

## On the Pharmacology of Oxidative Burst of Human Neutrophils

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### Summary

The effect of three therapeutically used drugs and five polyphenolic compounds on the mechanism of oxidative burst was compared in whole blood and isolated neutrophils at cellular and molecular level. In 10 µM concentration, the compounds investigated decreased the oxidative burst of whole blood in the rank order of potency: N-feruloylserotonin (N-f-5HT) > curcumin (CUR) > quercetin (QUER) > arbutin (ARB) > resveratrol (RES) > dithiaden (DIT) > carvedilol (CARV) > brompheniramine (BPA). The ratio between the percentage inhibition of extracellular versus intracellular chemiluminescence (CL) followed the rank order QUER > N-f-5HT > RES > CUR > DIT and is indicative of the positive effect of the compounds tested against oxidative burst of neutrophils, demonstrating suppression of reactive oxygen species extracellularly with minimal alteration of intracellular reactive oxygen species (ROS). Activation of protein kinase C was significantly decreased by DIT, CUR, QUER and N-f-5HT. CARV, DIT, QUER and ARB reduced activated neutrophil myeloperoxidase release more significantly compared with the effect on superoxide anion generation. All compounds tested increased the activity of caspase-3 in cell-free system. It is suggested that other regulatory mechanisms than protein kinase C might participate in the inhibition of neutrophil activation with the compounds tested. Different mechanisms are concerned in controlling the assembly of NADPH oxidase and the regulatory role of calcium ions is suggested. Compounds decreasing the amount of extracellular ROS generation, yet affecting but minimally intracellular ROS generation, are promising for further investigation *in vivo*.

### Key words

Human neutrophils • Oxidative burst • Chemiluminescence • Protein kinase C • Apoptosis

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### Introduction

Professional phagocytic cells play a central role in defending the host against microorganisms by producing reactive oxygen species (ROS) *via* the NADPH oxidase enzyme complex (Raad *et al.* 2009). When appropriately stimulated by a variety of agents, they undergo dramatic physiological and biochemical changes. These are resulting in phagocytosis, chemotaxis and degranulation with the activation of ROS production in a metabolic pathway known as the respiratory burst (O'Dowd *et al.* 2004). These radicals are involved in the elimination of pathogens, however an excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of cancer, diabetes mellitus, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, ischemia/reperfusion injury, obstructive sleep apnea, and other diseases (Droge 2002) and result in damage of surrounding tissues (Halliwell and Whiteman 2004, Lonkar and Dedon 2011). The intracellular oxidants fulfill a regulatory role and participate in the initiation

of neutrophil apoptosis. Protein kinase C (PKC) is required for full assembly of NADPH oxidase and activation of the respiratory burst in neutrophils (Bertram and Ley 2011). Phorbol esters stimulate the neutrophil respiratory burst through activation of cytosolic protein kinase C, which in turn activates either a regulatory constituent or the NADPH-oxidase directly in the plasma membrane creating an active  $O_2^-$  generating system (Cox *et al.* 1985). Under physiologic conditions, the balance between generation and elimination of ROS maintains the proper function of redox-sensitive signaling proteins. Normally, the redox homeostasis ensures that the cells respond properly to endogenous and exogenous stimuli. However, when the redox homeostasis is disturbed, oxidative stress may lead to aberrant cell death and contribute to disease development (Trachootham *et al.* 2008). Development and pharmacological use of polymorphonuclear-targeting agents may be a good therapeutic strategy for regulation of polymorphonuclear functions in control of inflammation and tissue injury (Kori *et al.* 2009). The development of pharmacological procedures to ameliorate undesirable ROS production may become one of the central issues in research on aging and oxidative stress-related diseases in the near future. A number of therapeutically used medicaments and natural products acted not only as extracellular scavengers but they also suppressed intracellular formation of ROS in human neutrophils (Drábiková *et al.* 2006, 2013, Jančinová *et al.* 2012a,b, 2015, Nosál *et al.* 2006, 2014, 2015, Pečivová *et al.* 2007, Perečko *et al.* 2012). Since the optimum therapy is expected to minimize tissue damage without reduction of neutrophil physiological function, separate analysis of extra- and intracellular effects of antioxidants is of particular importance (El-Benna *et al.* 2010).

The effect of three therapeutically used drugs and five polyphenolic compounds on the mechanism of oxidative burst was compared in whole human blood and isolated neutrophils at cellular and molecular level with respect to the ratio between extra- and intracellular suppression of ROS generation.

## Material and Methods

### Chemicals

Arbutin (hydroquinone  $\beta$ -D-glucopyranoside), quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one,3,3',4',5,6-pentahydroxyflavone,

PMA (phorbol-4 $\beta$ -12 $\beta$ -myristate-13 $\alpha$  acetate), luminol, isoluminol, superoxide dismutase, dextran (average MW 464,000), luciferase (from firefly *Photinus pyralis*), gallic acid, cytochalasin B, o-dianosidine and D-luciferin sodium salt were from Sigma Aldrich Chemie GmbH (Steinheim, Germany), horseradish peroxidase (HRP), catalase, cytochrome C and Folin Ciocalteu's phenol reagent were purchased from Merck (Darmstadt, German), Lymphoprep (density 1.077 g/ml) from NycomedPharma AS (Oslo, Norway), brompheniramine (BPA): 3-(4-bromophenyl)-N,N-dimethyl-3-pyridin-2-ylpropan-1-amine from Weyth (Austria), carvedilol ( $\pm$ )-[3-(9H-carbazol-4-yloxy)-2-hydroxypropyl][2-(2-methoxyphenoxy)ethyl]am from La Roche (Mannheim, Germany), N-feruloylserotonin (N-f-5HT) was isolated from the seeds of *Leuzea carthamoides* (Wild DC) by solvent extraction (Harmatha *et al.* 2007). Resveratrol (RES) was prepared by targeted regioselective synthesis purely as trans isomer (Šmidrkal *et al.* 2010). Picolinic acid was purchased from Fluka (Deisenhofen, Germany), and human purified caspase-3 was obtained from Enzo Life Sciences (Lausen, Switzerland). All other chemicals used were of analytical grade and obtained from commercial sources.

### Blood collection and neutrophil separation

Fresh human blood was obtained at the blood bank by venepuncture from healthy male volunteers (aged 20-50 years) who had not received any medication for at least 7 days. It was anticoagulated with 3.8 % trisodium citrate (blood:citrate ratio = 9:1). The Ethical Committee license for blood sampling was registered at the National Transfusion Service NTS-KRA/2012/SVI. Human neutrophils were isolated from whole blood, as described previously (Jančinová *et al.* 2009, Nosál *et al.* 2011). The blood was gently mixed and erythrocytes were allowed to sediment in 3 % dextran solution by centrifugation (10 $\times$ g, 25 min, 22 °C). The neutrophil suspension was layered on Lymphoprep (3 ml) and centrifuged (500 $\times$ g, 30 min, 22 °C). After hypotonic lysis and centrifugation (500 $\times$ g, 10 min, 22 °C), the neutrophils were resuspended in  $Ca^{2+}$ - $Mg^{2+}$ -free PBS. After counting, they were adjusted to a final concentration of 10<sup>5</sup> cells/ $\mu$ l (Coulter Counter, Coulter Electronics, England) and kept on ice. The final suspension of neutrophils contained more than 96 % of viable cells, as evaluated by trypan blue exclusion and was used within 2 h, as long as the control chemiluminescence remained constant.

### *Chemiluminescence (CL) assay of whole blood and isolated neutrophils*

The oxidative burst in whole blood was stimulated with phorbolmyristate acetate (PMA 0.05  $\mu$ M). CL was measured in 250  $\mu$ l samples consisting of 50  $\mu$ l aliquots that contained blood (50 $\times$  diluted), luminol (250  $\mu$ M), compounds tested (10  $\mu$ M) and phosphate buffer (Jančinová *et al.* 2009). Horseradish peroxidase (HRP 8 U/ml) was added to the system and maintained a sufficient extracellular peroxidase concentration. The effect of tested compounds on extra- and intracellular reactive oxygen species (ROS) production was measured in unstimulated and PMA (0.05  $\mu$ M)-stimulated neutrophils ( $5 \times 10^5$  per sample) by isoluminol/luminol-enhanced CL. Extracellular CL was determined in the system containing isoluminol (5  $\mu$ M) and HRP (8 U/ml). Intracellular CL was measured with luminol in the presence of extracellular scavengers – superoxide dismutase (100 U/ml) and catalase (2000 U/ml) (Drábíková *et al.* 2009). The CL of both whole blood and isolated neutrophils was evaluated in a microplate luminometer Immunotech LM-01T (Czech Republic) at 37 °C. The data were based on integral values of CL over 3600 s (whole blood) or 1800 s (isolated neutrophils) (RLU  $\times$  s; RLU, relative light units).

### *Protein kinase C activation*

Phosphorylation of protein kinase C (PKC) isoenzymes  $\alpha$  and  $\beta$ II was detected (for details see Jančinová *et al.* 2009). Isolated human neutrophils ( $5 \times 10^6$ ) were incubated at 37 °C with tested compounds for 1 min, stimulated with PMA (0.15  $\mu$ M, 1 min) and lysed by the addition of solubilization buffer. After sonication on ice, the samples were centrifuged to remove unbroken cells, the supernatant was boiled for 5 min with sample buffer and the samples were loaded on 9.8 % SDS polyacrylamide gels. Proteins were separated by electrophoresis, transferred to Immobilon-P Transfer Membrane (Millipore Corp., USA). From the two strips taken, one was detected for PKC and the second for  $\beta$ -actin, which represented the internal control. Membrane strips were blocked for 60 min with 1 % bovine serum albumin in Tris buffered saline. The membranes were subsequently washed six times with TBS and incubated for 60 min with the secondary antibody conjugated to horse-radish peroxidase. The activity of horseradish peroxidase was visualized using Enhanced Chemiluminescence Western Blotting

Detection Reagents (Amersham, UK), followed by autoradiography. The optical density of each PKC band was corrected by the optical density of the corresponding  $\beta$ -actin band.

### *Superoxide determination*

Superoxide formation (Pečivová *et al.* 2007) was measured in isolated human neutrophils as superoxide dismutase inhibitable reduction of cytochrome C. The suspension ( $1 \times 10^6$  neutrophils in PBS with 0.9 mmol/l CaCl<sub>2</sub>, 0.5 mmol/l MgCl<sub>2</sub>) was preincubated for 5 min at 37 °C with tested compounds (10  $\mu$ M) and subsequently stimulated by PMA (1  $\mu$ M) for 15 min at 37 °C. Controls were included for the effect of the stimulus of the drugs tested on cytochrome C reduction. After centrifugation (4200 $\times$ g for 4 min at 4 °C), absorbance was measured at 550 nm in a microplate spectrophotometer (Labsystem Multiscan RC, MTX LabSystems, Inc., Vienna, Wyoming, USA).

### *Myeloperoxidase (MPO) release*

For determination of MPO release (Pečivová *et al.* 2007), neutrophils were preincubated with cytochalasin B (5  $\mu$ g/ml) for 5 min at room temperature. Then the neutrophils ( $2 \times 10^6$ /sample) were preincubated with tested compounds (10  $\mu$ M) in a shaker bath at 37 °C for 5 min, followed by 15 min exposure to PMA (1  $\mu$ M). The activity of MPO was assayed in the supernatant after centrifugation (983 $\times$ g for 10 min at 4 °C) by determining the oxidation of o-dianisidine in the presence of hydrogen peroxide in a microplate spectrophotometer (Labsystem Multiscan RC, MTX LabSystems, Inc., Vienna, Wyoming, USA) at 450 nm.

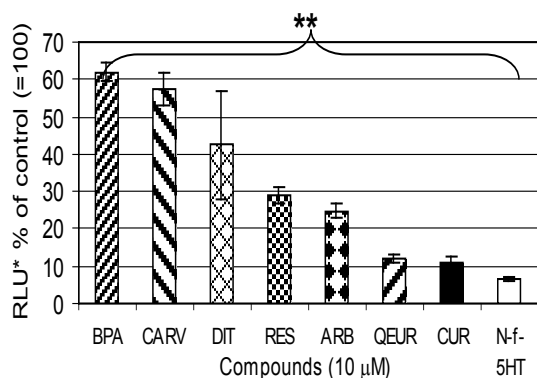
### *Recombinant caspase-3 activity*

To determine the caspase-3 activity, a modified method was applied (Perečko *et al.* 2010). Briefly, the cleavage of the Z-DEVD-amino-luciferin substrate by caspase releases amino-luciferin. The subsequent reaction with luciferase was detected by CL. The light production was measured in the Luminometer Immunotech LM-01T. According to the manufacturer's instructions, 10  $\mu$ l of 0.1 IU caspase was added to 20  $\mu$ l aliquots of tested compounds (10  $\mu$ M) and buffered solution. Finally, 50  $\mu$ l of Caspase-Glo 3/7 Reagent was added and the mixture was measured for 60 min to determine caspase-3 activity.

### *Statistical analysis*

Data represent the mean  $\pm$  SEM, unless stated

otherwise. Statistical analysis was performed using the ANOVA paired test to examine differences between the treatments and control. Differences were considered to be statistically significant when  $p \leq 0.05$  (\*) or  $p \leq 0.01$  (\*\*).

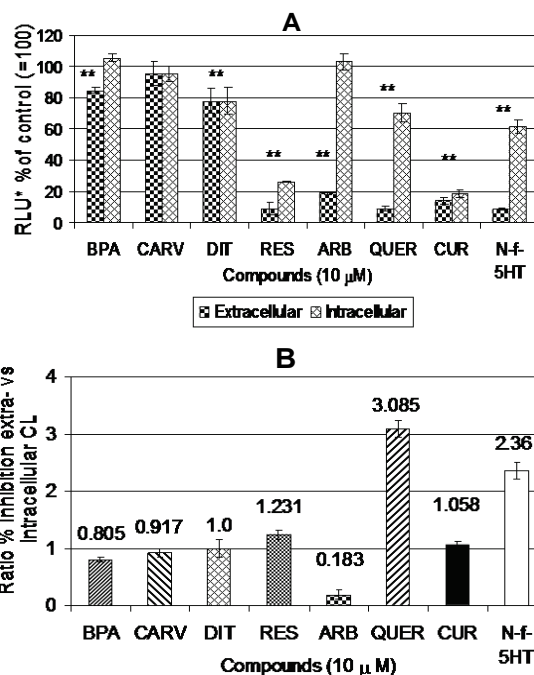


**Fig. 1.** Effect of the compounds tested on luminol-enhanced chemiluminescence expressed as relative luminescence units (RLU) in whole human blood pretreated with brompheniramine (BPA), carvedilol (CARV), dithiaden (DIT), resveratrol (RES), arbutin (ARB), quercetin (QUER), curcumin (CUR), and N-feruloylserotonin (N-F-5HT) in 10  $\mu\text{M}$  concentration and stimulated with phorbolmyristate acetate (0.05  $\mu\text{M}$ ).  $n=6$ ,  $x \pm \text{SEM}$ , \*\*  $p \leq 0.01$ .

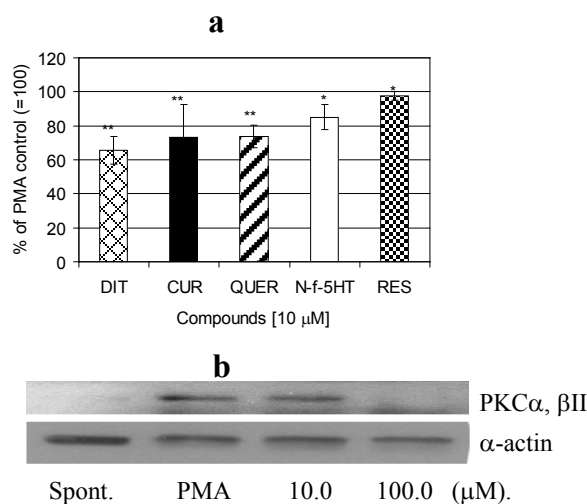
## Results

The effect of the compounds investigated on chemiluminescence (CL) of stimulated whole human blood is shown in Figure 1. In the concentration of 10  $\mu\text{M}$ , the compounds tested inhibited PMA (0.05  $\mu\text{M}$ ) stimulated oxidative burst of whole blood in the rank order of potency: N-feruloylserotonin > curcumin  $\geq$  quercetin > arbutin > resveratrol > dithiaden > carvedilol > brompheniramine by 93.5, 89.9, 88.1, 75.2, 71.0, 57.5, 42.5 and 38 %, respectively. The most effective was N-feruloylserotonin, the less potent was brompheniramine.

Figure 2A presents the effect of the compounds investigated on extra- and intracellular CL of isolated human neutrophils exposed to brompheniramine, carvedilol, dithiaden, resveratrol, arbutin, quercetin, curcumin and N-feruloylserotonin in 10  $\mu\text{M}$  concentration and subsequently stimulated with PMA (0.05  $\mu\text{M}$ ). The ratio percentage inhibition between extra- and intracellular CL is demonstrated in Figure 2B. It is evident that the most effective for inhibition of extracellular CL in comparison with intracellular CL inhibition was quercetin (ratio 3.085) followed by N-feruloylserotonin (ratio 2.36) and resveratrol (ratio 1.231). The lowest ratios were found for arbutin (0.183), brompheniramine (0.805) and carvedilol (0.917).

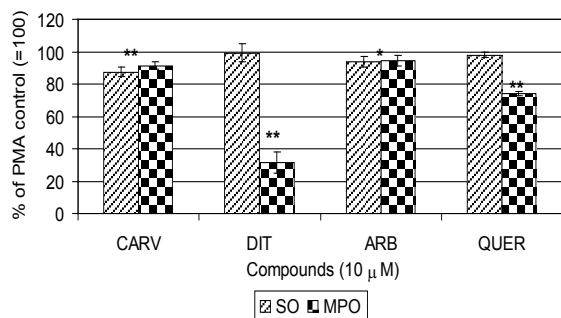


**Fig. 2. A.** The effect of brompheniramine, carvedilol, dithiaden, resveratrol, arbutin, quercetin, curcumin and N-feruloylserotonin in 10  $\mu\text{M}$  concentration on luminol-enhanced extracellular- and isoluminol-enhanced intracellular chemiluminescence (expressed as relative luminescence units – RLU) in isolated human neutrophils stimulated chemiluminescence with phorbolmyristate acetate (PMA 0.05  $\mu\text{M}$ ).  $n=6$ ,  $x \pm \text{SEM}$ , \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ . **B.** The ratio between percentage inhibition of extracellular versus intracellular chemiluminescence for brompheniramine (BPA), carvedilol (CARV), dithiaden (DIT), resveratrol (RES), arbutin (ARB), quercetin (QUER), curcumin (CUR) and N-feruloylserotonin (N-f-5HT) in 10  $\mu\text{M}$  concentration. Values are calculated from the data in Figure 2A.



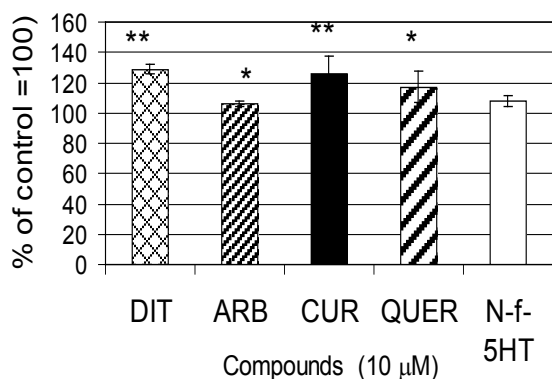
**Fig. 3.** Protein kinase C activation in isolated human neutrophils exposed to dithiaden (DIT), curcumin (CUR), quercetin (QUER), N-feruloylserotonin (N-f-5HT) and resveratrol (RES) in 10  $\mu\text{M}$  concentration and stimulated with PMA (0.15  $\mu\text{M}$ ). **(a)** measurements from four experiments,  $x \pm \text{SEM}$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ ; **(b)** Representative Western blotting analysis of protein kinase C activation in isolated human neutrophils pretreated with resveratrol (10 and 100  $\mu\text{M}$ ) and stimulated with PMA (0.15  $\mu\text{M}$ ) (Nosál *et al.* 2014).

The effect of the selected compounds dithiaden, curcumin, quercetin, N-feruloylserotonin and resveratrol in 10  $\mu$ M concentration on PMA-activated protein kinase C in isolated human neutrophils is presented in Figure 3. For resveratrol, the inhibition of PKC activation was dose-dependent. Of the selected compounds, the most effective was dithiaden, followed by curcumin, quercetin, N-feruloylserotonin and resveratrol.



**Fig. 4.** Generation of superoxide (SO) and liberation of myeloperoxidase (MPO) in isolated human neutrophils treated with carvedilol (CARV), dithiaden (DIT), arbutin (ARB) and quercetin (QUER) in the concentration of 10  $\mu$ M and stimulated with PMA (1  $\mu$ M). n=6,  $\bar{x}$ ±SEM, \* p<0.05, \*\* p<0.01.

Figure 4 demonstrates the effect of carvedilol, dithiaden, arbutin and quercetin in 10  $\mu$ M concentration on superoxide generation and myeloperoxidase liberation from isolated human neutrophils stimulated with PMA. The most effective for SO inhibition was carvedilol followed by arbutin. Dithiaden and quercetin were ineffective. The most effective for MPO release inhibition was dithiaden followed by quercetin, carvedilol and arbutin.



**Fig. 5.** The effect of dithiaden (DIT), arbutin (ARB), curcumin (CUR), quercetin (QUER) and N-feruloylserotonin (N-f-5HT) in 10  $\mu$ M concentration on caspase-3 activity shown as percentage of control sample (100 %). n=4-5,  $\bar{x}$ ±SEM, \* p<0.05, \*\* p<0.01.

The effect of dithiaden, arbutin, curcumin, quercetin and N-feruloylserotonin in 10  $\mu$ M concentration on human recombinant caspase-3 activity in cell free system is demonstrated in Figure 5. DIT, CUR, QUER, N-f-5HT and ARB increased significantly recombinant caspase-3 by 28.7, 25.7, 17.0, 7.6 and 5.8 %, respectively.

## Discussion

The results showed that the compounds tested inhibited oxidative burst in stimulated whole human blood as measured by luminol-enhanced chemiluminescence (CL). The inhibition of stimulated chemiluminescence was found to be dose dependent for H1 antihistamines (Nosál *et al.* 2015), carvedilol (Nosál *et al.* 2005) and many polyphenolic compounds (Jančinová *et al.* 2012a). Since the generation of reactive oxygen species was induced with PMA bypassing membrane receptors and stimulated protein kinase C, the inhibitory effect with three therapeutically used drugs (bromphenyramine, carvedilol and dithiaden) and five polyphenols (arbutin, curcumin, N-feruloylserotonin, quercetin and resveratrol) appeared intracellularly. This was confirmed in isolated neutrophils by measuring the intracellular chemiluminescence (Drábiková *et al.* 2009) decreasing it in the rank order of potency curcumin > resveratrol > N-feruloylserotonin > quercetin. The intracellular inhibition of luminol-enhanced CL with antioxidants is dose-dependent and differs for the substances investigated (Nosál *et al.* 2015, Jančinová *et al.* 2012a). On the other hand, all the compounds tested decreased extracellular isoluminol-enhanced CL corresponding to their scavenging activities for oxidants, most probably as a result of the physico-chemical structure of their molecules, as demonstrated for H1 antihistamines (Králová *et al.* 2009). Since intracellular ROS play an important role as regulatory mediators in signaling processes, many of the ROS-mediated responses actually protect the cells against oxidative stress and reestablish redox homeostasis (Droge 2002). PMN functions must be therefore appropriately regulated in order to achieve host defense and avoid tissue-damaging inflammation. Development and pharmacological use of PMN-targeting agents may in fact be a good therapeutic strategy for regulation of PMN functions in control of inflammation and tissue injury (Trachootham *et al.* 2008, Kori *et al.* 2009).

The calculated ratio between the percentage of extra- versus intracellular inhibition of CL indicates that the higher the ratio the lower the risk of the antioxidant tested to suppress intracellular regulatory ROS on the one side while decreasing the risk of extracellular ROS participating substantially in pathological processes (Valko *et al.* 2007).

Dithiaden, curcumin, quercetin, N-feruloylserotonin and resveratrol decreased the activation of PKC  $\alpha,\beta$  indicating interference with PMA activation of the NADPH oxidase system responsible for oxidative burst in neutrophils (Fialkow *et al.* 2007). The inhibition of PKC  $\alpha,\beta$  with several antioxidants in isolated neutrophils was dose-dependent, as found for dithiaden, N-f-5HT, curcumin, pinosylvin and resveratrol (Drábiková *et al.* 2012, Jančinová *et al.* 2012a, Nosál *et al.* 2011, 2014, 2015).

Protein kinase C (PKC) involved in the activation of NADPH oxidase in primary neutrophils is activated by exogenous phorbol esters (PMA – Tauber 1987, Dekker *et al.* 2000, Steinberg 2015). PKC isoforms are important intracellular signaling molecules in cell differentiation, migration, proliferation and activation (Bertram and Ley 2011).

Carvedilol and arbutin slightly decreased superoxide generation in PMA-stimulated neutrophils, dithiaden and quercetin were ineffective. On the other hand, all the compounds tested decreased significantly myeloperoxidase liberation in the same neutrophils with dithiaden being the most active. The kinetic profile of superoxide production varies significantly depending upon the agonists or combination of agonists-antagonists employed. The respiratory burst and production of superoxide in phagocytes is mediated by the NADPH-oxidase complex (Robinson 2009).

Neutrophils are the major source of myeloperoxidase in the circulation which is released during the respiratory burst and degranulation process when neutrophils infiltrate tissues. Both SO and MPO are constituents of ROS (Shukla and Walsh 2015).

Dithiaden, curcumin, quercetin and arbutin in concentration of 10  $\mu\text{M}$  increased the activity of human recombinant caspase-3 in cell free system significantly. The effect of N-feruloylserotonin was not significant. The caspase-3 activity increased with dithiaden was dose-

dependent (Nosál *et al.* 2015). Caspase-3 belongs to the effector group of caspases which are responsible for the executive phase of apoptosis (Fan *et al.* 2005). Activation of caspases from their pro-caspase form has been widely described in cells undergoing apoptosis, including neutrophils. Programmed cell death – apoptosis – is an important process for successful removal of recruited neutrophils (Fox *et al.* 2010, Witko-Sarsat *et al.* 2011). Activation of recombinant caspase-3 by the compounds tested might thus contribute to the inhibition of oxidative burst in human neutrophils resulting from suppression of PKC activation and to the antiinflammatory effect of the agents investigated.

An excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of many diseases (Droge 2002). PMN functions must be appropriately regulated in order to achieve host defense and avoid tissue-damaging inflammation. Development and pharmacological use of PMN-targeting agents may therefore be a good therapeutic strategy in regulating PMN functions for control of inflammation and tissue injury (Kori *et al.* 2009). Polyphenolic compounds have therapeutic value as antioxidants and anti-inflammatory agents in chronic inflammatory epigenetically regulated diseases (Rahman 2008). A growing body of evidence shows that within cells ROS act as secondary messengers in intracellular signaling cascades which induce and maintain the oncogenic phenotype of cancer cells and ROS can also induce cellular senescence and apoptosis and thus function as anti-tumorigenic species (Valko *et al.* 2007). Future studies on the bioavailability, absorption, tissue distribution, and understanding of the *in vivo* molecular effects of therapeutically used drugs and natural polyphenols are needed in order to consider these compounds as valuable antioxidants and natural therapy ‘nutraceuticals’ for chronic inflammatory disorders.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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