

# The Flavonols Quercetin, Myricetin, Kaempferol, and Galangin Inhibit the Net Oxygen Consumption by Immune Complex-Stimulated Human and Rabbit Neutrophils

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Stimulated human neutrophils exhibit increased net oxygen consumption (NOC) due to the conversion of  $O_2$  into the superoxide anion by the NADPH oxidase enzymatic complex during the respiratory burst. In several inflammatory diseases, overproduction of these oxidants causes tissue damage. The present study aims to: (a) optimize the experimental conditions used to measure the NOC in serum-opsonized zymosan (OZ)- and insoluble immune complex (i-IC)-stimulated human and rabbit neutrophils; and (b) compare the effect of four flavonols (quercetin, myricetin, kaempferol, and galangin) on this activity. We used a Clark-type oxygen electrode to measure the NOC of stimulated neutrophils. Eliciting the neutrophil respiratory burst with OZ and i-IC yielded similar maximum  $O_2$  uptake levels within the same species, but the human neutrophil NOC was almost four times higher than the rabbit neutrophil NOC. The optimal experimental conditions established for both cell types were  $4 \cdot 10^6$  neutrophils  $mL^{-1}$ ,  $2 \text{ mg } mL^{-1}$  OZ, and  $240 \mu g \text{ mL}^{-1}$  i-IC. Upon stimulation with OZ or i-IC, the tested flavonols reduced the human and rabbit neutrophil NOC in the same order of potency – quercetin and galangin were the most and the least potent, respectively. These compounds were around four times more effective in inhibiting the rabbit as compared to the human neutrophil NOC, respectively. The four flavonols were not toxic to human or rabbit neutrophils. The experimental conditions used are suitable for both the determination of human and rabbit neutrophil NOC and for the assessment of the modulatory effects of natural compounds on these activities. The relationship between the level of NOC and the inhibitory potency of the flavonols suggests that rabbit neutrophils can be useful experimental models to predict the effect of drugs on immune complex-stimulated human neutrophils.

**Key words:** NADPH Oxidase, Neutrophil, Flavonol

## Introduction

Chronic inflammatory diseases mediated by immune complexes (IC), such as rheumatoid arthritis, glomerulonephritis, and vasculitis, affect a significant percentage of the human population and diminish both life quality and expectancy (Németh and Mócsai, 2012). These diseases are associated with intense recruitment and activation of neutrophils in the tissues. Once at the site of inflammation, they recognize opsonized IC, microbes or particles mainly via the complement receptors (CR) CR1 and CR3, as well as the

receptors for the Fc-region of IgG ( $Fc\gamma R$ ), in particular  $Fc\gamma RIIa$  and  $Fc\gamma RIIIb$ . Neutrophil activation via CR and  $Fc\gamma R$  triggers the effector functions of these cells, like phagocytosis, degranulation, and production of reactive oxygen species (ROS) (Daëron, 1997; Wright *et al.*, 2010).

Rabbit neutrophils share some structural similarities with their human counterparts, especially with regard to  $Fc\gamma R$ . The ability of IC containing rabbit IgG to trigger the effector functions of both rabbit and human neutrophils is evidence of this homology (Lucisano and Mantovani, 1988; Marzocchi-

Machado *et al.*, 2002; Fairhurst *et al.*, 2007). In this context, studies have employed rabbit neutrophils as experimental models, to investigate the physiopathology of some rheumatic diseases and to assess the immunomodulatory effect of drugs (Cochrane and Koffler, 1973; Spilberg *et al.*, 1979; Podolin *et al.*, 2002; Kabeya *et al.*, 2008; Moreira *et al.*, 2007). Thus, comparative studies of the structural and functional properties of human and rabbit neutrophils are important to extend the results of animal studies to human diseases.

The first ROS produced during respiratory burst is the superoxide anion, generated after O<sub>2</sub> reduction by the NADPH oxidase (NADPHox) enzymatic complex. As a result, the net O<sub>2</sub> consumption (NOC) by the stimulated neutrophils increases (Cheung *et al.*, 1983; Segal, 2008). The importance of NADPHox for human host defence is evident in patients with chronic granulomatous disease, a syndrome characterized by the absence or reduction of NADPHox activity in phagocytes, which is clinically manifested by severe recurrent bacterial and fungal infections (Gallin and Zarember, 2007). In contrast, some inflammatory and autoimmune diseases involve neutrophil accumulation and overstimulation, with consequent increase in the production and release of ROS and other toxic products that contribute to tissue damage (Wright *et al.*, 2010; Németh and Mócsai, 2012).

In this sense, a therapeutic strategy to maintain body homeostasis is to downregulate excessive neutrophil-mediated ROS production by modulating the NADPHox activity and/or its activation using natural products (Tauber *et al.*, 1984; Paula *et al.*, 2009; Ciz *et al.*, 2012). Our research group has reported the immunomodulatory effect of a set of flavonoids (Kanashiro *et al.*, 2004) and highlighted that the flavonol moiety – the 2,3-double bond in conjugation with a 4-oxo group and a 3-hydroxy group – associated with the 5,7-dihydroxylation at ring A accounts for the significant inhibition of the IC-stimulated oxidative metabolism of rabbit neutrophils. Among the flavonols with these structural features, myricetin, quercetin, kaempferol, and galangin were selected for further studies (Fig. 1). These compounds bear the same substituents in rings A and C, but the hydroxylation patterns in ring B are different. These flavonols can inhibit neutrophil degranulation without eliciting cytotoxicity or impairing the phagocytic function (Kanashiro *et al.*, 2007; Moreira *et al.*, 2007). Quercetin and kaempferol inhibit the human neutrophil NOC *in vitro* and the NADPHox

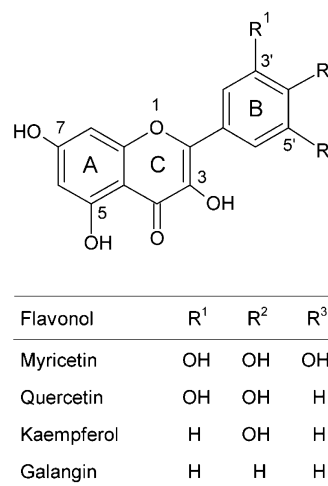


Fig. 1. Chemical structures of the flavonols tested in this study.

activity assayed in a cell-free system (Tauber *et al.*, 1984).

In this context, in this work we intended to (1) define the parameters to evaluate the direct effect of drugs on the human and rabbit neutrophil NOC, and (2) study a group of natural compounds, *i. e.* the flavonols myricetin, quercetin, kaempferol, and galangin (Fig. 1), using a polarographic method to measure the O<sub>2</sub> uptake. We used two types of IC to stimulate the human and rabbit neutrophils: insoluble IC (i-IC) composed of IgG and ovalbumin, and serum-opsonized zymosan (OZ).

## Materials and Methods

### Chemicals

Zymosan A (isolated from *Saccharomyces cerevisiae*), quercetin (3,3',4',5,7-pentahydroxyflavone dihydrate), kaempferol (3,4',5,7-tetrahydroxyflavone), myricetin (3,3',4',5,5',7-hexahydroxyflavone), galangin (3,5,7-trihydroxyflavone), diphenyleiiodonium chloride (DPI), ovalbumin (OVA; albumin from chicken egg), and sodium dithionite were acquired from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany). O<sub>2</sub> probe solution was provided by YSI Inc. (Yellow Springs, OH, USA), Difco™ gelatin (microbiological grade) was furnished by BD Biosciences (San Diego, CA, USA), and the LDH Liquiform test kit was obtained from Labtest Diagnostica (Lagoa Santa, MG, Brazil).

### *Healthy human subjects*

Twenty adult subjects (8 males and 12 females), aged between 18 and 40 years, were recruited according to the protocol approved by the local Research Ethics Committee (CEP/FCFRP-USP, protocol number 111). All participants were in good general health, as demonstrated by their medical history, and signed an informed consent prior to the performance of any study-related procedure. The exclusion criteria were: (1) history of any acute or chronic disease, (2) recent use of anti-inflammatory drugs, (3) recent history of alcohol or drug abuse, or (4) active smoking.

### *Animals*

New Zealand white rabbits (female adults, mean weight of 3 kg) were used for blood collection and anti-OVA antibody production. Animals were handled in accordance with the instructions issued by the Ethics Committee on Laboratory Animal Care and Use of the University of São Paulo, Campus of Ribeirão Preto, Ribeirão Preto, SP, Brazil. The experimental procedures were approved under protocol number 05.1050.53.9.

### *Isolation of neutrophils*

Blood was collected from the central artery in the rabbits' ears or drawn from human volunteers by venous puncture into Alsever solution (v/v), used as anticoagulant. Neutrophils were isolated by the method of Lucisano and Mantovani (1984). The cell pellets were suspended in Hank's balanced saline solution (HBSS) containing 0.1% (w/v) gelatin (HBSS-gel). The cell preparations contained 80–90% neutrophils with a viability higher than 95%, as established by exclusion with Trypan Blue.

### *Preparation of opsonized zymosan (OZ)*

Normal rabbit serum (NRS) and normal human serum (NHS) were obtained from blood samples collected in the absence of anticoagulant. The zymosan particles were prepared and opsonized with NRS or NHS according to the method of Cheung *et al.* (1983) with some modifications (Kanashiro *et al.*, 2004). Briefly, a zymosan ( $2 \text{ mg mL}^{-1}$ ) suspension in 0.15 M NaCl was boiled in a water bath for 30 min, cooled, and centrifuged at  $270 \times g$  for 5 min. The resulting pellet was washed with 0.15 M NaCl and the

mixture centrifuged in the same conditions. NRS or NHS was diluted 1:2 in complement fixation diluent (Weir, 1986) buffer containing 0.1% (w/v) gelatin and added to the pellet. The mixture was incubated at  $37^\circ\text{C}$  for 30 min and centrifuged at  $480 \times g$  for 5 min. The resulting pellet was washed twice with HBSS and the mixture centrifuged in the same conditions. Finally, the zymosan particles opsonized with NRS or NHS (OZ-NRS and OZ-NHS, respectively) were suspended in HBSS-gel for use.

### *Preparation of insoluble immune complexes (i-IC)*

The i-IC were prepared by mixing OVA and polyclonal rabbit anti-OVA IgG at equivalence, as determined on the basis of quantitative precipitin curves (Fahey *et al.*, 1964; Kabat, 1961). After incubation at  $37^\circ\text{C}$  for 30 min and maintenance at  $4^\circ\text{C}$  overnight, the i-IC were washed twice with cold 0.15 M NaCl ( $12,000 \times g$ , 15 min,  $4^\circ\text{C}$ ) and suspended in the same medium. The total protein concentration in the precipitates was calculated by absorbance readings at 280 nm and expressed as  $\mu\text{g mL}^{-1}$ . The i-IC were diluted in HBSS, pH 7.2, for use.

### *Determination of the neutrophil net $\text{O}_2$ consumption (NOC)*

#### *General procedure*

The NOC was assessed by a method modified from that of Chance and Williams (1956). The  $\text{O}_2$  uptake by the neutrophils was measured using an oxygraph equipped with a Clark-type oxygen electrode (Strathkelvin Instruments, Glasgow, Scotland, UK). The calibration of the equipment and the total  $\text{O}_2$  concentration in the reaction medium ( $37^\circ\text{C}$ , 0.5 mL) were first determined with sodium dithionite. The measuring chamber was thoroughly washed, the neutrophil suspension was transferred into it, and the "basal  $\text{O}_2$ " uptake – the  $\text{O}_2$  concentration that the cells needed to maintain their functions without NADPHox stimulation – was measured at  $37^\circ\text{C}$  for 7 min. Next, the cells were stimulated with OZ-NHS (for human neutrophils), OZ-NRS (for rabbit neutrophils), or i-IC (for both types of cell), and the  $\text{O}_2$  consumption was further measured for 7 min. This second measure represented the amount of  $\text{O}_2$  consumed by the cells during the respiratory burst and was designated "activated  $\text{O}_2$ ". The difference between the rates of "basal  $\text{O}_2$ " and "activated  $\text{O}_2$ " corresponds to the  $\text{O}_2$

consumption due to the NADPHox activity, expressed as  $\text{nmol of O}_2 \text{ min}^{-1} \text{ mL}^{-1}$ . DPI ( $20 \mu\text{M}$ ), which is an uncompetitive NADPHox inhibitor, was used as positive control.

#### Standardization of neutrophil number and concentration of OZ and i-IC

The  $\text{O}_2$  consumption by the neutrophils ( $2-8 \cdot 10^6 \text{ cells mL}^{-1}$ ) was calculated before and after the addition of OZ-NHS or OZ-NRS ( $1-4 \text{ mg mL}^{-1}$ ) or i-IC ( $60-480 \mu\text{g mL}^{-1}$ ). This assay allowed to establish the optimal number of neutrophils and the amount of stimulus.

#### Effect of the natural compounds on NOC

The NOC by neutrophils ( $4 \cdot 10^6 \text{ cells mL}^{-1}$ ) was determined before and after the addition of OZ-NHS or OZ-NRS ( $2 \text{ mg mL}^{-1}$ ) or i-IC ( $240 \mu\text{g mL}^{-1}$ ) in the presence of HBSS (negative control), DMSO [ $0.1\%$  (v/v); vehicle], the flavonols ( $2.5-60 \mu\text{M}$ ), or DPI ( $10 \mu\text{M}$ ; positive control). Final concentrations are given in parentheses. The inhibitory effect of each compound on the NOC was calculated based on DMSO as control.

#### Cytotoxicity

The cytotoxic effect of the flavonols on rabbit and human neutrophils was evaluated as described by Lucisano-Valim *et al.* (2002). Values in parentheses refer to the final concentrations in  $1.0 \text{ mL}$  of reaction mixture. Briefly, aliquots of neutrophils ( $1 \cdot 10^6 \text{ cells mL}^{-1}$ ) were incubated with the respective flavonol ( $50 \mu\text{M}$ ), DMSO [ $0.1\%$  (v/v); vehicle], HBSS-gel (negative control), or Triton X-100 [ $0.2\%$  (v/v); positive control] for 20 min at  $37^\circ\text{C}$ . The cell pellets were suspended in HBSS-gel after centrifugation ( $755 \times g$ , 10 min,  $4^\circ\text{C}$ ), and the cellular viability was determined by the Trypan Blue exclusion test, by counting a total of 200 cells for each sample. The activity of cytosolic lactate dehydrogenase (LDH) released into the supernatant was measured on the basis of absorbance changes at  $340 \text{ nm}$  for 2 min, at  $37^\circ\text{C}$ , using a DU-70 spectrophotometer (Beckman, Fullerton, CA, USA). The LDH Liquiform™ test kit was used in this assay.

#### Statistical analysis

Experimental data were processed and analysed with the aid of the GraphPad Prism Software (version 3.00 for Windows; GraphPad Software Inc., San

Diego, CA, USA). Statistical analysis was performed by analysis of variance (ANOVA) followed by the Tukey's or Dunnett's test, as indicated in the legends.  $p < 0.05$  was considered significant.

## Results and Discussion

It is possible to assess the neutrophil respiratory burst by measuring the  $\text{O}_2$  consumption or superoxide anion production after stimulating these cells with soluble or particulate agents (Tauber *et al.*, 1984; Sarna *et al.*, 2010). We measured the NOC by polarimetry using a Clark-type oxygen electrode, and investigated how the number of cells and the concentration of i-IC and OZ, representing different kinds of particulate stimuli, affected the  $\text{O}_2$  consumption during the respiratory burst of human and rabbit neutrophils.

In general, the NOC of human neutrophils stimulated with OZ or i-IC was significantly higher as compared to that of rabbit neutrophils. Only after stimulation with OZ was the increase in  $\text{O}_2$  uptake proportional to the number of human or rabbit neutrophils ( $p < 0.05$ ). We selected  $4 \cdot 10^6 \text{ neutrophils mL}^{-1}$  as the optimal cell density for the further study of the two stimuli (Fig. 2) – not the cell concentration that furnished maximum NOC – because it allowed us to obtain reliable results without using an excessive number of cells; thus, it was not necessary to collect large blood volumes from the volunteers.

In both types of neutrophils, the NOC augmented significantly with increasing concentration of the two stimuli, compared to the non-stimulated con-

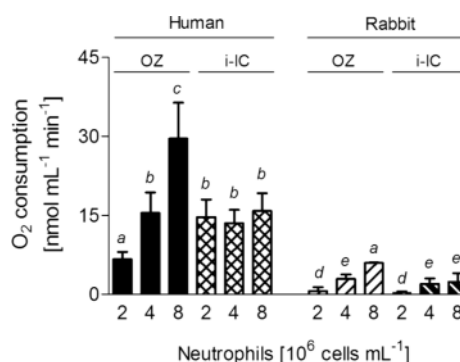


Fig. 2. Net  $\text{O}_2$  consumption by a different number of human and rabbit neutrophils stimulated by opsonized zymosan (OZ) ( $2 \text{ mg mL}^{-1}$ ) or insoluble immune complexes (i-IC) ( $120 \mu\text{g mL}^{-1}$ ). Data are expressed as the mean  $\pm$  SD of three experiments. Values not sharing the same letter (a–e) are significantly different from each other (ANOVA and Tukey's *post-hoc* test;  $p < 0.05$ ).

trols ( $p < 0.05$ ). Maximal responses were obtained with  $2 \text{ mg mL}^{-1}$  OZ (Fig. 3A) and  $240 \text{ } \mu\text{g mL}^{-1}$  i-IC (Fig. 3B), respectively. Increasing concentrations of the two stimuli did not enhance the NOC. Hence, we selected  $2 \text{ mg mL}^{-1}$  OZ and  $240 \text{ } \mu\text{g mL}^{-1}$  i-IC for further experiments.

Under the chosen conditions, the  $\text{O}_2$  uptake did not exceed the detection limits of the equipment employed (Fig. 4). Furthermore, the magnitude of the difference between  $\text{O}_2$  consumption by stimulated and non-stimulated neutrophils was large enough to allow us to test positive and negative modulators of the NOC related to the respiratory burst (Figs. 4 and 5). DPI completely inhibited the NOC, while DMSO was not inhibitory at all (Fig. 5).

The main advantage of this procedure is that polarimetry is a low-cost technique that directly analyses

the  $\text{O}_2$  concentration in the reaction medium; therefore, it is not necessary to use detection reagents that could interfere in the assay (Pouvreau *et al.*, 2008). Polarography has been used to determine the mitochondrial  $\text{O}_2$  consumption for over 50 years and still is the most reliable technique for this purpose (Chance and Williams, 1955; Li and Graham, 2012; Silva and Oliveira, 2012). Novel multi-well plate-based respirometry assays simultaneously measure the  $\text{O}_2$  consumption and extracellular flux of cellular metabolites. This technology increases the throughput and reduces the amount of biological material required for each assay (Ferrick *et al.*, 2008). However, it is an open or “semi-closed” system that allows the entry of  $\text{O}_2$  from the atmosphere and requires complex calculations to obtain the actual  $\text{O}_2$  consumption (Gerencser *et al.*, 2009). On the other hand, a Clark-type oxygen

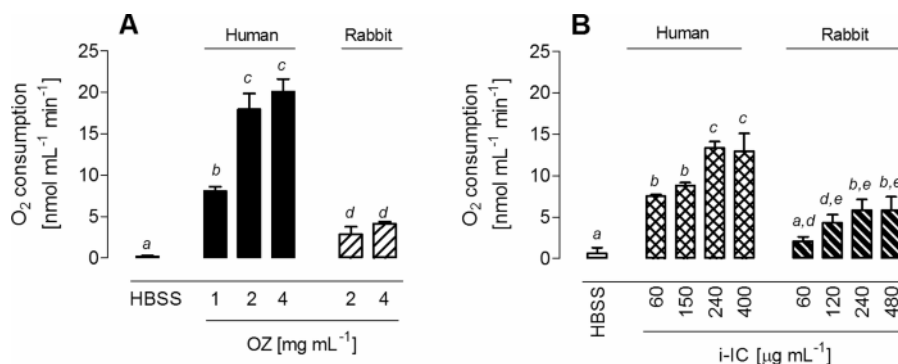


Fig. 3. Net  $\text{O}_2$  consumption by neutrophils ( $4 \cdot 10^6$  cells  $\text{mL}^{-1}$ ) stimulated with different concentrations of (A) opsonized zymosan (OZ) or (B) insoluble immune complexes (i-IC). HBSS: Hank's balanced saline solution (control representing the non-stimulated cells). Data are expressed as the mean  $\pm$  SD of three experiments. Values not sharing the same letter (a–e) are significantly different from each other (ANOVA and Tukey's *post-hoc* test;  $p < 0.01$ ).

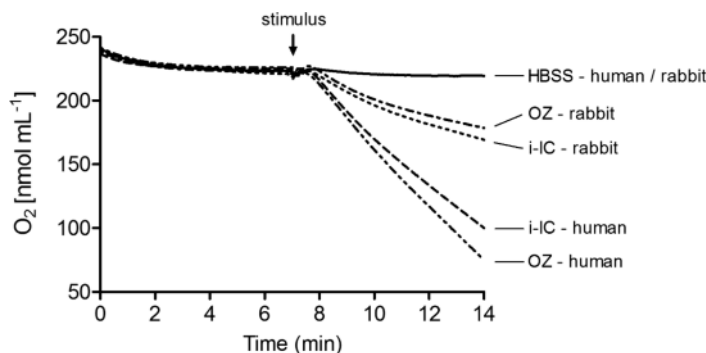


Fig. 4. Kinetics of the  $\text{O}_2$  consumption by human and rabbit neutrophils ( $4 \cdot 10^6$  cells  $\text{mL}^{-1}$ ) stimulated with opsonized zymosan (OZ) ( $2 \text{ mg mL}^{-1}$ ) or insoluble immune complexes (i-IC) ( $240 \text{ } \mu\text{g mL}^{-1}$ ). The  $\text{O}_2$  uptake was measured before and after addition of each stimulus to the reaction mixture. The traces representing  $\text{O}_2$  consumption by non-stimulated rabbit and human neutrophils (HBSS) are superimposed and appear as a single line. HBSS: Hank's balanced saline solution. Data are representative of three independent experiments with similar patterns.

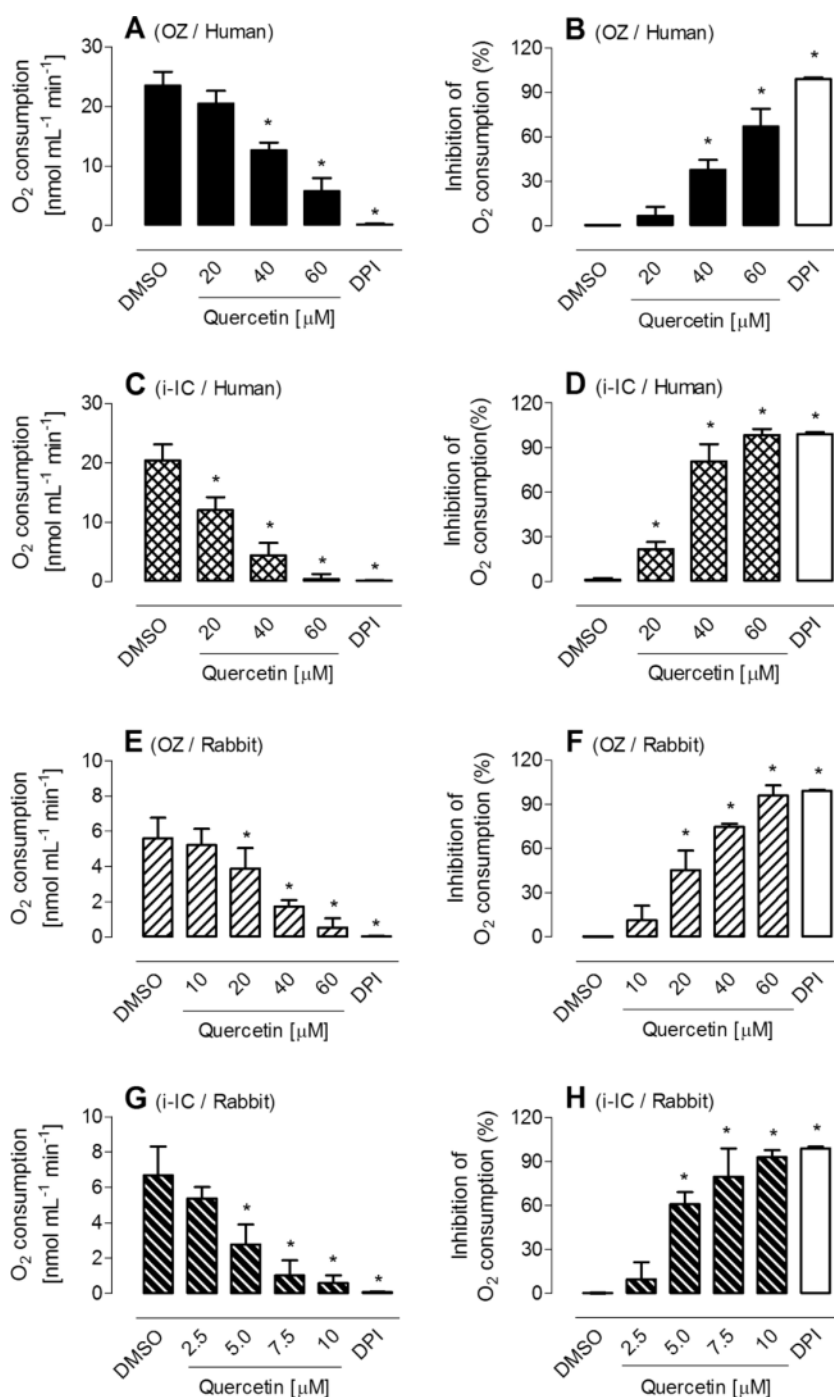


Fig. 5. Concentration-dependent inhibitory effect of quercetin on the net O<sub>2</sub> consumption of (A–D) human and (E–H) rabbit neutrophils ( $4 \cdot 10^6$  cells  $\text{mL}^{-1}$ ) stimulated with opsonized zymosan (OZ) ( $2 \text{ mg mL}^{-1}$ ; A, B, E, F) or insoluble immune complexes (i-IC) ( $240 \mu\text{g mL}^{-1}$ ; C, D, G, H). Diphenyleneiodonium chloride (DPI) ( $20 \mu\text{M}$ ) and dimethyl sulfoxide (DMSO) [ $0.1\%$  (v/v)] were used as positive and negative controls, respectively. Data are expressed as the mean  $\pm$  SD of four experiments. \* $p < 0.001$  vs. DMSO (ANOVA and Dunnett's *post-hoc* test).

electrode measures the concentration of dissolved O<sub>2</sub> in a closed system; thus, the decline of the O<sub>2</sub> concentration in the chamber directly relates to the actual O<sub>2</sub> uptake by the cells (Gerencser *et al.*, 2009; Marchandeu and Labbe, 2011). Measuring the O<sub>2</sub> uptake by lymphocytes, hepatocytes, and tissue extracts has helped to diagnose oxidative phosphorylation diseases, mitochondrial dysfunction, and liver diseases induced by xenobiotics (Artuch *et al.*, 2000; Marchandeu and Labbe, 2011).

OZ and i-IC comparably increased the NOC in both rabbit and human neutrophils, even though these stimuli trigger different classes of receptors. OZ consists of polysaccharide particles coated with C3b/C3bi proteins of the complement system, as well as naturally occurring IgG antibodies (Kemp and Turner, 1986). OZ can interact with and activate the complement receptors CR1 and CR3, FcγR, and lectin-like receptors, even though some studies have indicated that C3 fragments are the principal opsonins for zymosan in normal human serum (Ezekowitz *et al.*, 1985; Kemp and Turner, 1986). On the other hand, the structure of IC is that of an antibody bound to an antigen, and this complex activates Fc receptors (Daëron, 1997). In this work, we employed precipitated IC consisting of rabbit IgG antibodies and OVA as antigen. Such IC represent particulate stimuli that specifically activate Fcγ receptors.

Under identical experimental conditions, the human neutrophil NOC is almost four times greater than that of the rabbit neutrophils (Figs. 4 and 5). Therefore, the O<sub>2</sub> consumption by neutrophil NADPHox depends on the cell source. The greater range of absolute values of the O<sub>2</sub> uptake by the stimulated human neutrophils, as compared with rabbit neutrophils, is mathematically advantageous to distinguish the effect of samples with close inhibitory or stimulatory effects on the NOC in these cells. Because NADPHox and its component proteins are highly conserved in humans and rabbits (Hitt and Kleinberg, 1996), this fact might result from possible differences between rabbit and human neutrophils in terms of (i) the recognition pattern of stimuli by cellular receptors, (ii) the intracellular signaling pathways, (iii) the neutrophil activation efficiency (Löffler *et al.*, 2010), and (iv) the number of NADPHox complexes per neutrophil.

In the second part of this study, we investigated the inhibitory effect of the flavonols quercetin, myricetin, kaempferol, and galangin on the human and rabbit neutrophil NOC under the established experimental conditions. Quercetin had a concentration-dependent in-

hibitory effect on the NOC elicited by OZ and i-IC in both systems, the inhibition being more effective at all concentrations with the i-IC-stimulated NOC ( $p < 0.05$ ) (Fig. 5). We chose the quercetin concentrations that inhibited about 50% of the NOC to assess the modulatory effect of the flavonols myricetin, galangin, and kaempferol; these concentrations were 60 μM and 40 μM for human cells, and 20 μM and 5 μM for rabbit cells, stimulated with OZ and i-IC, respectively. At these concentrations, myricetin, kaempferol, and galangin inhibited the human (Figs. 6A–D) and rabbit (Figs. 6E–H) neutrophil NOC to a less extent compared to quercetin; galangin was the least active flavonol.

Inhibition of the neutrophil NOC can be mediated by a combination of different mechanisms, including cell death, inhibition of the NADPHox activity, and inhibition of the NADPHox activation. These possibilities will be discussed below. Compared with the control, the four flavonols did not induce significant LDH release or decrease the neutrophil viability (Table I). Thus, the reduction of the NOC was not the result of a general toxicity of the four flavonols to the neutrophils.

Interestingly, O<sub>2</sub> uptake by rabbit neutrophils was almost four times lower than that by human neutrophils after stimulation. Also, rabbit neutrophils were around four times more sensitive to the inhibitory effect of the tested flavonols than human neutrophils. Thus, the inhibitory potency of these compounds seems to correlate negatively with the intrinsic ability of OZ- or i-IC-stimulated neutrophils of a given species to consume O<sub>2</sub>. It reinforces that rabbit neutrophils are useful experimental models for a preliminary study of the modulatory effect of natural compounds on neutrophils in some human inflammatory and autoimmune diseases.

Considering structure-activity relationships, we found that quercetin, which bears the 3',4'-*ortho*-dihydroxy substitution in ring B, displayed the highest inhibitory activities on the human and rabbit neutrophil NOC. Quercetin suppresses the NOC by OZ-stimulated human neutrophils, as well as the NADPHox activity in a cell-free system, more strongly than kaempferol (Tauber *et al.*, 1984). Quercetin was more effective than myricetin to inhibit the NADPHox p47<sup>phox</sup> subunit gene expression in HL-60-derived neutrophils and the NADPHox activity of phorbol-12-myristate-13-acetate-stimulated human neutrophils (Dávalos *et al.*, 2009).

Flavonols decrease the superoxide anion levels of activated neutrophils by scavenging this free radical

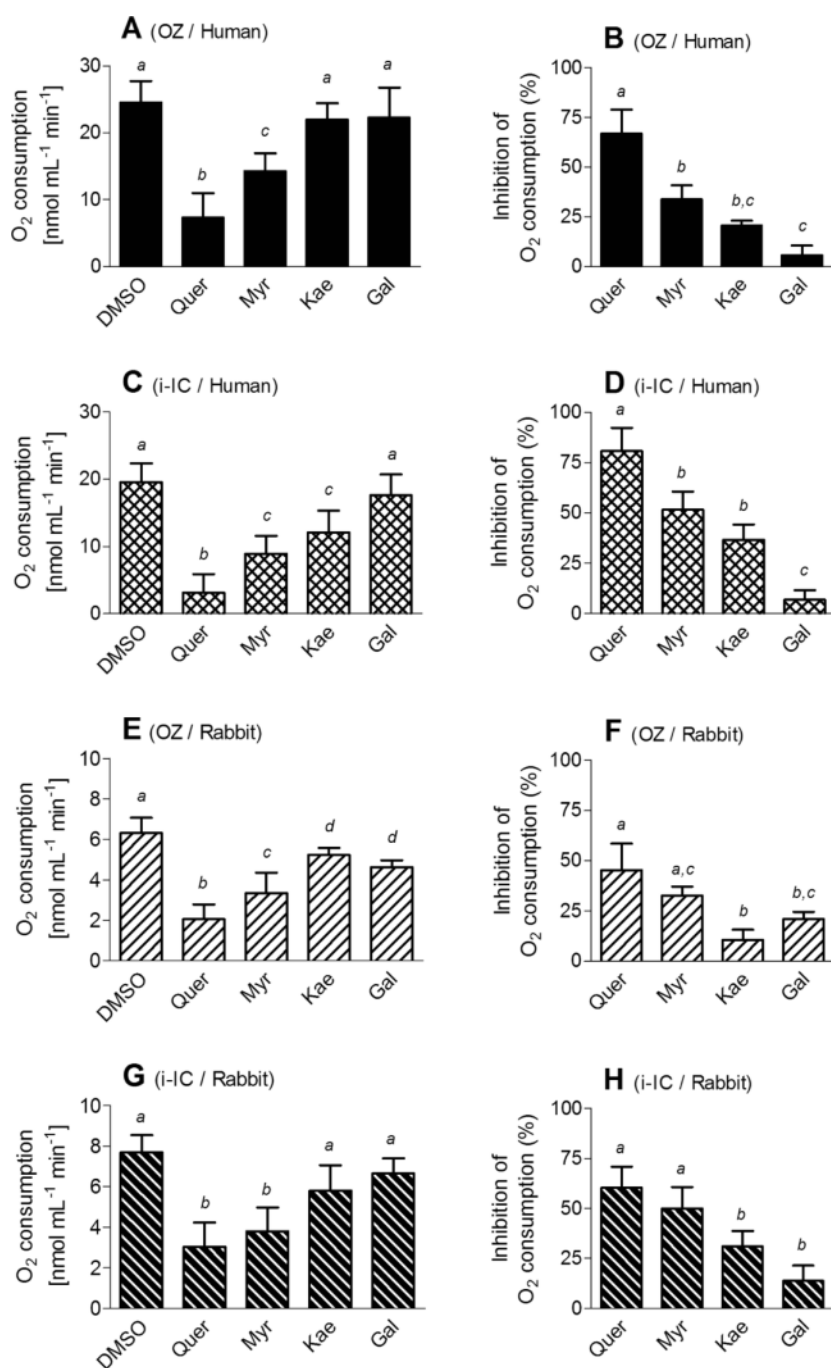


Fig. 6. Inhibitory effect of flavonols on the net O<sub>2</sub> consumption of (A–D) human and (E–H) rabbit neutrophils ( $4 \cdot 10^6$  cells mL<sup>-1</sup>) stimulated with opsonized zymosan (OZ) ( $2 \text{ mg mL}^{-1}$ ; A, B, E, F) or insoluble immune complexes (i-IC) ( $240 \text{ } \mu\text{g mL}^{-1}$ ; C, D, G, H). The flavonols quercetin (Quer), myricetin (Myr), kaempferol (Kae), and galangin (Gal) were added to the medium at the following concentrations: (A, B)  $60 \text{ } \mu\text{M}$ ; (C, D)  $40 \text{ } \mu\text{M}$ ; (E, F)  $20 \text{ } \mu\text{M}$ ; (G, H)  $5 \text{ } \mu\text{M}$ . Data are expressed as the mean  $\pm$  SD of three to four experiments. Values not sharing the same letter (*a–d*) are significantly different from each other (ANOVA and Tukey's *post-hoc* test; *p* < 0.05).

Table I. Viability of neutrophils after treatment with flavonols<sup>a</sup>.

Compound <sup>b</sup>	Rabbit neutrophils <sup>c</sup>		Human neutrophils	
	Viable cells (%) <sup>d</sup>	Released LDH (%) <sup>e</sup>	Viable cells (%) <sup>d</sup>	Released LDH (%) <sup>e</sup>
HBSS	91.1 ± 2.7	6.9 ± 0.8	98.3 ± 2.1	4.0 ± 5.5
DMSO	92.7 ± 2.1	8.7 ± 1.2	96.3 ± 3.4	4.8 ± 2.0
Myricetin	92.7 ± 2.3	8.9 ± 1.3	94.5 ± 6.8	5.7 ± 2.5
Quercetin	91.2 ± 1.2	10.5 ± 1.1	96.0 ± 3.2	3.5 ± 1.4
Kaempferol	91.3 ± 2.7	9.9 ± 1.3	99.0 ± 0.8	4.0 ± 2.2
Galangin	91.1 ± 1.7	10.5 ± 1.2	94.3 ± 4.3	7.3 ± 2.3

<sup>a</sup> Data represent the mean ± SD of three independent experiments performed in duplicate.

<sup>b</sup> HBSS, Hank's balanced saline solution (untreated cells); DMSO, dimethyl sulfoxide [0.1% (v/v); vehicle control]; flavonols tested at 50 μM.

<sup>c</sup> Data reproduced from Moreira *et al.* (2007) with permission.

<sup>d</sup> Cell viability determined by the Trypan Blue exclusion test with a total of 200 cells counted for each sample.

<sup>e</sup> Values represent relative amounts of lactate dehydrogenase (LDH) released into the supernatant compared with neutrophils completely lysed by Triton X-100.

and inhibiting NADPHox assembly and activity (Ciz *et al.*, 2012). These polyphenolic compounds can inhibit the protein kinase C and phospholipase D signaling pathways, which mediate the NADPHox assembly and degranulation process (Ciz *et al.*, 2012). The *ortho*-dihydroxy group is the structural requirement to significantly suppress neutrophil degranulation (Kanashiro *et al.*, 2007) and NADPHox activity, thus suggesting that quercetin and myricetin interfere in the aforementioned intracellular signaling pathways.

Measuring the neutrophil O<sub>2</sub> consumption in the presence of flavonols sometimes does not give their true effect on the NADPHox activity and/or activation, and also does not exclude their free radical scavenging properties. These compounds can participate in downstream O<sub>2</sub>-generating reactions involving the ROS produced during the respiratory burst, in particular the superoxide anion and H<sub>2</sub>O<sub>2</sub>, as well as the enzymes catalase and myeloperoxidase (Meotti *et al.*, 2008; Ciz *et al.*, 2012).

## Conclusion

The experimental conditions established in this work were applicable to the assay of human and rabbit neutrophil NOC and to evaluate the modulatory ef-

fect of natural compounds. When comparing the results obtained with rabbit and human neutrophils, it is vital to consider the interspecific differences in the O<sub>2</sub> uptake and how much they affect the inhibitory potency of a given compound – in the present study, OZ- and i-IC-stimulated rabbit neutrophils consumed less O<sub>2</sub> but were more easily inhibited by the flavonols, as compared to the human neutrophils. Furthermore, the structure-activity relationships of the flavonols screened in this study provide preliminary information that can contribute to the development of new modulators of neutrophil NADPHox activity and/or activation implicated in inflammatory and autoimmune diseases.

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Artuch R., Colomé C., Playán A., Alcaine M. J., Briones P., Montoya J., Vilaseca M. A., and Pineda M. (2000), Oxygen consumption measurement in lymphocytes for the diagnosis of pediatric patients with oxidative phosphorylation diseases. *Clin. Biochem.* **33**, 481–485.

Chance B. and Williams G. R. (1955), A simple and rapid assay of oxidative phosphorylation. *Nature* **175**, 1120–1121.

Chance B. and Williams G. R. (1956), The respiratory chain and oxidative phosphorylation. *Adv. Enzymol. Relat. Subj. Biochem.* **17**, 65–134.

- Cheung K., Archibald A. C., and Robinson M. F. (1983), The origin of chemiluminescence produced by neutrophils stimulated by opsonized zymosan. *J. Immunol.* **130**, 2324–2329.
- Ciz M., Denev P., Kratchanova M., Vasicek O., Ambrozova G., and Lojek A. (2012), Flavonoids inhibit the respiratory burst of neutrophils in mammals. *Oxid. Med. Cell. Longev.* **2012**, article ID 181295.
- Cochrane C. G. and Koffler D. (1973), Immune complex disease in experimental animals and man. *Adv. Immunol.* **16**, 185–264.
- Daéron M. (1997), Fc receptor biology. *Annu. Rev. Immunol.* **15**, 203–234.
- Dávalos A., de la Pena G., Sanchez-Martin C. C., Guerra M. T., Bartolome B., and Lasuncion M. A. (2009), Effects of red grape juice polyphenols in NADPH oxidase subunit expression in human neutrophils and mononuclear blood cells. *Br. J. Nutr.* **102**, 1125–1135.
- Ezekowitz R. A., Sim R. B., MacPherson G. G., and Gordon S. (1985), Interaction of human monocytes, macrophages, and polymorphonuclear leukocytes with zymosan *in vitro*. Role of type 3 complement receptors and macrophage-derived complement. *J. Clin. Invest.* **76**, 2368–2376.
- Fahey J. L., Wunderlich J., and Mishell R. (1964), The immunoglobulins of mice. I. Four major classes of immunoglobulins: 7S gamma-2-, 7S gamma-1-, gamma-1A (beta-2A)-, and 18S gamma-1M-globulins. *J. Exp. Med.* **120**, 223–242.
- Fairhurst A. M., Wallace P. K., Jawad A. S., and Goulding N. J. (2007), Rheumatoid peripheral blood phagocytes are primed for activation but have impaired Fc-mediated generation of reactive oxygen species. *Arthritis Res. Ther.* **9**, R-29.
- Ferrick D. A., Neilson A., and Beeson C. (2008), Advances in measuring cellular bioenergetics using extracellular flux. *Drug Discov. Today* **13**, 268–274.
- Gallin J. I. and Zarembek K. (2007), Lessons about the pathogenesis and management of aspergillosis from studies in chronic granulomatous disease. *Trans. Am. Clin. Climatol. Assoc.* **118**, 175–185.
- Gerencser A. A., Neilson A., Choi S. W., Edman U., Yadava N., Oh R. J., Ferrick D. A., Nicholls D. G., and Brand M. D. (2009), Quantitative microplate-based respirometry with correction for oxygen diffusion. *Anal. Chem.* **81**, 6868–6878.
- Hitt N. D. and Kleinberg M. E. (1996), Identification of neutrophil NADPH oxidase proteins gp91-phox, p22-phox, p67-phox, and p47-phox in mammalian species. *Am. J. Vet. Res.* **57**, 672–676.
- Kabat E. (1961), *Experimental Immunochemistry*, 2nd ed. Blackwell Scientific Publications, Oxford, UK.
- Kabeya L. M., da Silva C. H., Kanashiro A., Campos J. M., Azzolini A. E. C. S., Polizello A. C., Pupo M. T., and Lucisano-Valim Y. M. (2008), Inhibition of immune complex-mediated neutrophil oxidative metabolism: a pharmacophore model for 3-phenylcoumarin derivatives using GRIND-based 3D-QSAR and 2D-QSAR procedures. *Eur. J. Med. Chem.* **43**, 996–1007.
- Kanashiro A., Kabeya L. M., Polizello A. C., Lopes N. P., Lopes J. L., and Lucisano-Valim Y. M. (2004), Inhibitory activity of flavonoids from *Lychnophora* sp. on generation of reactive oxygen species by neutrophils upon stimulation by immune complexes. *Phytother. Res.* **18**, 61–65.
- Kanashiro A., Souza J. G., Kabeya L. M., Azzolini A. E. C. S., and Lucisano-Valim Y. M. (2007), Elastase release by stimulated neutrophils inhibited by flavonoids: importance of the catechol group. *Z. Naturforsch.* **62c**, 357–361.
- Kemp A. S. and Turner M. W. (1986), The role of opsonins in vacuolar sealing and the ingestion of zymosan by human neutrophils. *Immunology* **59**, 69–74.
- Li Z. and Graham B. H. (2012), Measurement of mitochondrial oxygen consumption using a Clark electrode. *Methods Mol. Biol.* **837**, 63–72.
- Löffler B., Hussain M., Grundmeier M., Bruck M., Holzinger D., Varga G., Roth J., Kahl B. C., Proctor R. A., and Peters G. (2010), *Staphylococcus aureus* panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathog.* **6**, 1–12.
- Lucisano Y. M. and Mantovani B. (1984), Lysosomal enzyme release from polymorphonuclear leukocytes induced by immune complexes of IgM and of IgG. *J. Immunol.* **132**, 2015–2020.
- Lucisano Y. M. and Mantovani B. (1988), The role of complement in the stimulation of lysosomal enzyme release by polymorphonuclear leukocytes induced by immune complexes of IgG and of IgM. *Immunology* **65**, 171–175.
- Lucisano-Valim Y. M., Kabeya L. M., Kanashiro A., Russo-Carbolante E. M. S., Polizello A. C. M., Azzolini A. E. C. S., Silva S. C., Lopes J. L. C., Oliveira C. A., and Mantovani B. (2002), A simple method to study the activity of natural compounds on the chemiluminescence of neutrophils upon stimulation by immune complex. *J. Pharmacol. Toxicol. Methods* **47**, 53–58.
- Marchandeu J.-P. and Labbe G. (2011), Evaluation of mitochondrial respiration in cultured rat hepatocytes. *Methods Mol. Biol.* **691**, 243–253.
- Marzocchi-Machado C. M., Alves C. M., Azzolini A. E. C. S., Polizello A. C., Carvalho I. F., and Lucisano-Valim Y. M. (2002), Fcγ and complement receptors: expression, role and co-operation in mediating the oxidative burst and degranulation of neutrophils of Brazilian systemic lupus erythematosus patients. *Lupus* **11**, 240–248.
- Meotti F. C., Senthilmohan R., Harwood D. T., Missau F. C., Pizzolatti M. G., and Kettle A. J. (2008), Myricitrin as

- a substrate and inhibitor of myeloperoxidase: implications for the pharmacological effects of flavonoids. *Free Radical Biol. Med.* **44**, 109–120.
- Moreira M. R., Kanashiro A., Kabeya L. M., Polizello A. C., Azzolini A. E. C. S., Curti C., Oliveira C. A., and Lucisano-Valim Y. M. (2007), Neutrophil effector functions triggered by Fc-gamma and/or complement receptors are dependent on B-ring hydroxylation pattern and physico-chemical properties of flavonols. *Life Sci.* **81**, 317–326.
- Németh T. and Mócsai A. (2012), The role of neutrophils in autoimmune diseases. *Immunol. Lett.* **143**, 9–19.
- Paula F. S., Kabeya L. M., Kanashiro A., de Figueiredo A. S., Azzolini A. E. C. S., Uyemura S. A., and Lucisano-Valim Y. M. (2009), Modulation of human neutrophil oxidative metabolism and degranulation by extract of *Tamarindus indica* L. fruit pulp. *Food Chem. Toxicol.* **47**, 163–170.
- Podolin P. L., Bolognese B. J., Foley J. J., Schmidt D. B., Buckley P. T., Widdowson K. L., Jin Q., White J. R., Lee J. M., Goodman R. B., Hagen T. R., Kajikawa O., Marshall L. A., Hay D. W., and Sarau H. M. (2002), A potent and selective nonpeptide antagonist of CXCR2 inhibits acute and chronic models of arthritis in the rabbit. *J. Immunol.* **169**, 6435–6444.
- Pouvreau L. A., Strampraad M. J., Van Berloo S., Kattenberg J. H., and de Vries S. (2008), NO, N<sub>2</sub>O, and O<sub>2</sub> reaction kinetics: scope and limitations of the Clark electrode. *Methods Enzymol.* **436**, 97–112.
- Sarna L. K., Wu N., Hwang S.-Y., Siow Y. L., and O. K. (2010), Berberine inhibits NADPH oxidase mediated superoxide anion production in macrophages. *Can. J. Physiol. Pharmacol.* **88**, 369–378.
- Segal A. W. (2008), The function of the NADPH oxidase of phagocytes and its relationship to other NOXs in plants, invertebrates, and mammals. *Int. J. Biochem. Cell Biol.* **40**, 604–618.
- Silva A. M. and Oliveira P. J. (2012), Evaluation of respiration with Clark type electrode in isolated mitochondria and permeabilized animal cells. *Methods Mol. Biol.* **810**, 7–24.
- Spilberg I., Mandell B., Mehta J., Simchowit L., and Rosenberg D. (1979), Mechanism of action of colchicine in acute urate crystal-induced arthritis. *J. Clin. Invest.* **64**, 775–780.
- Tauber A. L., Fay J. R., and Marletta M. A. (1984), Flavonoid inhibition of the human neutrophil NADPH-oxidase. *Biochem. Pharmacol.* **33**, 1367–1369.
- Weir D. M. (1986), *Immunochemistry and Molecular Immunology*, 5th ed. Blackwell Scientific Publications, Oxford, UK.
- Wright H. L., Moots R. J., Bucknall R. C., and Edwards S. W. (2010), Neutrophil function in inflammation and inflammatory diseases. *Rheumatology* **49**, 1618–1631.