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4-Fluoro-2-methoxyphenol, an apocynin analog with enhanced inhibitory effect on leukocyte oxidant production and phagocytosis

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ABSTRACT

Apocynin, a methoxy-substituted catechol (4-hydroxy-3-methoxyacetophenone), originally extracted from the roots of *Picrorhiza kurroa*, has been extensively used as a non-toxic inhibitor of the multienzymatic complex NADPH oxidase. We discovered that the analogous methoxy-substituted catechol, 4-Fluoro-2-methoxyphenol (F-apocynin), in which the acetyl group present in apocynin was changed to a fluorine atom, was significantly more potent as an inhibitor of NADPH oxidase activity, myeloperoxidase (MPO) chlorinating activity and phagocytosis of microorganisms by neutrophils; it was also as potent as apocynin in inhibiting tumor necrosis factor-alpha (TNFα) release by peripheral blood mononuclear cells. We attribute the increased potency of F-apocynin to its increased lipophilicity, which could facilitate the passage of the drug through the cell membrane. The inhibition of MPO chlorination activity, phagocytosis and TNFα release shows that apocynin and F-apocynin actions are not restricted to reactive oxygen species inhibition, but further studies are needed to clarify if these mechanisms are related. Like apocynin, F-apocynin did not show cell toxicity, and is a strong candidate for use in the treatment of inflammatory diseases.

1. Introduction

Reactive oxygen species play a central role in host defense by killing microbes in phagocytic cells. NADPH oxidase is a multienzymatic complex formed by cytochrome b558 linked to the membrane, three cytosolic factors (p47phox, p67phox e p40phox) and the small GTPase Rac2 (in neutrophils) or Rac1 (in monocytes). When cells are activated by stimuli like opsonized particles or phorbol myristate acetate, a cascade of events is initiated that results in migration of the NADPH oxidase cytosolic components to the membrane and the assembly of the enzymatic complex that initiates O_2^- production (Cathcart, 2004; Groemping and Rittinger, 2005; Ushio-Fukai, 2009), which, spontaneously or catalyzed by superoxide dismutase, dismutates to hydrogen peroxide. In this sequence, oxidant compounds like hydroxyl radicals, singlet oxygen, ozone and hypochlorous acid can be generated and constitute the microbicidal arsenal of phagocytes. Hypochlorous acid (HOCl) is generated by chloride anion oxidation by H_2O_2 via catalytic action of myeloperoxidase (MPO), which is also released into the phagolysosome during cell activation (Hansson et al., 2006; Klebanoff, 2005).

However, excessive production of ROS is harmful to the adjacent tissue and is involved in the genesis and/or progression of several inflammatory diseases, including vascular diseases with an inflammatory component (vasculitis, arteritis, atherosclerosis, hypertension and myocardial infarction, for example). This fact has stimulated the development of drugs targeting the NADPH oxidase system (Heumuller et al., 2008; Jo et al., 2006; Sun et al., 2007). In the same way, a growing body of literature implicates MPO-derived HOCl in the progression of inflammatory diseases, such as atherosclerosis, asthma, rheumatoid arthritis, cystic fibrosis, kidney diseases and some cancers (Malle et al., 2007). Increasing efforts have been devoted towards the development of a therapeutically useful myeloperoxidase inhibitor.

Apocynin is a methoxy-substituted catechol (4-hydroxy-3-methoxyacetophenone), originally extracted from the roots of *Picrorhiza kurroa*, from the Himalayas, that has demonstrated anti-inflammatory properties (Engels et al., 1992). Currently, apocynin is used as an inhibitor of the multienzymatic NADPH oxidase complex and concomitant reactive oxygen species production in experimental models involving phagocytic cells (Lafeber et al., 1999; Stolk et al., 1994; Zhang et al., 2005). The mechanism of NADPH oxidase inhibition by apocynin is not fully understood, but seems to involve the reduction of p47phox expression and membrane translocation (Barbieri et al., 2004), inhibition of NF-κB activation and expression, interference in arachidonic acid metabolism and reduction of cytokine

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release by monocytes and T cells (Engels et al., 1992; Lafeber et al., 1999; Mazor et al., 2010).

The multiple side effects of steroidal drugs make the discovery and development of a safer anti-inflammatory drug the goal of many researchers. Considering the growing evidence of the anti-inflammatory effects of apocynin, and its low cost and toxicity, we aimed to study the effect of the analogous methoxy-substituted catechol, 4-Fluoro-2-methoxyphenol (F-apocynin). The choice of this compound was based on its structural similarity, but increased hydrophobicity compared to apocynin, which could facilitate its penetration in the cell membrane. On the other hand, the fluorine atom is an electron-withdrawing group, which is supposed to be important for the inhibitory effect of apocynin analogous (Kanegae et al., 2007). These compounds were compared regarding inhibition of reactive oxygen species production, HOCl production, opsonized microorganism phagocytosis and tumor necrosis factor-alpha (TNFα) release.

2. Materials and methods

2.1. Chemicals

Apocynin, F-apocynin, 5-fluorotryptamine, catalase, dimethyl sulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), taurine, dihydrorhodamine 123, paraformaldehyde, zymosan, sodium azide, 3,3,5,5-tetramethylbenzidine (TMB), lucigenin, calcium chloride, magnesium chloride, glucose, dextran, and RPMI-1640 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ficoll-Paque was purchased from GE Healthcare. Myeloperoxidase was purchased from Planta Natural Products (Vienna, Austria) and Red Blood Cell (RBC) Lysis Buffer was purchased from eBioscience. FITC-labeled *Candida albicans* was provided by Dr. Sônia Jancar and Mariana Morato, and PI-labeled *Staphylococcus aureus* was provided by Dr. Alice Maria Melville Paiva Della. Hydrogen peroxide was prepared by diluting a 30% stock solution and calculating its concentration using its absorption at 240 nm (240 nm = 43.6 M^{-1} cm^{-1}). Hypochlorous acid was prepared by diluting a concentrated commercial chlorine bleach solution and calculating its concentration using its absorption at 292 nm (292 nm = 350 M^{-1} cm^{-1}). Apocynin and F-apocynin stock solutions were prepared by dissolving the compounds in dimethyl sulfoxide (DMSO). Cells incubated with apocynin or F-apocynin were always compared to cells incubated with the same volume of DMSO used as vehicle for the drugs (control group). PMA was dissolved in DMSO at 10 μg/ml. TMB solution was prepared by dissolving 10 mM TMB and 100 μm potassium iodide in 50% dimethylformamide and 50% acetic acid (400 mM). Human serum opsonized zimosan was prepared as described (Brochetta et al. 2003) in a final concentration of 10 mg/ml. Briefly, 10 mg of zymosan was suspended in PBS, boiled for 30 min and washed once with PBS. The pellet was then suspended in 1.0 ml of human serum pool, kept at 37 °C for 30 min, washed once with PBS and suspended in 1.0 ml of PBS for a final concentration of 10 mg/ml.

2.2. Isolation of human neutrophils and peripheral blood mononuclear cells

Polymorphonuclear (PMN) and peripheral blood mononuclear cells were separated by Ficoll-Paque (1.119 and 1.077 g/ml, respectively) density gradient centrifugation from 20 ml of blood from healthy donors. After isolation, the cells were resuspended in phosphate buffered saline (PBS) supplemented with 1.0 mM calcium chloride, 0.5 mM magnesium chloride, and 1.0 mg/ml glucose (supplemented PBS) or RPMI-1640 supplemented with 10% fetal bovine serum, 2.0 mM L-glutamine, 100 U/ml streptomycin and 100 U/ml penicillin for longer culture periods. Flow cytometric assays were developed with total blood or total leukocytes, in which erythrocytes were lysed with RBC lysis buffer, according to the needs of the evaluation.

2.3. Cytotoxicity

Cytotoxicity analysis was performed by Annexin-V/propidium iodide (PI) assay. Annexin-V and PI staining were performed to distinguish apoptotic cells from necrotic cells after apocynin or F-apocynin incubation. Annexin-V has a high affinity for phosphotidyl serine (PS), which translocates from the inner to the outer leaflet of the plasma membrane at an early stage of apoptosis. Annexin-V was conjugated with the fluorescent probe FITC, which allowed measurement by flow cytometry. PI staining distinguishes between apoptosis and necrosis due to the difference in the permeability of PI through the cell membranes of live and damaged cells. PI is able to emit fluorescence at fluorescence channel (FL) 2, and is also used in flow cytometric analysis. Total leukocytes were incubated with apocynin or F-apocynin (1.0 mM), or with the same volume of the vehicle for 6 h (control group), and an FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) was used to complete the staining (Vassena et al., 2007), according to the manufacturer's instructions. Fluorescence of gated PMN was detected in a FACS Canto Flow Cytometer (BD, Franklin Lakes, NJ, USA). Data was analyzed using the Flowjo Flow Cytometry Analysis Software (Treestar Inc., Ashlan, OR, USA) and statistical data were gained from the dot plots based on the proportions of unstained cells (viable cells), FITC fluorescent-stained cells (apoptotic cells), PI fluorescent-stained cells and dual stained cells (necrotic cells).

2.4. Determination of hypochlorous acid production by myeloperoxidase (cell-free system)

The determination of MPO chlorinating activity was based on the reaction of the produced HOCl with taurine to produce taurine chloramines, which oxidizes TMB (Malle et al., 2007). The TMB assay is extremely sensitive and was used to quantitate the production of HOCl by MPO catalyzed oxidation of chlorine (Malle et al., 2007). The reactions were performed in a 96-well plate containing PBS, taurine (5.0 mM), apocynin or F-apocynin (10 or 100 μM, respectively), purified MPO (50 nM) and H_2O_2 (50 μM). The reactions were triggered by adding H_2O_2 and incubated at 37 °C. The final volume was 200 μl. After 10 min, the reactions were stopped by adding catalase (20 μg/ml) and the accumulated taurine chloramine was measured by adding 50 μl TMB solution. The resulting blue product was detected spectrophotometrically at 655 nm using a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA).The chlorinating inhibitory potency was calculated by accounting for the amount of taurine chloramine produced in the control, in which the tested substances were absent.

2.5. Determination of hypochlorous acid production by neutrophils

The determination of hypochlorous acid production by neutrophils was also based on the reaction of the produced HOCl with taurine to produce taurine chloramines, which oxidizes TMB (Malle et al., 2007). The difference in this case is that MPO and H_2O_2 will be released by stimulated neutrophils instead of being added to the reaction. Neutrophils (5.0×10^6 cells/ml) were preincubated at 37 °C in supplemented PBS, containing 5.0 mM taurine and apocynin, F-apocynin or 5-fluorotryptamine (100 or 10 μM, respectively), a compound considered an excellent inhibitor of MPO (Jantschko et al., 2005) for 10 min. Next, the cells were stimulated by the addition of PMA (100 nM) or opsonized zymosan (1.0 mg/ml) and incubated for an additional 30 min at 37 °C for MPO and H_2O_2 release. The reactions were stopped by adding catalase (20 μg/ml) and centrifuged at 2000 rpm. The supernatants (200 μl) were transferred to a 96-well plate and 50 μl TMB was added, as above. The inhibitory potency was calculated by accounting for the amount of taurine chloramine produced in the control, in which the cells were incubated in the absence of the tested compounds.

2.6. Chemiluminescence assay

Lucigenin-dependent chemiluminescence is a sensitive, reproducible and reliable technique for superoxide detection. In this reaction, superoxide reduces lucigenin to its cation radical, which reacts with a second superoxide anion to form the energy-rich dioxetane molecule emitting a photon (Munzel et al., 2002). In our assay, PMN cells and peripheral blood mononuclear cells (1×10^6 cells/ml) were pre-incubated with apocynin or F-apocynin (10 or 100 μM, respectively) for 15 min in supplemented PBS. Next, lucigenin (5.0 μM) and opsonized zymosan (1.0 mg/ml) were added and the light emission was measured for 30 min at 37 °C (Centro Microplate Luminometer LB960, Berthold Technologies, TN, USA). The integrated light emission was used as an analytical parameter. The inhibitory potency was calculated by accounting for the light emission generated by the control, in which the cells were incubated in the absence of the tested compounds.

2.7. Cytometry assay

Dihydrorhodamine 123 (DHR) is largely used in the detection of intracellular oxidant species production by cell systems. DHR oxidation by reactive oxygen species results in the formation of rhodamine, a highly fluorescent component. Total leukocytes were incubated with apocynin or F-apocynin (1.0 mM) for 2 h and then stimulated with PMA (400 nM) for 10 min. After PMA stimulation, cells were incubated with DHR (10 mg/ml) for 5 min, washed once with PBS and suspended in PBS/BSA/azide buffer (sodium azide 0.1 g, fetal bovine serum 1.0 ml, PBS q.s.p. 100 ml). Fluorescence of gated PMN was detected at FL1, counting 30,000 events/gate, in a FACS Canto Flow Cytometer (BD, Franklin Lakes, NJ, USA). Data was analyzed using the Flowjo Flow Cytometry Analysis Software (Treestar Inc., Ashlan, OR, USA) and results were recorded as fluorescence intensity and percentage of positive cells in the sample.

2.8. Phagocytosis

Phagocytosis assay of *S. aureus* and *C. albicans* was performed by flow cytometry, using microorganisms labeled with FITC (*C. albicans*) or PI (*S. aureus*). *C. albicans* labeling was performed by incubating the yeast in carbonate buffer ($NaHCO_3$ 840 mg in 100 ml of MilliQ water, pH 9.5) with FITC (5.0 mg/ml) at 37 °C for 30 min; cells were then washed twice in carbonate buffer, twice in PBS and stored at −80 °C. *S. aureus* labeling with PI was performed using the technique described by Hasui et al. (1989) with some modifications; briefly, the bacteria were suspended in sterile saline solutions and incubated at 60 °C for 30 min; in the sequence, cells were washed twice in sterile saline solutions and incubated with propidium iodide (0.1%) for 30 min in the dark; cells were washed twice with sterile saline solution, suspended in glucose solution (5.0 mM glucose, 0.1% gelatin) and stored at −80 °C. Phagocytosis assay was performed according to the method described by White-Owen et al. (1992), with some modifications. Briefly, total blood (100 μl) was used to maintain the serum fraction for pathogen opsonization. Blood of healthy donors was incubated with apocynin or F-apocynin (1.0 mM) for 2 h and the cells were allowed to phagocyte labeled microorganisms for 1 h in a proportion of 10 microorganisms to 1 leukocyte. After this, erythrocytes were lysed and leukocytes were fixed in 2% paraformaldehyde. Fluorescence of gated PMN was detected at FL2 for *S. aureus* and FL1 for *C. albicans* in FACS Canto Flow Cytometer (BD, Franklin Lakes, NJ, USA). As for DHR assay, data was analyzed using the Flowjo Flow Cytometry Analysis Software (Treestar Inc., Ashlan, OR, USA) and results were recorded as fluorescence intensity and percentage of positive cells in the sample.

2.9. TNFα release

TNFα, a primary regulator of inflammatory responses, was quantified in the supernatant of peripheral blood mononuclear cells and PMN cells of healthy donors incubated with lipopolysaccharide (LPS — 10 μg/ml) and apocynin or F-apocynin (1.0 mM) for 4 or 24 h (peripheral blood mononuclear cells only). Cells were cultured in supplemented RPMI-1640 medium at 37 °C in an atmosphere of 5% CO_2. Cytokine quantification was performed by Enzyme-linked Immunosorbent Assay (ELISA), using a BD OptiEIA Human TNF ELISA Kit II (Cat. n° 550610), according to the manufacturer's instructions. Briefly, a monoclonal antibody specific for TNFα was coated on a 96-well plate. Dilutions of the TNFα of the standard curve and the supernatant of cells cultured as mentioned were added to the wells, and the TNFα present in the sample was allowed to bind to the immobilized antibody. The wells were washed and a streptavidin–horseradish peroxidase conjugate mixed with a biotinylated antihuman TNFα antibody was added, producing an antibody–antigen–antibody "sandwich". The wells were again washed and TMB substrate solution was added, which produced a blue color in direct proportion to the amount of TNFα present in the initial sample. The Stop Solution changed the color from blue to yellow, and color intensity was detected spectrophotometrically at 450 nm using a plate reader (SpectraMax plus 384, Molecular Devices, Sunnyvale, CA, USA).

2.10. Statistical analysis

Considering the small size of our samples ($n<10$), groups were compared using Mann–Whitney test for independent and asymmetric samples and results were expressed as median with range. Results were considered significant with a P value <0.05 (Montgomery, 1991).

3. Results

3.1. Apocynin versus 4-Fluoro-2-methoxyphenol

The molecular structure of apocynin and F-apocynin is depicted in Fig. 1. The change of the acetyl group for fluorine was chosen to maintain the characteristic electron-withdrawing substituent on the benzene ring, which seems to be important for the inhibitory property of apocynin (Kanegae et al., 2007), and to increase its lipophilicity, hence potentially increasing its accessibility to the intracellular medium. The octanol–water partition coefficient (logP) was calculated using the ChemDraw Ultra® 7.0 software. LogP is the ratio of the concentration of a chemical in octanol and in water at equilibrium and at a specified temperature. Oil–water partition coefficients in general, and the octanol–water partition coefficient in particular, are widely

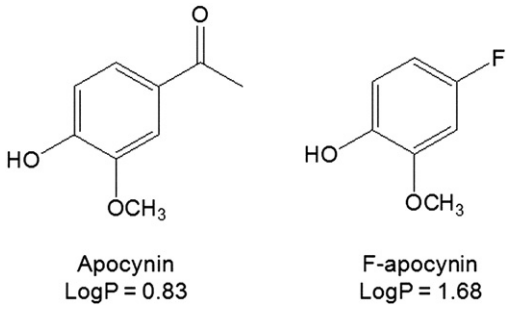

Fig. 1. The molecular structure of apocynin and F-apocynin, and the octanol–water partition coefficient (logP) calculated using the ChemDraw Ultra® 7.0 software. The higher the logP, the more hydrophobic the molecule becomes.

used as a measure of lipophilicity (for revision, Poole and Poole, 2003).

3.2. Cytotoxicity of apocynin and F-apocynin

Since this was the first study evaluating the biological properties of F-apocynin, the first step was to determine whether this chemical could be cytotoxic at the concentrations used in our experiments. Therefore, FITC-labeled Annexin-V and PI staining were performed to distinguish viable cells from apoptotic and necrotic cells by flow cytometry. At early stages of apoptosis, phosphatidyl serine translocates from the inner to the outer surface of the plasma membrane, and binds with high affinity to Annexin-V. Conversely, late apoptotic (or necrotic) cells present increased permeability to PI, and damaged cells will be stained in red. Our experiments show that neither apocynin nor F-apocynin (1.0 mM) changed the percentage of either unstained cells or live cells. Additionally, the percentages of Annexin-V and PI-labeled PMN cells were not altered by the drugs studied in comparison to the control group (Fig. 2). In short, neither apocynin nor F-apocynin was cytotoxic to PMN cells, under the conditions studied.

3.3. Effect of apocynin and F-apocynin on superoxide production by leukocytes

We determined and compared the efficiencies between apocynin and F-apocynin as inhibitors of superoxide anion release by stimulated PMN and peripheral blood mononuclear cells. In this study, the cells were previously incubated with apocynin or F-apocynin for 15 min and the reaction triggered by adding 10 mg/ml opsonized zymosan. The production of superoxide anion was measured by the lucigenin-dependent light emission. The concentration of lucigenin in the reaction mixture (5 μM) was purposely used to guarantee the specificity for detection of superoxide anion released to the extracellular medium (Munzel et al., 2002). The results in Fig. 3A show that F-apocynin was significantly more effective than apocynin in both concentrations tested in PMN cells. Similar results were obtained when peripheral blood mononuclear cells were stimulated by opsonized zymosan (Fig. 3B).

We also compared the abilities of apocynin and F-apocynin as inhibitors of intracellular production of reactive oxygen species by PMN cells assessed by flow cytometry, in which the non-fluorescent DHR is oxidized by reactive oxygen species giving rise to the fluorescent rhodamine. As observed in superoxide production, reactive oxygen species production was also inhibited by F-apocynin to a higher extent than apocynin, after 2 h of incubation (Fig. 4).

3.4. Effect of apocynin and F-apocynin on the yields of HOCl produced by isolated MPO and stimulated PMN

The effect of apocynin and F-apocynin on the production of HOCl through H_2O_2 mediated oxidation of chloride at physiological levels and catalyzed by MPO was determined by trapping the produced HOCl with taurine to form the taurine chloramine, which was assayed with TMB (Malle et al., 2007). The results depicted in Fig. 5A show that F-apocynin was also a stronger inhibitor of the chlorinating activity of MPO than apocynin. In the same way, the inhibitory potency of F-apocynin was higher compared to apocynin regarding the HOCl

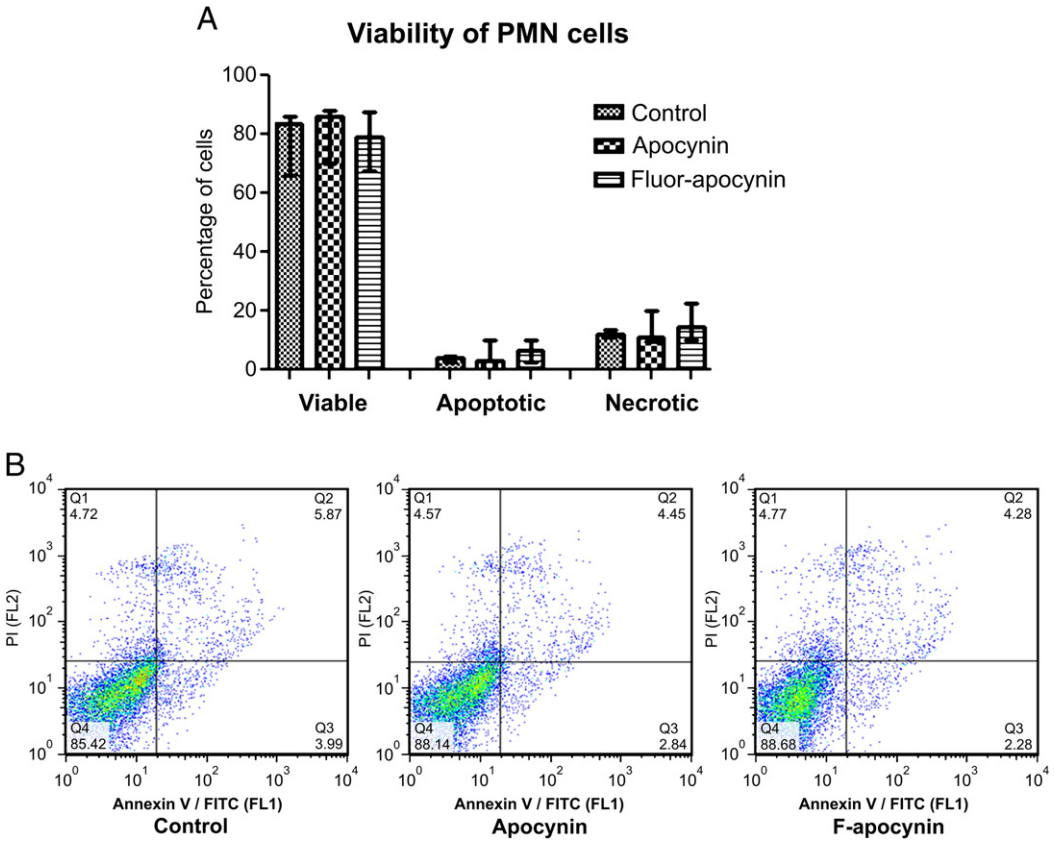

Fig. 2. The effect of apocynin and F-apocynin on PMN cell viability determined by Annexin V/PI flow cytometry. A: Percentage of viable, apoptotic and necrotic PMN cells in the control group and after 6 h of incubation with apocynin or F-apocynin (1.0 mM) ($P>0.05$, Mann–Whitney test, $n=4$). B: A dot plot display of Annexin V-FITC fluorescence (FL1) versus PI fluorescence (FL2) is shown in logarithmic scale. Upper left quadrant (PI-stained cells) and upper right quadrant (double stained cells) represented necrotic cells, lower left quadrant (unstained cells) were vital cells, and lower right quadrant (Annexin-V positive and PI negative cells) were apoptotic cells. PMN: polymorphonuclear, PI: propidium iodide, FITC: fluorescein isothiocyanate, FL: fluorescence channel.

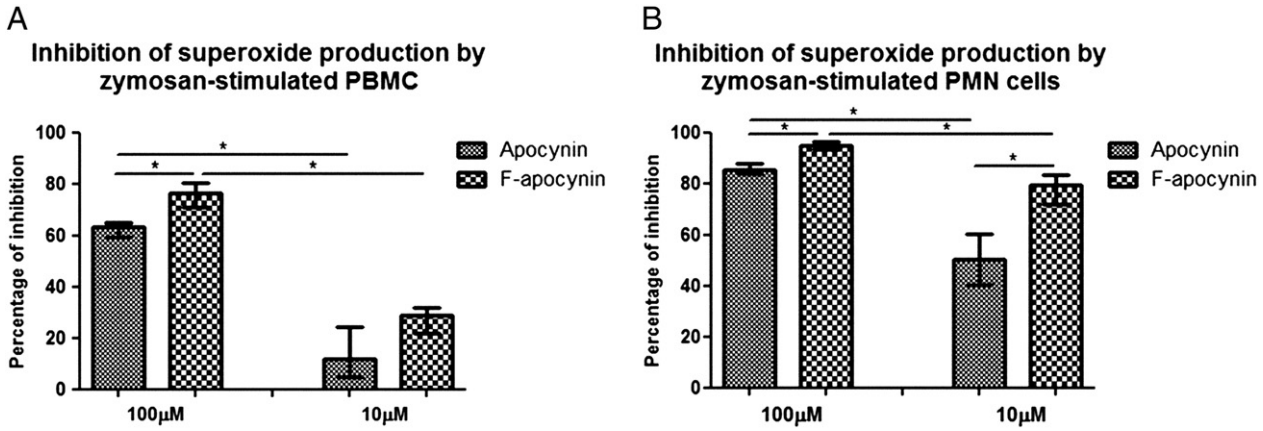

Fig. 3. Inhibition of superoxide production by apocynin or F-apocynin. Peripheral blood mononuclear cells (A) or PMN cells (B) were pre-incubated with 100 or 10 µM of apocynin or F-apocynin for 15 min and then superoxide production was stimulated by opsonized zymosan. Lucigenin was used as a probe for superoxide detection and light emission was measured for 30 min at 37 °C. Percentage of inhibition was calculated in comparison to the control group. Data represents at least four experiments (*P<0.05, Mann–Whitney test). PMN: polymorphonuclear.

produced by PMA- or opsonized zymosan-stimulated PMN (Fig. 5B and C). In fact, the inhibitory effect in the cell systems of both apocynin and F-apocynin was higher compared to the cell-free system. This result can be easily explained considering that the cells were affected in both the production of superoxide anion and the chlorinating activity of MPO. Specifically, the inhibitory capacity of F-apocynin was equivalent to 5-fluorotryptamine, a compound considered an excellent inhibitor of MPO (Jantschko et al., 2005).

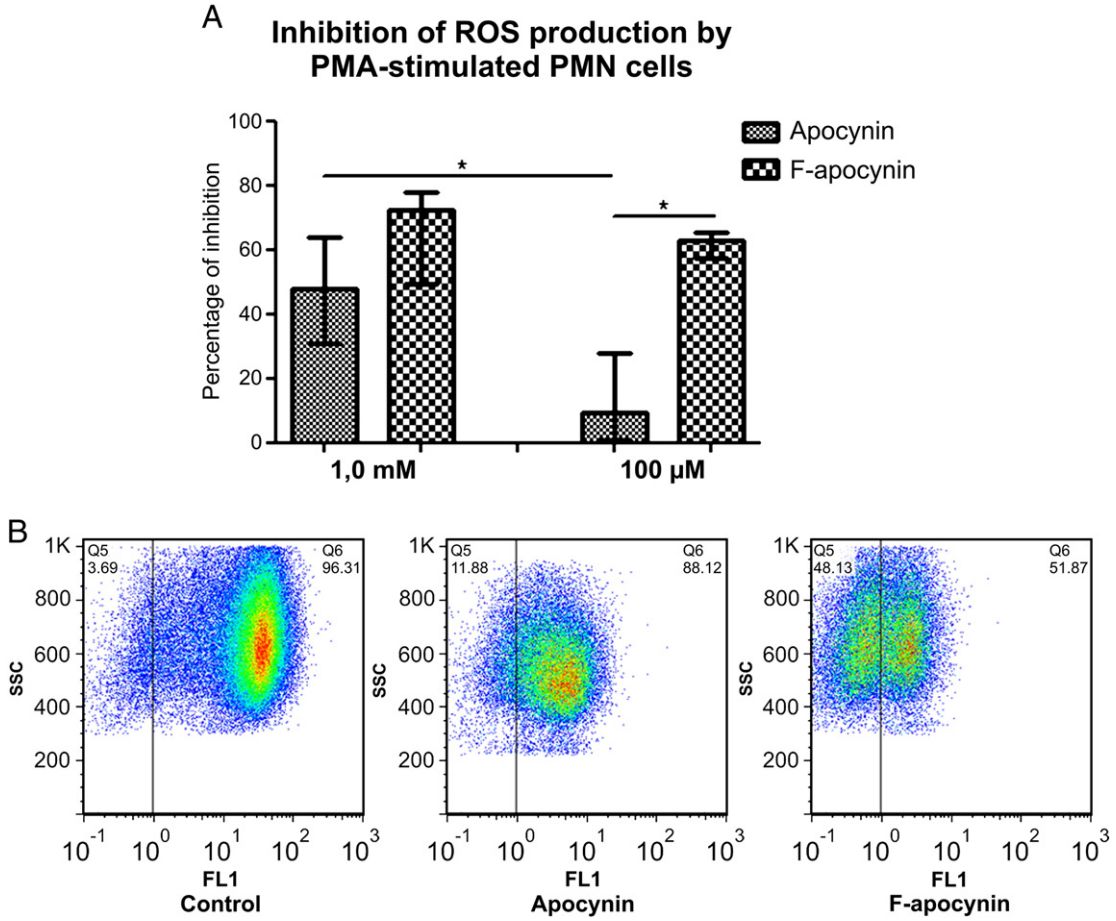

Fig. 4. The effect of apocynin and F-apocynin on reactive oxygen species production by PMN cells. PMN cells were pre-incubated with 1.0 mM or 100 µM of apocynin or F-apocynin, stimulated with PMA, and intracellular reactive oxygen species production was determined by flow cytometry using DHR as a probe. A: Inhibition of reactive oxygen species production by apocynin or F-apocynin. Percentage of inhibition was calculated in comparison to the control group. Data represents at least four experiments (*P<0.05, Mann–Whitney test). B: A dot plot display of FL1 (green fluorescence) versus side scatter (SSC, that represents cell granularity) is shown in logarithmic scale. In this example, cells were cultured with 1.0 mM of apocynin or F-apocynin, or in the absence of the studied compounds. Left quadrant represented rhodamine-negative cells, while right quadrant represented rhodamine-positive cells. The closer the cells were of the left quadrant, the lower was their fluorescence or reactive oxygen species production. PMA: phorbol myristate acetate, PMN: polymorphonuclear, SSC: side scatter channel, FL: fluorescence channel.

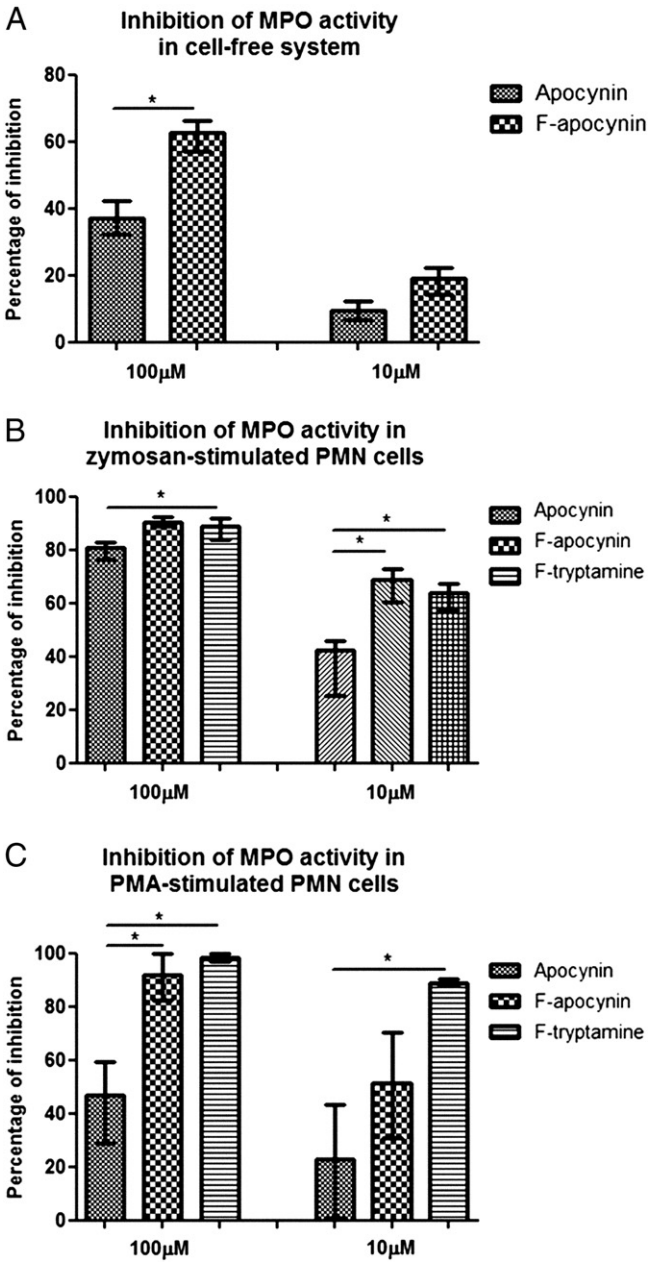

Fig. 5. The effect of apocynin and F-apocynin on MPO chlorinating activity. A: Inhibition of MPO chlorinating activity by apocynin or F-apocynin (100 or 10 μM, respectively) in a cell-free system. Apocynin or F-apocynin was incubated in supplemented PBS with taurine (5.0 mM), purified MPO (50 mM) and H_2O_2 (50 μM). HOCl production took place for 10 min, and reactions were stopped by adding catalase (20 mg/ml) and TMB was used to detect the accumulated taurine chloramine. B and C: Inhibition of MPO chlorinating activity by apocynin, F-apocynin or 5-fluorotryptamine (100 or 10 μM, respectively), a potent inhibitor of MPO activity, in PMN cells. Neutrophils in PBS/taurine were pre-incubated with apocynin or F-apocynin (100 or 10 μM, respectively) and MPO release was stimulated by opsonized zymosan (B) or PMA (C). After 30 min, the reaction was stopped by catalase addition and TMB was added to detect the accumulated taurine chloramine. Percentage of inhibition was calculated in comparison to the control group. Data represents at least four experiments (*$P<0.05$, Mann–Whitney test). MPO: myeloperoxidase, PMN: polymorphonuclear, PMA: phorbol myristate acetate.

3.5. Effect of apocynin and F-apocynin on phagocytosis of C. albicans and S. aureus

The effects of apocynin and F-apocynin on phagocytosis ability by PMN leukocytes were determined by flow cytometry using labeled microorganisms. Our results showed that F-apocynin and, to a lesser extent apocynin, significantly decrease the ability of PMN cells to phagocytose *C. albicans*. Apocynin and F-apocynin decreased the fluorescence intensity of positive cells, meaning that cells internalized a smaller number of microorganisms after incubation with the drugs (Fig. 6), but neither apocynin nor F-apocynin decreased the number of positive cells in comparison to the control group (data not shown). The phagocytosis of *S. aureus* was also impaired by F-apocynin, but not by apocynin, although this drug subtly decreased *S. aureus* phagocytosis by PMN cells (Fig. 6). In contrast to the phagocytosis observed in *C. albicans*, F-apocynin decreased fluorescence intensity and the percentage of *S. aureus* positive cells. In other words, the drug decreased the number of PMN cells that are able to phagocytose *S. aureus* and the relative number of microorganisms per cell, and, once again, the inhibition caused by F-apocynin was significantly stronger compared to apocynin (Fig. 6).

3.6. Effect of apocynin and F-apocynin on TNFα release by peripheral blood mononuclear cells and PMN cells

The release of TNFα by LPS-stimulated PMN and peripheral blood mononuclear cells incubated with apocynin or F-apocynin was determined by ELISA. Peripheral blood mononuclear cells were incubated with apocynin or F-apocynin for 4 and 24 h while PMN cells, which have shorter life, were cultured for 4 h only. In our experiments, apocynin and F-apocynin significantly inhibited TNFα release by peripheral blood mononuclear cells in both 4 and 24 h of culture. However, the drugs had no effect on TNFα release by PMN cells after 4 h of culture. Different from the previous results, the inhibition of TNFα observed after incubation with F-apocynin was the same as observed after incubation with apocynin (Fig. 7).

4. Discussion

The clinical applicability of apocynin as an anti-inflammatory drug has been studied by several groups, which showed that apocynin efficiently decreased signs and symptoms of rheumatoid arthritis, inflammatory bowel disease, atherosclerosis and asthma (Van den Worm, 2001; Stefanska and Pawliczak, 2008; Pandey et al., 2009; Tang et al., 2010). Here, we found that the analogous compound F-apocynin is even more efficient than apocynin at inhibiting NADPH oxidase-mediated reactive oxygen species generation in neutrophils and mononuclear cells. This is the first work evaluating the anti-inflammatory effects of F-apocynin and its choice was based in its structural similarity, but increased lipophilicity, compared to apocynin, which could facilitate the passage of the drug through the cell membrane. Moreover, in substitution to the acetyl group, the fluorine atom provides the electron-withdrawing characteristic which seems important for the mechanism of action of apocynin (Kanegae et al., 2007). Hence, we propose that a similar working mechanism is responsible by the inhibitory effects of F-apocynin. An important point regarding the application of apocynin is its low toxicity, enabling its use *in vivo*. Like apocynin, F-apocynin was not cytotoxic for the cells used here. Therefore, we suggest that F-apocynin will also be applicable in animal models.

In addition to efficiently inhibit the NADPH oxidase system, F-apocynin significantly decreased HOCl production in a cell-free system and in stimulated PMN. MPO performs a crucial role in the microbicidal system of neutrophils and monocytes through the generation of reactive oxidants, in particular HOCl, and radical species (Van der Veen et al., 2009). In health, MPO-catalyzed formation of highly reactive halide-derived oxidants is an important component of the armament of neutrophils and monocytes needed to kill and digest phagocytosed pathogens (Van der Veen et al., 2009). HOCl, the main product of the MPO–H_2O_2–chloride system is a membrane-permeant potent oxidant with a broad range of potential targets, including thiols,

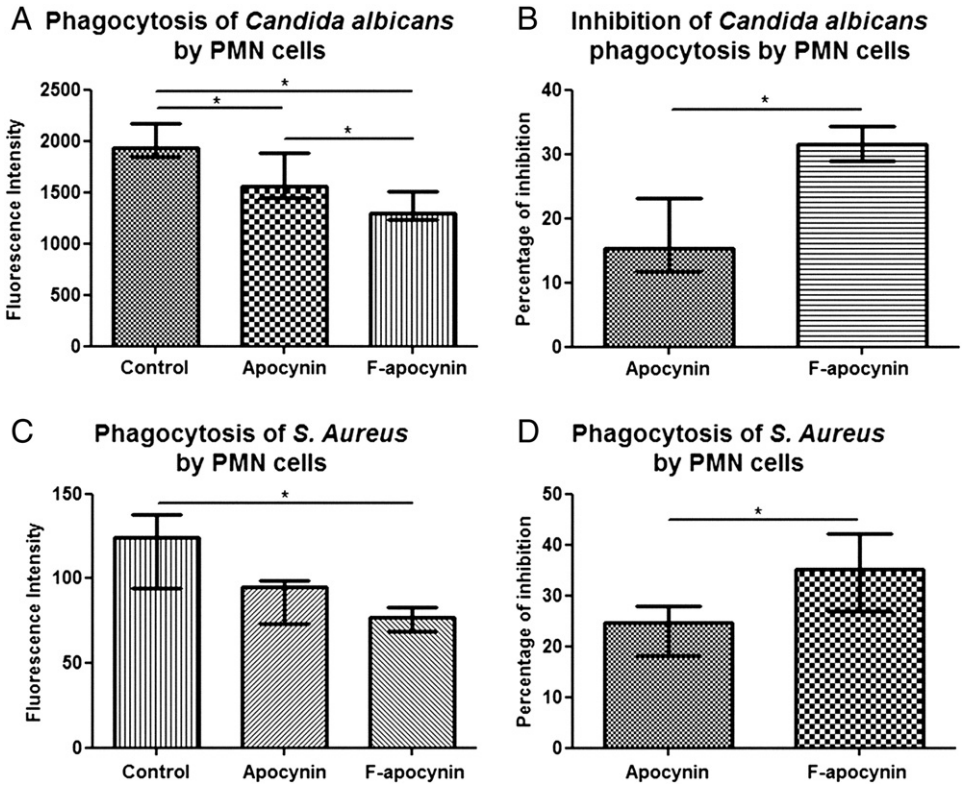

Fig. 6. Effect of apocynin and F-apocynin on *Candida albicans* (A and B) and *Staphylococcus aureus* (C and D) phagocytosis by PMN cells. Cells were pre-incubated with 1.0 mM of apocynin or F-apocynin for 2 h, PI-labeled *S. aureus* or FITC-labeled *C. albicans* were added, phagocytosis took place for 1 h, and the measurement of *S. aureus* and *C. albicans* phagocytosis by PMN cells was determined by flow cytometry. Fluorescence intensity was used as a parameter of the amount of microorganism ingested by the cells (A and C), and percentage of inhibition of *S. aureus* and *C. albicans* phagocytosis by apocynin or F-apocynin was calculated in comparison to the control group (B and D). Data represents six experiments (*$P<0.05$, Mann–Whitney test). PMN: polymorphonuclear.

phenols, and iron centers. Furthermore, HOCl can react with amines to generate chloramines and thereby spawn a wave of toxic species that have relatively long half-lives and can propagate additional tissue damage (Nauseef, 2001). In this way, the direct toxic effects of MPO-derived oxidants, together with its noncatalytic immune-modulating properties, have been implicated in the induction of malignancies and tissue injury, as well as in the progression of the inflammatory response in multiple diseases. Specifically, MPO is directly involved in the induction and progression of cardiovascular diseases, endothelial dysfunction (by inhibiting NO), atherosclerosis, carcinogenesis, neurodegenerative, renal and lung diseases, and chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease (Nauseef, 2001; Malle et al., 2007; Van der Veen et al., 2009). Thus, we conclude that the inhibition of HOCl production after incubation with F-apocynin and, to a lesser extent, with apocynin, can consequently decrease the damage induced by these oxidants. Adding to it the property of inhibiting NADPH oxidase-mediated reactive oxygen species production, F-apocynin has a great potential to be used as non-steroidal anti-inflammatory drug.

We also compared F-apocynin and apocynin in regard to their respective capacities to interfere with phagocytosis of opsonized microorganisms. In contrast with the literature (Stefanska and Pawliczak, 2008), we found that apocynin significantly inhibited *C. albicans* and *S. aureus* phagocytosis by PMN cells. In both assays, F-apocynin was more efficient than apocynin in the inhibition of phagocytosis. F-apocynin was 100% more efficient than apocynin in the inhibition of *C. albicans* phagocytosis and 45% more efficient in the inhibition of *S. aureus* phagocytosis. Stolk et al. (1994) showed that apocynin, in a concentration of 300 μM, selectively inhibits NADPH oxidase activity but does not inhibit phagocytosis ability by neutrophils. Our experiments were developed with a high dose of apocynin and F-apocynin (1.0 mM) which explains why we observed a reduction in phagocytosis ability.

TNFα, a cytokine produced for many cell types, including macrophages, neutrophils and keratinocytes, mediates various activities in different cells (Weinstein and Kirsner, 2010). The effect of apocynin on TNFα release, along with other cytokines, is well established. For example, in both *in vivo* and *in vitro* assays, apocynin inhibits TNFα release in cultured peripheral blood mononuclear cells of rheumatoid arthritis patients (Van den Worm, 2001). Paterniti et al. (2010) showed that apocynin attenuates TNFα production in the ileum of splanchnic artery occlusion-shocked rats, and suggested that this effect is a result of the inhibitory effect of the activation of NF-κB. Using the Sabra rat model of salt-sensitive hypertension, Mazor et al. (2010) showed that inhibition of NADPH oxidase activity by apocynin is followed by a reduction in the levels of systemic oxidative stress and TNFα, events related to the development of hypertension. The authors propose that the reduction in TNFα release is secondary to the inhibition of oxidative stress and inflammation triggered by the active enzyme. Although the mechanism by which apocynin inhibits TNFα release is not clear, the usefulness of this inhibition in the treatment of inflammatory diseases is unquestionable. In agreement with the literature, apocynin significantly decreased TNFα release by LPS-stimulated peripheral blood mononuclear cells in 4 and 24 h of culture, but no effect was observed on LPS-stimulated PMN cells cultured for 4 h. The same effect was observed using F-apocynin, which inhibited TNFα release by mononuclear cells but not by PMN, but, in this case, the F-apocynin effect was comparable to apocynin, and not stronger. It is interesting to note that, different than the observations in TNFα release experiments, apocynin and F-apocynin effects on NADPH oxidase and MPO activity are mainly associated with PMN cells, while little or no effect was observed in mononuclear cells or monocytes (data not shown). We suggest that the mechanism involved in NADPH oxidase activity inhibition by apocynin, which is MPO-dependent and, for this reason, more intense in MPO-rich cells, is different than the mechanism of TNFα release inhibition. In other

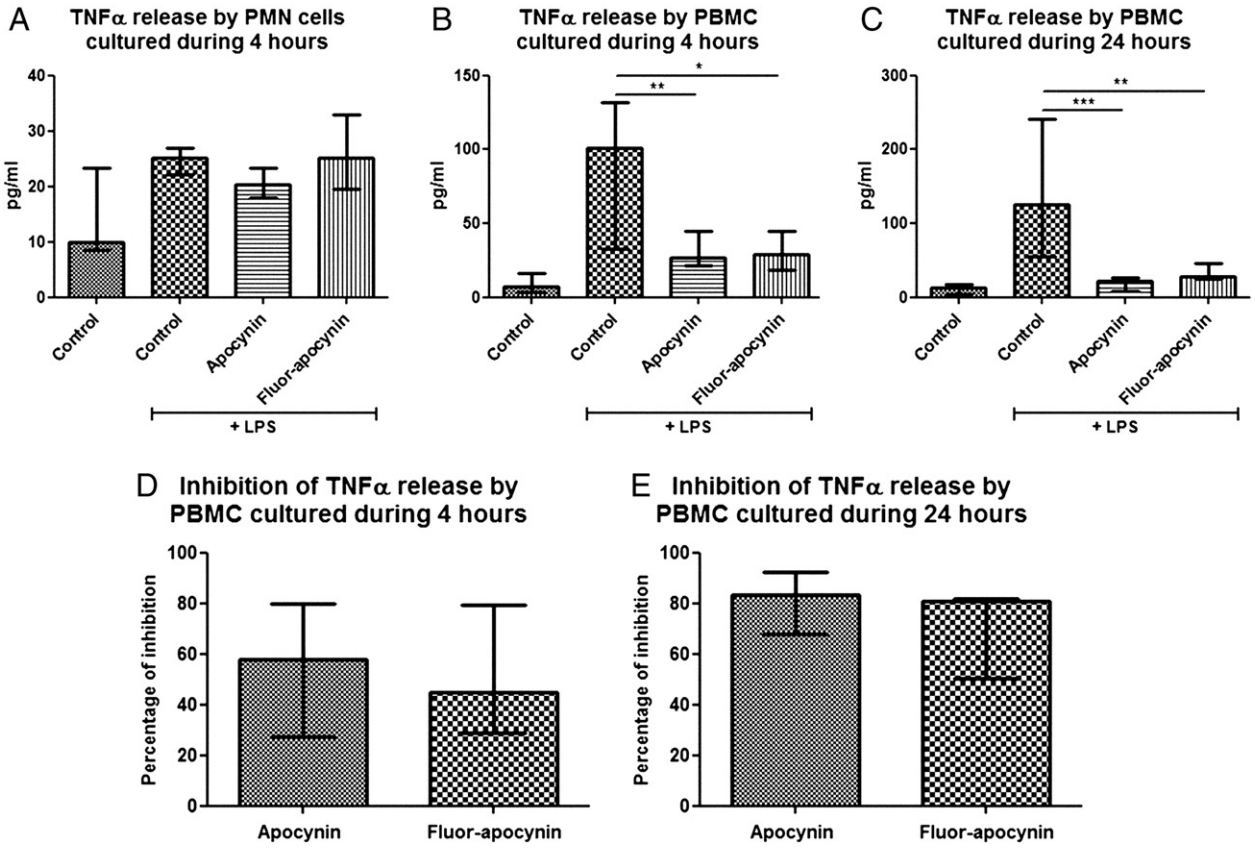

Fig. 7. The effect of apocynin and F-apocynin on TNFα release by PMN (A) or peripheral blood mononuclear cells (B and C). A, B and C: Cells were incubated with LPS (10 μg/ml) and 1.0 mM of apocynin or F-apocynin for 4 and 24 h (peripheral blood mononuclear cells only), and TNFα release was quantified in the supernatant using Enzyme-linked Immunosorbent Assay (ELISA). D and E: Percentage of inhibition of TNFα release by peripheral blood mononuclear cells incubated with apocynin or F-apocynin for 4 (D) or 24 h (E) was calculated in comparison to the control group. Data represents seven experiments (*P<0.05, **P<0.01, ***P<0.001, Mann–Whitney test). TNFα: tumor necrosis factor alpha, PMN: polymorphonuclear.

words, TNFα release inhibition by apocynin or F-apocynin does not seem to be a result of the redox imbalance resulting from NADPH oxidase activity inhibition. Recently, Dharmarajah et al. (2010) showed that the anti-platelet actions of apocynin are not the result of an inhibitory effect on NADPH oxidase or reactive oxygen species scavenging, as proposed by others (Allen and Bayraktutan, 2008), and suggested that this effect reinforces the indication of this drug as an anti-thrombotic agent. It confirms that the effects of apocynin extend beyond NADPH oxidase inhibition and consequent redox imbalance.

In summary, owing to the involvement of MPO in the pathophysiology of many diseases, several research groups are striving to develop an effective and safe MPO inhibitor. The same is observed within the research of NADPH oxidase inhibitors and the search for more selective and less toxic molecules. In this regard, the efficacy and lack of toxicity in different *in vivo* and *in vitro* assays raised apocynin to the rank of a lead compound in the search for new, non-steroidal anti-inflammatory compounds. In this work, we have demonstrated that F-apocynin was more efficient than apocynin in NADPH oxidase, MPO activity and phagocytosis inhibition. Since F-apocynin exhibits low toxicity and efficiently inhibits reactive oxygen species and HOCl production and TNFα release, it is a strong candidate for use in the treatment of inflammatory diseases. Additional research is needed to clearly define its mechanism.

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