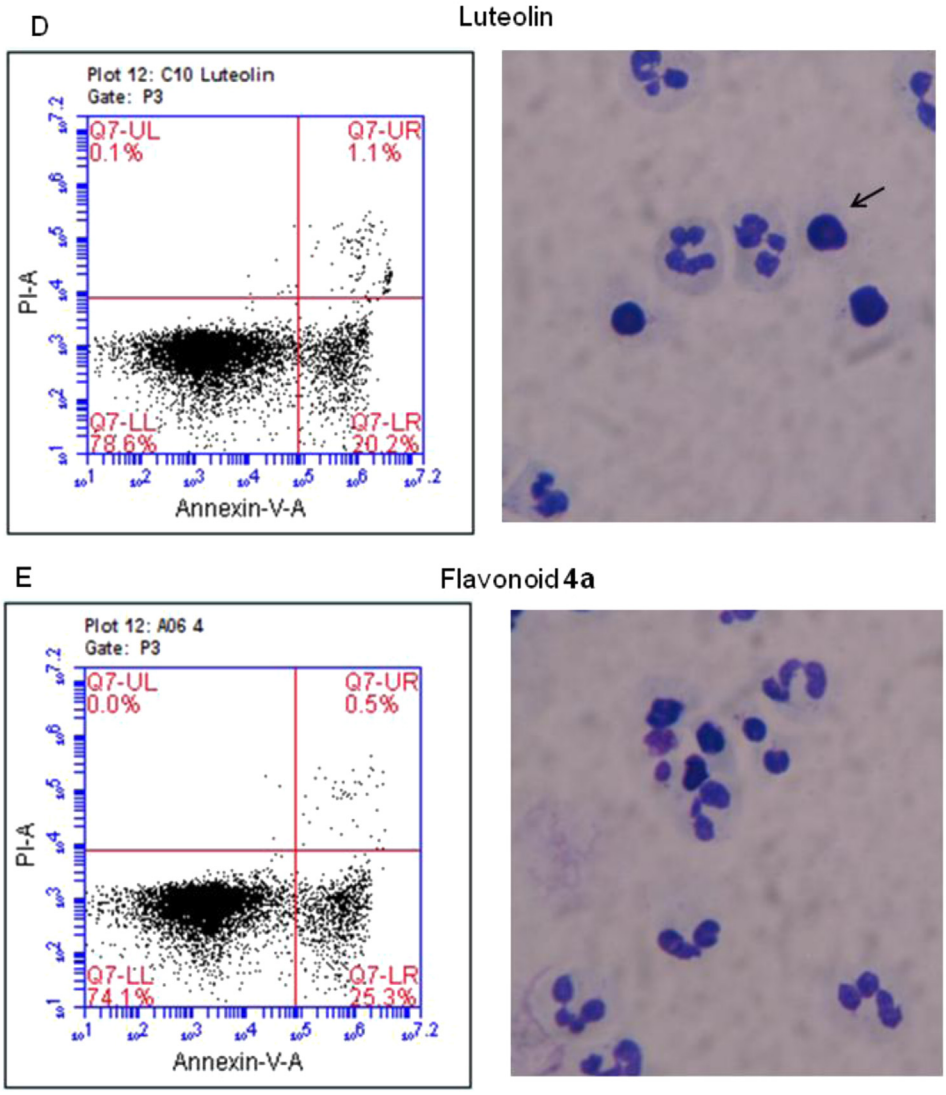


Fig. 2. (*continued*).

tested flavonoids to induce apoptosis. As it can be seen in Fig. 3 A, the inhibitor of caspase 3 decreased the apoptosis values close to the obtained in the control assay (without flavonoids). Fig. 3 B–D comprises a representative flow cytometry plots of annexin-V (annexin-V; *x* axis)/PI (*y* axis) and its analysis allows the conclusion that the caspase 3 inhibitor effectively decreased the apoptosis induced by flavonoid **4a**. This effect of the inhibitor indicates that the flavonoids induced neutrophils' apoptosis by caspase 3 enzyme activation.

4. Discussion

4.1. Chemistry

3′,4′,5,7-Tetramethoxyflavone (**3a**) was prepared by the base-catalysed aldol condensation of 2′-hydroxycetophenone (**1a**) with 3,4-dimethoxybenzaldehyde leading to 2′-hydroxy-3,4,4′,6′-tetramethoxychalcone (**2a**), which was then subjected to oxidative cyclization with a catalytic amount of iodine in refluxing DMSO (Scheme 1). The chlorination of flavone **3a** was performed by two different methods depending on the aimed chlorination pattern. Flavones **3d**,**e** were synthesised by using NCS as the chlorination agent in refluxing THF (44% and 50% yields, respectively). This method preferentially halogenates the position 3 of flavone **3a**. The synthesis of flavone **3b** can be accomplished both by using NaClO, that preferentially chlorinates position 8 of flavone **3a** (32%), via the *in situ* generation of HOCl; or by chlorination prior to cyclization (54%), in significantly different yields. After several chlorination attempts, we concluded that by using NCS or NaClO chlorination agents, it is not possible to chlorinate position 6 of flavone **3a**, disallowing the synthesis of chloroflavone **3c** by its direct chlorination being necessary to find an alternative method in which we consider the dichlorination of the starting 2′-hydroxyacetophenone **1a**.

Chlorination of 2′-hydroxyacetophenone **1a** was performed by using NCS as the chlorination agent affording mixtures of chloro-derivatives **1b**–**d**, which composition mixture depends on the used molar equiv of NCS (1.1 equiv gave **1b** and **1d** in 35% and 39% yields; 2.1 equiv yielded **1c** in 49% yield). The low chlorination selectivity of 2′-hydroxyacetophenone **1a** explains the limited yields of **1b**–**d**. Then we build the expected chloromethoxyflavones **3b**,**c** as depicted in Scheme 1.

The final synthetic step in the synthesis of chlorinated 3′,4′,5,7-tetrahydroxyflavones **4a**–**d** consisted on the cleavage of the methyl

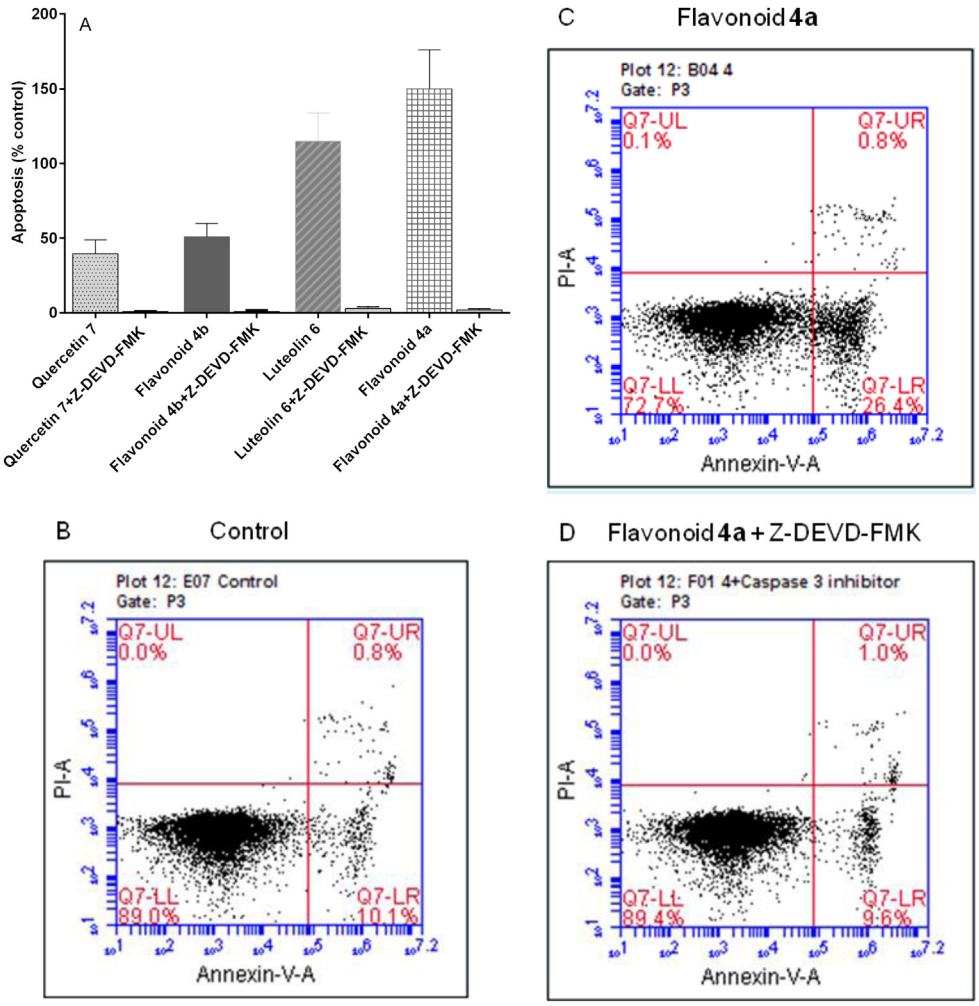
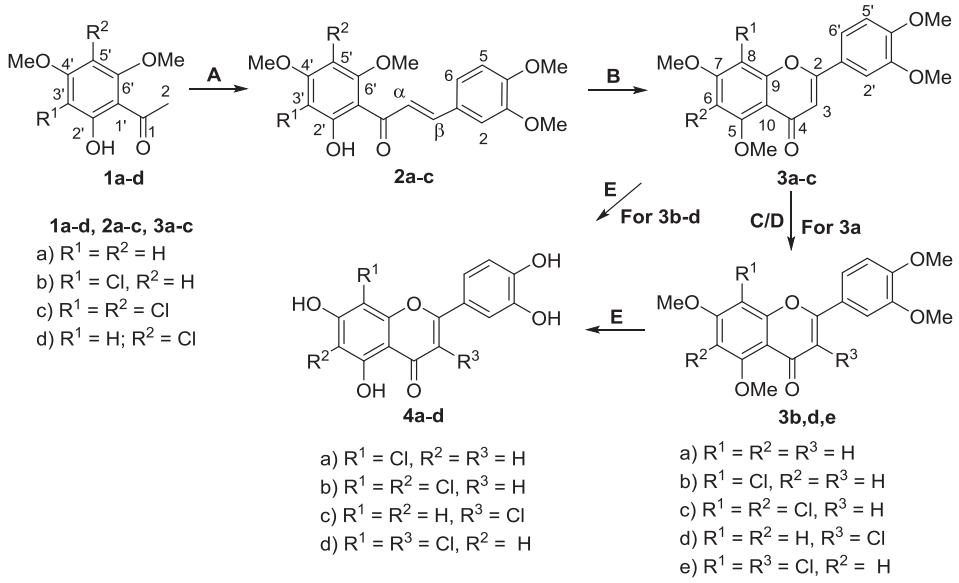


Fig. 3. Caspase 3 activity in flavonoids induced human neutrophils' apoptosis. Neutrophils were cultured in the presence of quercetin (**7**), luteolin (**6**) and flavones **4a** and **4b** at 50 μM with or without the caspase 3 inhibitor Z-DEVD-FMK (10 μM) before neutrophil apoptosis assessment by flow cytometric analysis (A). The values are given as the mean ± SEM ($n \geq 6$). Representative flow cytometry plots of annexin-V (annexin-V; *x* axis)/PI (*y* axis) binding at 4 h for control (without flavonoids) (B), for 50 μM of flavone **4a** (C) and 50 μM of flavone **4a** plus Z-DEVD-FMK (10 μM) (D).

protecting groups of the prepared chloromethoxyflavones **3b**–**e** by treatment with boron tribromide (2.5 equiv per methyl group) in dichloromethane, affording the expected compounds **4a**–**d** in moderate to good yields (42–86%). The lower yields of chlorohydroxyflavones **4b**,**d** are due to the required extensive reaction time (15 days) that besides affording the expected compounds also originates degradation products.

4.2. Biological evaluation

It was already reported that reactions between flavonoids and reactive species occur [12], although the effect of their derived products has not been studied. As example, it was reported that isoflavones in presence of HOCl could be chlorinated and the chlorinated products will modify inherent properties of the initial flavonoids. As such, in this work we intended to study the modulation of neutrophils activities by chlorinated flavones. The group of flavonoids evaluated in the present study **4a**–**d** and **5**–**7**, enabled the establishment of important structure/activity relationships and the finding of potent flavonoids able to modulate neutrophil's oxidative burst and apoptosis. This work revealed, for the first time, that chlorinated flavones are most effective than their parent flavonoids in modulating neutrophils' oxidative burst and apoptosis. It is important to refer that the viability of neutrophils remained unaltered after 4 h of incubation with the tested flavonoids at the maximum concentration tested, 50 μM. In this study PMA was used as neutrophil stimulator because its effect is closely analogous to that obtained during the phagocytic process [20] with an activation of protein kinase C, which results in NADPH oxidase activation, with subsequent production of $O_2^{\bullet -}$. This radical undergoes either spontaneous- or enzyme catalysed dismutation (through SOD from ingested microorganisms) to H_2O_2. H_2O_2 may generate HO• via Haber Weiss or Fenton reaction. Concomitantly, MPO, a heme protein present in azurophil granules of neutrophils, is released upon cell activation into the phagolysosome or into the extracellular space. This protein contributes considerably to the bactericidal capabilities of these cells via formation of HOCl from H_2O_2 and chloride ions. Since the main ROS produced by neutrophils are $O_2^{\bullet -}$, H_2O_2 and HOCl, it was of our great interest to evaluate the scavenging effect of chlorinated flavones against these species. We started our study using a chemiluminescent probe, luminol, that unspecifically detects $O_2^{\bullet -}$, H_2O_2, HO•, HOCl, •NO and $ONOO^-$ [5]. Among the tested flavonoids, flavone **5** was the less effective. What distinguish flavone from the other tested flavonoids



Scheme 1. Synthesis of chlorohydroxyflavones **4a**–**d**.

is the absence of a catechol group in the B-ring and the hydroxyl group in the 5- and 7-positions of the A-ring. This comes in agreement with previous studies about structure – activity relationships of flavonoids, which indicated that the number and the position of the hydroxyl groups are very relevant to the effect of flavonoids, namely the presence of a 3′,4′-dihydroxyl substitution in the B-ring [13,21]. In addition, Ribeiro et al. [12] reported that the substitution pattern in A-ring did not seem determinant for a modulatory effect of neutrophils' oxidative burst, since when a catechol group was present in the B-ring, the 5,7-$(OH)_2$ did not favour the activity of the tested flavonoids.

Flavone **4c**, with a 3-Cl substituent, was the one that demonstrated the best inhibition of the chemiluminescence ($IC_{50} = 2.0 \pm 0.4$ μM), followed by quercetin (**7**) ($IC_{50} = 2.8 \pm 0.4$ μM) (Table 2). The highest IC_{50} of luteolin (**6**) (5.2 ± 0.9 μM), without substitution in carbon 3, showed that the modulatory activity of the tested flavonoids may be determined by the C-ring substitution. According to the previously described the combination of 3- and 5-OH groups with the 4-oxo group, allows electron delocalisation from the 4-oxo group to both substituents, favouring the free radical scavenging by flavonoids [10,13]. Taking into account that the flavonoids with a 3-Cl or a 3-OH in C-ring presented almost the same effect, the electron delocalisation could be favoured not only by the presence of an OH in position 3 but also by a Cl in the same position.

Since luminol detects various reactive species, to assess the flavonoids activity in more detail it was necessary to use different and more specific probes. As such, we used amplex red, a highly specific and sensitive fluorogenic probe for the measurement of H_2O_2, to understand the involvement of this reactive species in flavonoids inhibition of oxidative burst [5]. Once again, 3-chloro-3′,4′,5,7-tetrahydroxyflavone **4c** was the most effective with an $IC_{50} = 1.4 \pm 0.1$ μM. The presence of a 3-chloro substituent of C-ring, revealed to be most important than the 3-OH group for the modulatory effect of the tested flavonoids, since the flavonoid **4c** was more active than quercetin (**7**) (IC_{50} = 2.7 ± 0.2 μM). The presence of 3-Cl in C-ring or a 8-Cl in A-ring or simultaneously 3,8-$(Cl)_2$ presented very similar ICs_{50}, although 6,8-dichloro-3′,4′,5,7,-tetrahydroxyflavone **4b** was the less effective flavonoid ($IC_{50} = 6.2 \pm 1.0$ μM) (Table 2).

It is important to note that HOCl generated by the heme peroxidase enzyme MPO has been reported to play a role in a wide range of human inflammatory diseases including atherosclerosis, asthma, rheumatoid arthritis, cystic fibrosis and some cancers [22]. As such, we intended to study the effect of chlorinated flavonoids in the scavenging HOCl. For that purpose, we used APF, a fluorescence probe with high sensitivity to HOCl. Flavonoid **4c** was the most effective, almost five times more active than quercetin (**7**) and luteolin (**6**). These results corroborate the results obtained with the other probes, showing that the 3-Cl substituent of the C-ring is relevant for the modulatory effect of flavonoids in neutrophils' oxidative burst. Once again, flavonoid **4b**, with a chloro substituent in positions 6 and 8 in A-ring, was the less effective ($IC_{50} = 7.2 \pm 0.6$ μM) (Table 2).

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 of the A-ring, each product potentially having a unique reactivity [23–25]. Inclusively, D'Alessandro et al. [23] reported that conversion of the native isoflavones to modified products was rapid and complete within 60 min of incubation with human neutrophils. By 30 min, 95% of the added genistein was recoverable as chlorinated products in the cell medium [23]. As such, it is important to understand the effect of the chlorinated flavones **4a**–**d** comparing it with their native compounds. In this work it was clearly proven that the chlorinated compounds were more active than the original ones. This is in agreement with Binsack et al. [24] that reported that mono- and dichlorinated quercetin derivatives were more effective (approximately 2- to 3-fold) at inhibiting copper-mediated LDL oxidation than the unmodified form. The authors concluded that the chlorination of quercetin occurred only at two sites, C-6 and C-8, and they tested 6-chloroquercetin and 6,8-dichloroquercetin showing that these two derivatives were more efficient than quercetin. Our results indicated that 6,8-dichloro-3′,4′,5,7,-

tetrahydroxyflavone (**4b**) was three times less effective than luteolin (**6**). The less reactivity of the flavonoid chlorinated in C-6 and C-8 could be related to the fact that the occupation of positions 6 and 8 of the A-ring prevents the reaction of flavonoids with HOCl, yielding a higher IC_{50} value.

Neutrophils have the shortest half-life of any blood leucocyte and rapidly undergo apoptosis *in vitro* (within 24 h) and *in vivo* (8–20 h) [26]. The regulation of neutrophil lifespan is a critical determinant of a circumscribed neutrophilic inflammatory response. Accordingly, uncontrolled neutrophil recruitment or inappropriate neutrophil longevity is pathophysiologically involved in inflammatory diseases, including arthritis, asthma, cancer, cardiovascular and periodontal diseases [9]. As such, resolution of inflammation depends on the clearance of neutrophils from tissues via apoptosis and macrophage phagocytosis, being this effect an attractive avenue for development of novel proresolution anti-inflammatory treatments [27].

Apoptosis may be initiated by two distinct but interlinked pathways, the extrinsic pathway upon ligation of a death receptor (such as TNF-α, Fas Ligand or TRAIL), or the intrinsic pathway in response to diverse cellular stressors which is associated with loss of mitochondrial membrane potential [27]. The convergence of the extrinsic and intrinsic pathways occurs at externalization of phosphatidylserine (PS) residues from the inner leaflet of the plasma membrane to the outer leaflet of apoptotic cells. Annexin V has a strong and specific affinity for PS, being an excellent probe to monitor the PS translocation that occurs due to apoptosis [28]. Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI.

Our results clearly demonstrated that some of tested flavonoids were able to induce primary neutrophil apoptosis *in vitro*. To our knowledge our study is the first to characterize the direct effects of chlorinated flavonoids on the regulation of neutrophil lifespan.

Flavones **4c**,**d** and **5** have no effect on human neutrophils' apoptosis up to the highest tested concentration (50 μM). It is interesting to note that flavonoid **4c** which was one of the most effective in decreasing human neutrophils' oxidative burst did not show any effect on the neutrophils' spontaneous apoptosis. Luteolin (**6**) and flavonoid **4a** were the flavonoids which more efficiently induced neutrophils' apoptosis, in a concentration and time-dependent manner (data not shown). As example, flavonoid **4a** increased 150 ± 26% the apoptotic neutrophils when compared with control (without flavonoid) (Fig. 1). It seems that the presence of 8-chloro substituent is important for the modulation of neutrophils lifespan. Also the presence of OH in C-3 instead of a Cl revealed to be important, since quercetin (**7**) induced neutrophils' apoptosis in contrast with flavonoid **4c**. Nevertheless, it is most efficient the flavonoid without substitution in C-3 as luteolin (**6**), which was one of the most active compounds. Our results were corroborated by Lucas and colleagues [9] who also reported that effect of luteolin (**6**) in neutrophils lifespan.

The pro-apoptotic role of selected plant flavonoids in various human and animal normal and cancer cell lines was already described [9,29–31]. However, it is clear that pharmacological induction of apoptosis is a highly cell type specific.

Liu et al. [32] demonstrated that quercetin (**7**) inhibited LPS-induced delay in spontaneous apoptosis of neutrophils after 24 h of incubation. These results suggest that quercetin (**7**) may decrease the susceptibility of neutrophils to pro-inflammatory factors. This effect of quercetin (**7**) could be related with the ability of quercetin (**7**) to induce neutrophils' apoptosis, corroborating our results.

The intrinsic and extrinsic pathways conducting apoptosis eventually act through activation of enzymes called caspases [28]. In neutrophil apoptosis, caspases play a central role in apoptotic cell death by the degradation of regulatory and structural proteins essential for cell survival and by the activation of nucleases and caspase 3, which is considered one of the most important apoptosis executors [27,33]. It was our intent to study the participation of caspase 3 enzyme in the pro-apoptotic effect of the tested flavonoids. The use of a specific inhibitor of caspase 3 activity allowed the conclusion that the pro-apoptotic flavonoids quercetin (**7**), flavones **4a**,**b** and luteolin (**6**) induced neutrophils' apoptosis via activation of caspase 3 (Fig. 3). Zielinska-Przyjemska et al. [30] corroborate our results by showing that the flavonoids naringin, naringenin and hesperidin induced neutrophil spontaneous apoptosis by activation of caspase 3. The authors reported that neither 2 h nor 24 h of PMA stimulation of neutrophils caused significant changes of the caspase-3 activity in the cells, suggesting that the production of reactive species by neutrophils prevented caspase-3 activation. The authors also demonstrated that NADPH oxidase inhibition induced a marked elevation of the caspase-3 activity in cells. These results suggested that ROS may inhibit apoptosis via inhibition of caspase 3 activation and the antioxidant action of the tested flavonoids may reverse this process. Similar results were reported by Fadeel et al. [34] and Arroyo et al. [35]. Lucas and colleagues [9] also reported that flavones, apigenin, luteolin, and wogonin induced apoptosis via caspase enzyme activation, as the coincubation of neutrophils with a caspase inhibitor abolished the ability of the flavones to induce apoptosis.

In conclusion, chlorinated flavonoids revealed to be more efficient than their parent compounds in modulate neutrophils' oxidative burst and lifespan. We have demonstrated that the tested flavonoids drive neutrophil apoptosis in a caspase-dependent fashion. The present data showed that among the tested flavonoids, 8-cloro-3′,4′,5,7-tetrahydroxiflavone (**4a**) constitute an alternative anti-inflammatory therapy, due to the proven ability to suppress mechanisms engaged at the onset and progression of inflammation.

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