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ARTICLE

Oenothera paradoxa Defatted Seeds Extract and Its Bioactive Component Penta-*O*-galloyl-*β*-D-glucose Decreased Production of Reactive Oxygen Species and Inhibited Release of Leukotriene B_4, Interleukin-8, Elastase, and Myeloperoxidase in Human Neutrophils

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In this study, we analyzed ex vivo the effect of an aqueous extract of *Oenothera paradoxa* defatted seeds on the formation of neutrophil-derived oxidants. For defining active compounds, we also tested lypophilic extract constituents such as gallic acid, (+)-catechin, ellagic acid, and penta-*O*-galloyl-*β*-D-glucose and a hydrophilic fraction containing polymeric procyanidins. The anti-inflammatory potential of the extract and compounds was tested by determining the release from activated neutrophils of elastase, myeloperoxidase, interleukin-8 (IL-8), and leukotriene B_4 (LTB_4), which are considered relevant for the pathogenesis of cardiovascular diseases. The extract of *O. paradoxa* defatted seeds displays potent antioxidant effects against both 4*β*-phorbol-12*β*-myristate-α13-acetate- and formyl-met-leu-phenylalanine-induced reactive oxygen species production in neutrophils with IC_{50} values around 0.2 *μ*g/mL. All types of polyphenolics present in the extract contributed to the extract antioxidant activity. According to their IC_{50} values, penta-*O*-galloyl-*β*-D-glucose was the more potent constituent of the extract. In cell-free assays, we demonstrated that this effect is partially due to the scavenging of O_2^- and H_2O_2 oxygen species. The extract and especially penta-*O*-galloyl-*β*-D-glucose significantly inhibit elastase, myeloperoxidase IL-8, and LTB_4 release with an IC_{50} for penta-*O*-galloyl-*β*-D-glucose of 17 ± 1, 15 ± 1, 6.5 ± 2.5, and around 20 *μ*M, respectively. The inhibition of penta-*O*-galloyl-*β*-D-glucose on reactive oxygen species and especially on O_2^- production, myeloperoxidase, and chemoattractant release may reduce the interaction of polymorphonuclear leukocyte with the vascular endothelium and by that potentially diminish the risk of progression of atherosclerosis development.

KEYWORDS: *Oenothera paradoxa*; penta-*O*-galloyl-*β*-D-glucose; polyphenols; neutrophils; inflammation; reactive oxygen species; atherosclerosis development

INTRODUCTION

Oenothera sp. (Oenotheraceae) are native to Central and South America, where they have been widely used for their sedative, analgesic, astringent, and wound-healing properties (*1*). Today, *Oenothera biennis* L. and *Oenothera paradoxa* Hudziok are also cultivated in Europe for the production of seeds as a source of oil, the main source of *γ*-linolenic acid (GLA). In the past few years, there has been a growing interest in the evening primrose due to its polyphenolics content. The plant contains flavonoids, phenolic acids, and hydrolyzable tannins (*2*, *3*). Fractionation of the seed extracts, obtained from the defatted pulp, showed the presence of hydrolyzable and condensed tannins. Gallic acid, (+)-catechin, (−)-epicatechin, and a tetrameric procyanidin gallate were isolated as pure compounds from *O. biennis* seeds (*4*, *5*). In our previous study, we have additionally identified (−)-epicatechin, (−)-epicatechin gallate, ellagic acid, caffeic acid, quercetin, penta-*O*-galloyl-*β*-D-glucose (PGG), and procyanidins in *O. paradoxa* seeds (*6*).

Pharmacological investigations have revealed that *Oenothera* (*O. biennis* and *O. paradoxa*) defatted seed extracts demonstrate antioxidant and iron(II) chelating activities (*7*−*9*) and the inhibition of vasopeptidase activity (*6*). The phenolic fraction purified from defatted seeds of *O. biennis* and *O. paradoxa* promoted selective apoptosis of human bone marrow-derived cell lines and human skin melanoma cells with a higher activity than against normal cells. Analysis of the fractions has showed the presence of gallic acid and PGG (*10*, *11*). PGG, one of the main constituents of the *O. paradoxa* seed extract, itself demonstrated a significant antiproliferative activity against several cancer cell lines: prostate, breast, lung, and liver (*12*). PGG also displays a number of biological activities related to inflammation: inhibition of

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tumor necrosis factor α (TNF-α), interleukin-6, and interleukin-12 production/release (*13*, *14*), inhibition of nitric oxide (NO) production and inducible NO syntase expression (*15*), and inhibition of COX-2 activity (*16*).

Taking into account the antioxidant and vasopeptidase inhibitory activities of the *O. paradoxa* extracts and the anti-inflammatory activity of its constituent PGG, we expect that the extract may play a potential role in cardiovascular disorders, especially related to inflammation and reactive oxygen species (ROS) production. In this study, we analyzed ex vivo the effect of the aqueous extract of *O. paradoxa* defatted seeds on the formation of neutrophil-derived oxidants. For defining active compounds, we also tested lypophilic extract constituents such as gallic acid, (+)-catechin, ellagic acid, and PGG and a hydrophilic fraction containing polymeric procyanidins (PAs). The anti-inflammatory potential of the extract and compounds, especially PGG, was tested by determining the release from activated neutrophils of elastase, myeloperoxidase (MPO), interleukin-8 (IL-8), and leukotriene B_4 (LTB_4), which are considered relevant to the pathogenesis of cardiovascular diseases.

MATERIALS AND METHODS

Chemicals. Luminol, PMA (4β-phorbol-12β-myristate-α13-acetate), f-MLP (formyl-met-leu-phenylalanine), vitamin C, indomethacin, DCFH-DA (2′,7′-dichlorodihydrofluorescein-diacetate), NBT (nitrobluetetrazolium), xanthine, xanthine oxidase, HRP (horseradish peroxidase), SAAVNA (*N*-succinyl-alanine-alanine-valinine-*p*- nitroanilide), LPS (lipopolysacharide), cytochalasin B, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], taurine, and TMB (3,3′,5,5′-tetramethylbenzidine) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Lucigenin, (+)-catechin, and quercetin were purchased from Carl Roth (Karlsruhe, Germany). NaClO was purchased from POCH (Gliwice, Poland). Gallic acid and ellagic acid were purchased from ChromaDex (Santa Ana, United States). PGG was isolated as described previously (*6*). All substances used were of >95% purity. Phosphate-buffered saline (PBS) was purchased from Biomed (Lublin, Poland). Hanks' balanced salt solution (HSSB) and RPMI 1640 medium were purchased from Sigma-Aldrich Chemie GmbH.

Plant Material and Preparation of PA-Rich Extract. The dried aqueous extract of defatted seeds of *O. paradoxa* containing ca. 45% of phenolic compounds was obtained from Agropharm S.A. (Tuszyn, Poland). The seeds were obtained from crops cultivated according to the GACP guidelines (EMEA/HMPWP/31/99 Rev. Three of 2002). The extract contained 3.70 ± 0.13 mg/g of gallic acid, 23.43 ± 0.82 mg/g of (+)-catechin, 1.48 ± 0.02 mg/g of ellagic acid, and 12.05 ± 0.53 mg/g of pentagalloyloglucose, as determined previously (*11*). An accurately weighted 20 g amount of aqueous extract was dissolved in water (200 mL) and extracted with ethyl acetate (3 × 200 mL) to clean the extract of PGG, (+)-catechin, ellagic acid, and gallic acid. The aqueous residue was lyophilized, giving a residue of 16.2 g containing PAs. Before each experiment, the extracts/compounds were dissolved in the appropriate buffer.

Isolation of Human Neutrophils. Peripheral venous blood was taken from a healthy human donor (20−35 years old) in the Warsaw Blood Donation Centre. Donors did not smoke or take medication. They were clinically confirmed to be healthy, and a routine laboratory test showed values within the normal range. The study conformed to the principles of the Declaration of Helsinki. Neutrophils were isolated with a standard method of dextran sedimentation prior to hypotonic lysis of erythrocytes and to centrifugation in a Ficoll Hypaque gradient (*17*). The purity of neutrophils preparation was >97%, and viability measured by tryptan blue exclusion was >98%. Neutrophils were resuspended in Ca^{2+}-free HSSB and Ca^{2+}-free PBS buffers at pH 7.4 or RPMI 1640 medium and were maintained at 4 °C before use.

Evaluation of ROS Production by Chemiluminescence. Oxidant generation by f-MLP or by PMA-stimulated neutrophils was measured using luminol- and lucigenin-dependent chemiluminescence. A cell suspension (3.5×10^5) was incubated with 50 μL of extracts/compounds at concentrations of 0.2, 2, 20, and 50 μg/mL and luminol (100 μM) or lucigenin (200 μM) in a 96-well plate. ROS production was initiated by the addition of f-MLP (0.1 μg/mL) or PMA (1 μg/mL) to obtain 200 μL/well. Changes in chemiluminescence at 37 °C were measured immediately for 45 min at intervals of 2 min in a microplate reader (Biotek). Background chemiluminescence produced by nonstimulated cells was also determined.

Evaluation of Intercellular ROS Production with DCFH-DA. Intracellular oxidant generation by f-MLP-stimulated neutrophils was measured using DCFH-DA as described by Bland et al. (*18*). A cell suspension (5×10^5) was incubated with 50 μL of extract at concentrations of 0.2, 2, 20, and 50 μg/mL and DCFH-DA (100 μM) in a 96-well plate. The ROS production was started by the addition of f-MLP (0.1 μg/mL) to obtain 200 μL/well. Changes in fluorescence at 37 °C were measured immediately for 90 min at intervals of 5 min in a microplate reader (BioTek) at 485 nm excitation and 530 nm emission. Background fluorescence produced by nonstimulated cells was also determined.

Evaluation of ROS Scavenging in Cell-Free Systems. Scavenging of superoxide anion (O_2^-) was performed using a xanthine−xanthine oxidase system with the NBT reduction method as described by Choi et al. (*19*). Fifty microliters of extracts or PGG at concentrations of 0.2, 2, 20, and 50 μg/mL, 50 μL of xantine oxidase (0.1 mU in PBS), and 100 μL of 0.4 mM xantine and 0.24 mM NBT solution in PBS were added to a 96-well plate. The absorbance at 560 nm was measured in a microplate reader (BioTek) over a 30 min period. The percent of inhibition of the xantine/xantine oxidase system was calculated in comparison to the control without test extracts/compound. To evaluate whether extracts or PGG affected the O_2^- generation by direct interaction with xanthine oxidase, the enzyme activity was determined by monitoring the uric acid formation at 295 nm (*20*).

Scavenging of hydrogen peroxide (H_2O_2) was performed with modification as described by O'Dowd et al. (*21*). Fifty microliters of extracts or PGG at concentrations of 0.2, 2, 20, and 50 μg/mL, 50 μL of HRP (5 mU in PBS), and 50 μL of 0.03% H_2O_2 solution in PBS were added to a 96-well plate. The chemiluminescence was measured in a microplate reader (BioTek) after the addition of 50 μL of luminol solution (10 μM) over a 10 min period. The percent of inhibition of the HRP/hydrogen peroxide system was calculated in comparison to the control without test extracts/compound.

Scavenging of hypochlorous acid (HClO) was performed by the chlorination of taurine with modification as described by Daels-Rakotoarison et al. (*22*). Fifty microliters of extracts or PGG at concentrations of 0.2, 2, 20, and 50 μg/mL, 100 μL of taurine (150 mM in PBS), and 50 μL of 0.05% NaClO solution in PBS were added to a 96-well plate. After 5 min, the absorbance was measured in a microplate reader (BioTek) at 350 nm after the addition of 10 μL of KI solution (20 mM in PBS). The percent of inhibition of chlorination of taurine was calculated in comparison to the control without inhibitors, taking into account the absorbance of light by test extracts/compound.

Elastase Release. Neutrophil elastase release was determined using SAAVNA as a substrate, and *p*-nitrophenol was measured spectrophotometrically. The cell suspension (5×10^5) was preincubated with 50 μL of extracts/compounds at concentrations of 5, 10, 20, and 50 μg/mL for 15 min at 37 °C and then stimulated with cytochalasin B (5 μg/mL) and f-MLP (0.1 μg/mL) for 15 min. After the addition of SAAVNA (100 μM), the extent of *p*-nitrophenol was measured spectrophotometrically, for 1 h at intervals of 20 min, at 412 nm using a microplate reader (BioTek).

MPO Release. Neutrophil MPO release was determined using TMB as a substrate. The assay is based on the oxidation of TMB by MPO in the presence of H_2O_2 (*23*). A cell suspension (2×10^6) was preincubated with 50 μL of extracts/compounds at concentrations of 5, 10, 20, and 50 μg/mL for 15 min at 37 °C and then stimulated with cytochalasin B (5 μg/mL) and f-MLP (0.1 μg/mL) for 10 min. After centrifugation (3000 rpm; 10 min; 4 °C), 75 μL of supernatant was incubated with 100 μL of the reaction mixture (1 mg/mL TMB in citrate phosphate buffer, pH 5, supplemented with 0.015% H_2O_2). The reaction was terminated after 5 min by the addition of 150 mM sodium acetate (pH 3), and the absorbance was measured at 655 nm using a microplate reader (BioTek).

IL-8 Production. Neutrophils (2×10^6/mL) were cultured in a 24-well plate in RPMI 1640 medium with 10% FBS, 10 mM HEPES, and 2 mM L-glutamine in the absence or presence of LPS (100 ng/mL) for 12 h at 37 °C with 5% CO_2 in the absence or presence of extracts or PGG at concentrations of 5, 10, 20, and 50 μg/mL added 1 h before the stimuli. The released

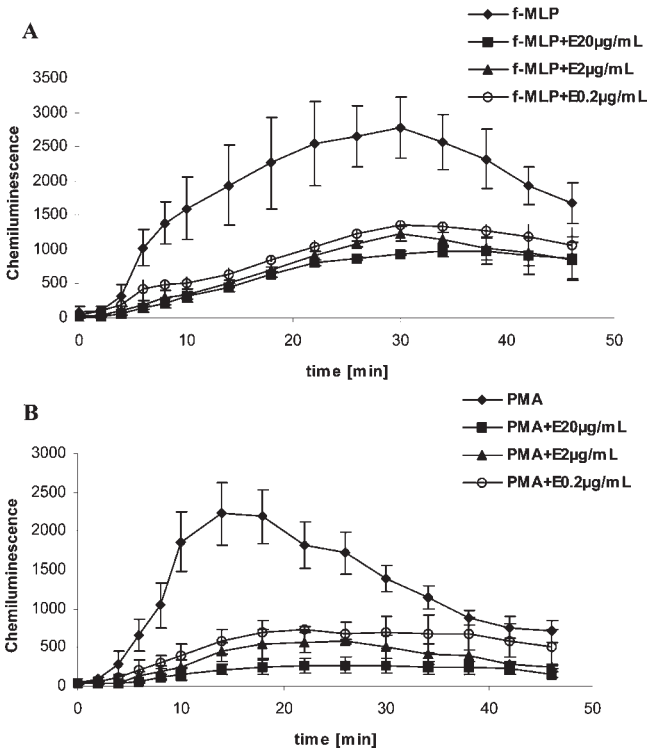

Figure 1. Inhibitory effects of aqueous extract (E) of *O. paradoxa* at concentrations of 0.2, 2, 20 μg/mL on the luminol-enhanced chemiluminescence response of f-MLP (**A**) and lucigenin-enhanced chemiluminescence response of PMA (**B**)-stimulated human neutrophils. Data were expressed as means ± SDs; $n = 6$, assayed in triplicate. Experiments were performed using cells of different donors.

IL-8 into cell supernatants was measured by enzyme-linked immunoabsorbent assay (ELISA) following the indications of the manufacturer (R&D Systems).

LTB_4 Production. Neutrophils (2×10^6 cells) were preincubated at 37 °C for 15 min before the stimuli in the absence or presence of extracts or PGG at concentrations of 20 and 50 μg/mL. After this, cytochalasin B (5 μg/mL) and f-MLP (0.1 μg/mL) were added for a further 10 min of incubation. The released LTB_4 into cell supernatants was measured by ELISA following the indications of the manufacturer (R&D Systems).

Cytotoxicity Assay. Extracts and PGG cytotoxicity for neutrophils were tested by MTT colorimetric assay. A cell suspension (5×10^5 in RPMI 1640 medium) with 50 μL of extracts or PGG at concentrations of 20, 50, and 100 μg/mL were incubated in a 96- well plate for 1 or 3 h at 37 °C, and then, MTT (0.5 mg/mL) was added and incubated at 37 °C for an additional 1 h. The insoluble formazan product was dissolved in 200 μL of 0.04 M HCl in isopropanol and measured spectrophotometrically at 570 nm using a microplate reader (BioTek).

Statistical Analysis. The results were expressed as means ± standard deviations (SDs) of the indicated number of experiments. The statistical significance of differences between means was established by analysis of variance with Tukey's posthoc test. P values below 0.05 were considered statistically significant. All analyses were performed using Statistica 8.

RESULTS

Effects of Aqueous Extracts of *O. paradoxa*, PAs Fraction, and PGG, (+)-Catechin, Ellagic Acid, and Gallic Acid on PMA and f-MLP-Induced ROS Production in Human Neutrophils . Activated neutrophils produce a high amount of ROS, depending on the stimuli; bacterial-derived peptides f-MLP, which act by a specific receptor or PMA, a direct protein kinase C activator, cells produce O_2^-, H_2O_2, HClO, or only O_2^- and H_2O_2.

The aqueous extract of *O. paradoxa* (0.2−20 μg/mL) reduced ROS production in concentration-dependent manner (**Figure 1**)

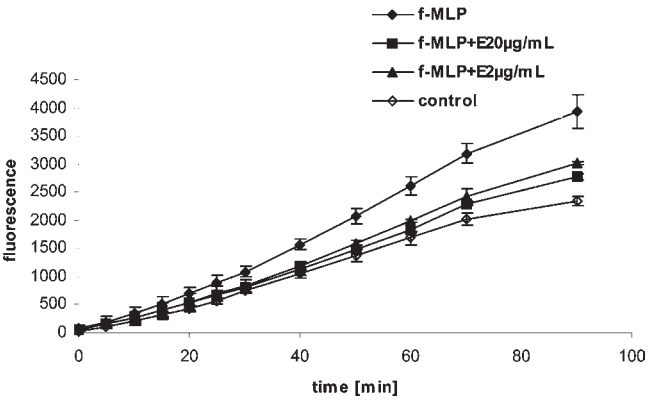

Figure 2. Inhibitory effects of aqueous extract (E) of *O. paradoxa* at concentrations of 2 and 20 μg/mL on the intracellular ROS production of f-MLP-stimulated human neutrophils in comparison to unstimulated cells (control) .Data were expressed as means ± SDs; $n = 3$, assayed in triplicate. Experiments were performed using cells of different donors.

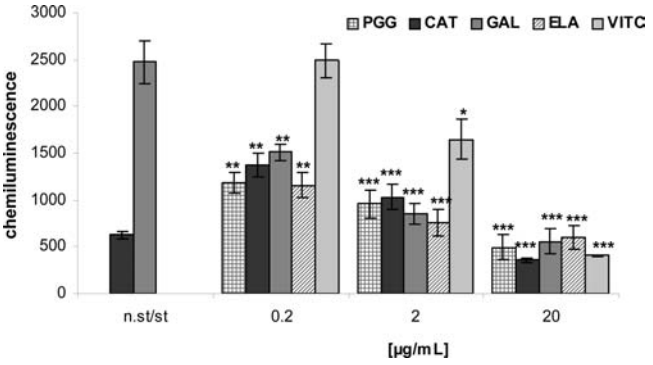

Figure 3. Inhibitory effects of polyphenols [PGG; CAT, (+)-catechin; GAL, gallic acid; and ELA, ellagic acid] from *O. paradoxa* and vitamin C (VIT C) at concentrations of 0.2, 2, and 20 μg/mL on the luminol-enhanced chemiluminescence response of f-MLP-stimulated human neutrophils (n.st., not stimulated cells; and st., stimulated cells). Data were expressed as means ± SDs; $n = 6$, assayed in triplicate. The reduction of chemiluminescence by testes compounds was demonstrated at the maxinal luminescence of stimulated cells. Experiments were performed using cells of different donors. *$P < 0.05$, **$P < 0.02$, and ***$P < 0.005$.

with a high potency IC_{50} value of around 0.2 μg/mL. No significant difference between the f-MLP and the PMA stimuli suggested no activity of the extract on their signal transduction pathways. The effect of the extract on the production of intracellular ROS was affected to a lesser extent (**Figure 2**). The PAs fraction showed significant inhibitory activity against ROS species, especially against stimulated PMA ($IC_{50} = 2.0 \pm 0.5$ μg/mL). Other polyphenolics present in an aqueous extract such as PGG, (+)-catechin, ellagic acid, and gallic acid strongly inhibited ROS production in comparison with vitamin C (**Figure 3**). Among tested compounds, PGG was the most potent with $IC_{50} = 0.2 \pm 0.1$ μM; however, other compounds were also very active: ellagic acid, $IC_{50} = 0.6 \pm 0.1$ μM; (+)-catechin, $IC_{50} = 1.7 \pm 0.7$ μM; and gallic acid, $IC_{50} = 4.2 \pm 1.4$ μM. On the other hand, PGG did not affect the intracellular ROS production tested in the DCFH-DA system (not shown).

Effects of Aqueous Extract of *O. paradoxa*, PAs Fraction, and PGG on ROS (O_2^-, H_2O_2, and HClO) Scavenging Activity in a Cell-Free System. Polyphenols are well-known radical scavengers, and to further elucidate whether the extract, procyanidins, and PGG are able to scavenge O_2^-, H_2O_2, or HClO, we

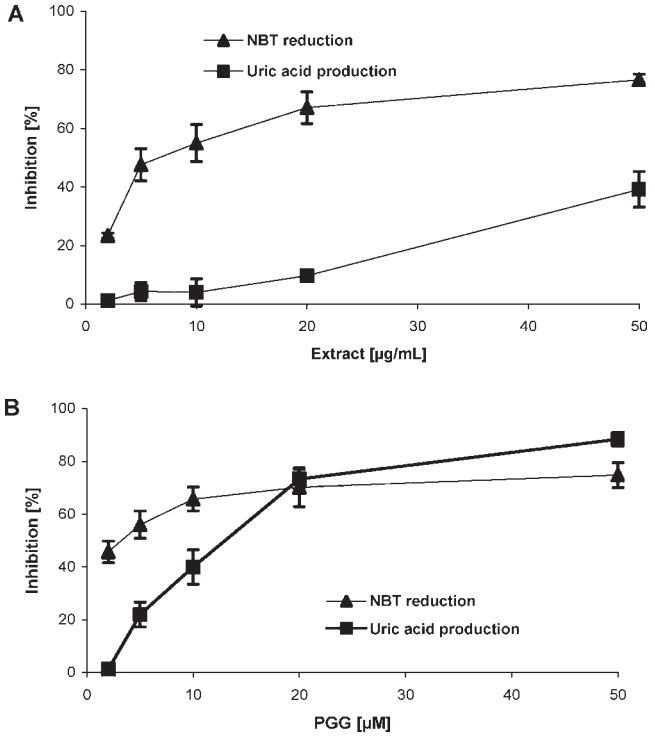

Figure 4. Inhibitory effects of aqueous extract of *O. paradoxa* (**A**) and PGG (**B**) on the xanthine/xanthine oxidase system. For analysis of scavenging of O_2^-, NTB reduction was determined. For analysis of oxidase inhibition, uric acid production was determined. Data were expressed as means ± SDs; $n = 3$, assayed in triplicate.

performed a cell-free system, which generated selected ROS. The aqueous extract of *O. paradoxa* showed significant H_2O_2 scavenging properties with $IC_{50} = 1.0 \pm 0.3$ μg/mL. The PAs fraction also shown significant inhibitory activity against H_2O_2. The O_2^- production was significantly reduced by PGG ($IC_{50} = 2.2 \pm 0.5$ μM), and the extract and procyanidins fraction were also active with IC_{50} below 10 μg/mL. Interestingly, the activity of PGG was partly due the inhibition of oxidase activity (**Figure 4**) with $IC_{50} = 10 \pm 2$ μM, while both extract and fraction affect the enzyme activity only in higher concentrations up to 50 μg/mL. The extract and PGG were also HClO scavengers.

PGG scavenged all types of ROS with the pronounced specifity to H_2O_2 and O_2^- (**Table 1**). The scavenging property of the extract and its constituents partly contribute to the inhibition of ROS production in human neutrophils (**Table 1**).

Effects of Aqueous Extract of *O. paradoxa* and PGG on Elastase Release in Human Neutrophils. Incubation of stimulated neutrophils with the *O. paradoxa* extract (5−50 μg/mL) resulted in statistically significant reduction in elastase release by the cells. PGG (5−50 μg/mL) reduced in a concentration-dependent manner the release of this enzyme (**Figure 5**) with an IC_{50} value of 17 ± 1 μM. The potency of PGG was comparable to the known inhibitor of elastase release quercetin, which showed $IC_{50} = 20 \pm 3$ μM. The procyanidins fraction, as well as (+)-catechin, ellagic acid, and gallic acid, were inactive.

Effects of Aqueous Extract of *O. paradoxa*, PAs Fraction, and PGG, (+)-Catechin, Ellagic Acid, and Gallic Acid on MPO Release in Human Neutrophils. Incubation of stimulated neutrophils with the *O. paradoxa* extract and procyanidins fraction (10−50 μg/mL) resulted in statistically significant reduction in MPO release from the cells. PGG (5−50 μg/mL) reduced in a concentration-dependent manner the release of this enzyme (**Figure 6**) with an IC_{50} value of 15 ± 1 μM. Catechin and gallic acid were even more

Table 1. Summary of the IC_{50} Values for Inhibiting f-MLP- and PMA-Induced ROS Production on Human Neutrophils and Scavenging Selected Radicals in Cell-Free Systems by Aqueous Extract, PAs-Rich Extract, and PGG[a]

	aqueous extract (μg/mL)	PA (μg/mL)	PGG (μM)
f-MLP induction	0.2 ± 0.1	6.5 ± 3.0	0.2 ± 0.1
PMA induction	<0.2	2.0 ± 0.5	1.0 ± 0.5
O_2^-	7.0 ± 2.5	9.5 ± 2.7	2.2 ± 0.5
H_2O_2	1.0 ± 0.3	1.5 ± 0.5	0.8 ± 0.2
HClO	20.7 ± 2.5	>50	11.2 ± 0.1

[a] Data were calculated as 50% inhibitory concentration (IC_{50}). Experiments were performed in triplicate in three independent experiments. Experiments with neutrophils were performed using cells of different donors.

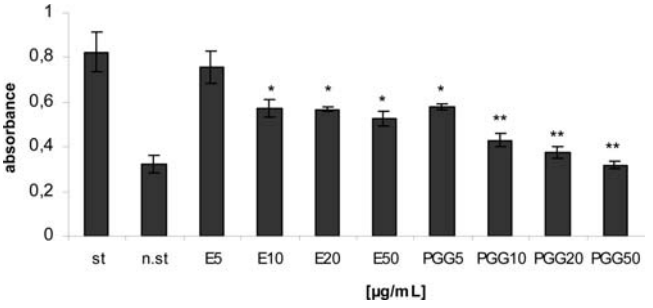

Figure 5. Inhibitory effects of aqueous extract (E) of *O. paradoxa* and PGG at concentrations of 5, 10, 20, and 50 μg/mL on the elastase release of f-MLP-stimulated human neutrophils (n.st., not stimulated cells; and st., stimulated cells). Data were expressed as means ± SDs; $n = 6$, assayed in triplicate. Experiments were performed using cells of different donors. *$P <$ 0.05, and **$P < 0.02$.

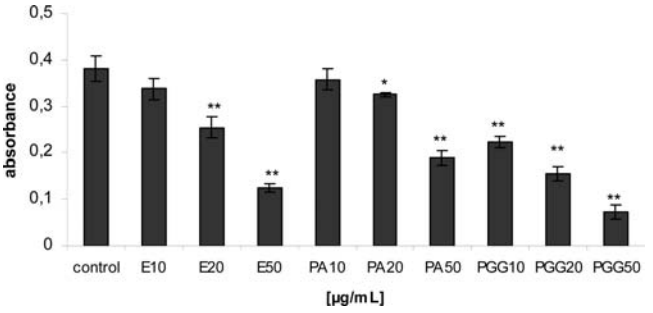

Figure 6. Inhibitory effects of aqueous extract (E), PAs fractions of *O. paradoxa*, and PGG at concentrations of 10, 20, and 50 μg/mL on the MPO release of f-MLP-stimulated human neutrophils (control). Data were expressed as means ± SDs; $n = 6$, assayed in duplicate. Experiments were performed using cells of different donors. *$P < 0.005$, and **$P < 0.002$.

active with IC_{50} 12.0 ± 3.5 and 2.8 ± 0.8 μM, respectively. The potency of gallic acid was higher than that of indomethacin, which showed $IC_{50} = 5.5 \pm 1$ μM. Ellagic acid was inactive.

Effects of Aqueous Extract of *O. paradoxa* and PGG on IL-8 Release in Human Neutrophils. Activation of neutrophils by LPS resulted in release of IL-8 to the level of 329 ± 66 pg/2 × 10^6 cells in comparison to the level of 14 ± 7 pg/2 × 10^6 cells in the untreated control. Incubation for 20 h of LPS-stimulated neutrophils with the *O. paradoxa* extract (10−50 μg/mL) resulted in statistically significant reduction in IL-8 release by the cells. PGG (5−50 μg/mL) strongly reduced in a concentration-dependent manner the release of this cytokine (**Figure 7**) with an IC_{50} value of 6.5 ± 2.5 μM. The procyanidins fraction, as well as (+)-catechin, ellagic acid, and gallic acid, were inactive.

Effects of Aqueous Extract of *O. paradoax*, PAs Fraction, and PGG on LTB_4 Release in Human Neutrophils. Activation of

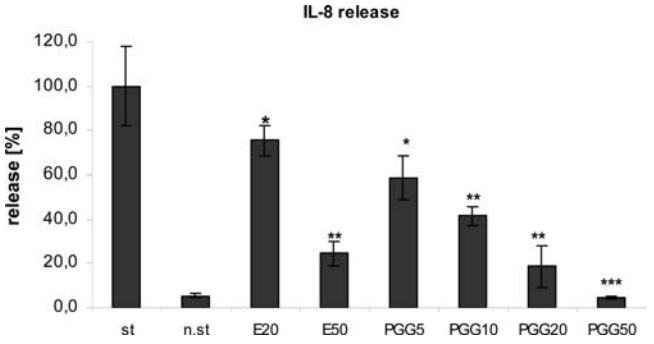

Figure 7. Inhibitory effects of aqueous extract (E) of *O. paradoxa* and PGG at concentrations of 5, 10, 20, and 50 μg/mL on the IL-8 release of LPS-stimulated human neutrophils (n.st., not stimulated cells; and st., stimulated cells). Data were expressed as means ± SDs; $n = 3$, assayed in duplicate. Experiments were performed using cells of different donors. $^*P < 0.05$, $^{**}P < 0.02$, and $^{***}P < 0.005$.

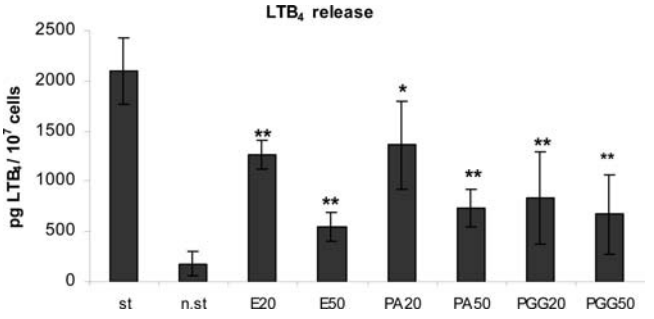

Figure 8. Inhibitory effects of aqueous extract (E), PAs fraction of *O. paradoxa*, and PGG at concentrations of 20 and 50 μg/mL on the LTB_4 release of f-MLP-stimulated human neutrophils (n.st., not stimulated cells; and st., stimulated cells). Data were expressed as means ± SDs; $n = 4$, assayed in duplicate. Experiments were performed using cells of different donors. $^*P < 0.05$, and $^{**}P < 0.01$.

neutrophils resulted in release of LTB_4 to the level of 2099 ± 326 pg/10^7 cells in comparison to the level of 177 ± 119 pg/2 × 10^6 cells in the untreated control. Incubation of stimulated neutrophils with the *O. paradoxa* extract and PAs fraction (20 and 50 μg/mL) resulted in statistically significant reduction of LTB_4 release by the cells. PGG (20 and 50 μg/mL) also reduced the release of this eicosanoid (**Figure 8**), while (+)-catechin, ellagic acid, and gallic acid were inactive.

Effects of Aqueous Extract of *O. paradoxa*, PAs Fraction, and PGG on Cell Viability. The aqueous extract of *O. paradoxa*, PAs fraction, and PGG, up to the concentrations of 100 μg/mL or 100 μM, respectively, had no adverse effect on the cell viability after 1 (**Table 2**) or 3 h treatment (not shown).

Table 2. Evaluation of Cytotoxicity on Human Neutrophils of Aqueous Extract, PAs-Rich Extract, and PGG by MTT Test[a]

	viable cells (%)		
concentration	aqueous extract (μg/mL)	PA (μg/mL)	PGG (μM)
100	100 ± 13	108 ± 24	102 ± 3
50	101 ± 8	96 ± 13	104 ± 7
20	102 ± 6	98 ± 8	99 ± 7

[a] Data were expressed as means ± SEMs; $n = 3$, assayed in triplicate using cells of different donors.

DISCUSSION

The present investigation demonstrated, for the first time, that *O. paradoxa* aqueous defatted seed extract attenuates ex vivo the neutrophils activation and that these effects can be correlated with the presence of polyphenols, especially with the presence of PGG.

Neutrophils activation results in a rapid increased formation of ROS, O_2^-, H_2O_2, and HClO extracellulary, as well as released proteolitic enzymes (e.g., elastase) and other inflammatory mediators (e.g., IL-8 and LTB_4), and increased cell mobility and adhesion. It has been shown that the excessively activated neutrophils can contribute to a variety of chronic inflammatory diseases including all stages of atherosclerosis (*24*, *25*).

For this study, we used f-MLP (a synthetic peptide that activates neutrophils through a receptor-mediated pathway) and PMA (a direct PKC activator). The f-MLP-mediated stimulation contrary to PMA stimulation led to the release of MPO, which is responsible for the HClO production from H_2O_2. For this reason, luminol was used together with f-MLP, because of its greater specificity to enhance HClO-mediated luminescence. The lucigen chemiluminescence is generally attributed to O_2^- generation (*26*). However, to a lesser extent, both compounds are also able to enhance H_2O_2-mediated luminescence (*27*, *28*). For determining the intracellular antioxidant activity, we used the DCFH-DA method. DCFH-DA is a nonfluorescent that can pass through cell membranes, and then, esterases transformed DCFH-DA to DCFH, which reacts with ROS (mainly H_2O_2) to form a highly fluorescent product: 2′,7′-dichlorofluorescein (DCF) (*26*). The aqueous extract of *O. paradoxa* significantly inhibited both f-MLP and PMA-induced ROS production, while the intracellular level of ROS was affected to a lesser extent. All types of polyphenols present in the extract (PAs, gallic acid, ellagic acid, (+)-catechin, and PGG) contributed to the extract antioxidant activity and showed a stronger antioxidant activity in comparison with vitamin C. According to their IC_{50} values, PGG was the most potent constituent of the extract. Taking into account these results and the fact that the antioxidative activity of PGG was only slightly examined until now (*29*, *30*), we further examined the direct scavenging abilities of PGG and the extract. We used specific cell-free systems for testing O_2^-, H_2O_2, and HClO scavenging abilities. The aqueous extract of *O. paradoxa* and the PA fraction showed similar scavenging abilities with the strongest activity against H_2O_2. PGG appeared as a strong antioxidant agent, especially against $O^-{}_2$ and H_2O_2. However, the inhibition of O_2^- production by PGG is not only due to its scavenging activity but also due to the inhibition of oxidase activity. At the same time, in our study, both extract and procyanidins fraction and PGG at s concentration up to 100 μg/mL and 100 μM did not affect the viability of neutrophils during 1 and 3 h incubation periods.

In addition to respiratory burst, granule exocytosis also plays a pivotal role in neutrophil function. During inflammation, neutrophils migrate and release destructive enzymes such as MPO, elastase, and proinflammatory factors LTB_4 and IL-8, which unlike ROS have relatively long half-lives. Neutrophil elastase, which catalyzes the breakdown of collagen and fibronectin, is a major enzyme secreted by stimulated neutrophils and a major contributor to the destruction of tissue. The level of elastase is increased in unstable angina pectoris and acute myocardial infraction (*25*), while MPO levels are associated with the presence of coronary artery disease and MPO considerably contributes to plaque vulnerability (*31*). MPO plays a key role in atherogenesis by affecting lipid peroxidation and the generation of reactive nitrogen species, which in turn convert low-density lipoprotein into an atherogenic form (*32*). HClO, as a product of the MPO

chlorchination activity, easily reacts with ether-phospholipids resulting in 2-chlorhexadecanal (2-CIHDA) formation. 2-CIHDA is a potent neutrophil chemoattractant, which promotes endothelial dysfunction (*31*). IL-8 and LTB_4 are also chemoattractants that amplify the recruitment of neutrophils to the site of inflammation. IL-8 and LTB_4 may also induce the formation of oxygen radicals and increase vascular permeability (*33*, *34*). In our study, we have shown for the first time that PGG significantly inhibits elastase, MPO, and LTB_4 release. The inhibition of IL-8 release by PGG from human monocytic cells has been demonstrated by Oh et al. (*35*); in both cases, the IC_{50} was below 10 μM. The inhibition of IL-8 in monocytes was associated with the inhibitory effect of PGG on TNF-α mediated NF-κB activation (*35*). The effect of the *O. paradoxa* extract was weaker in comparison to the isolated compound. The procyanidin-rich fraction showed even lower or no activity. Carini et al. (*36*) described a strong inhibition of elastase release (IC_{50} = 5.4 μM) by procyanidins from *Vitis vinifera* seeds. Procyanidins in *O. paradoxa* seeds are mainly type B polymers of (+)-catechin (*6*), while *V. vinifera* seeds contain both type A and type B galloyated procyanidins (*37*). Other phenolics present in the aqueous extract of *O. paradoxa* showed weaker activity in comparison with PGG, and gallic acid and (+)-cathechin were only able to inhibit the MPO released as it was described by other authors (*36*, *38*).

In conclusion, our results indicate that the aqueous extract of *O. paradoxa* defatted seeds displays a strong antioxidative activity, mainly due to the ROS scavenging ability. This property is mostly due to the presence of different classes of polyphenolic compounds: phenolic acids, procyanidins, and PGG. The latter compound also appears as a major anti-inflammatory constituent of the extract. The inhibiting influence of PGG on ROS and especially on O_2^- production, MPO, and chemoattractant release may reduce the interaction of polymorphonuclear leukocyte with the vascular endothelium, reduce the peroxynitrite ($ONOO^-$) formation, and by that diminish the risk of progression of atherosclerosis development. To assess the therapeutic relevance of the neutrophils activity modulation by *O. paradoxa* aqueous defatted seed extract and PGG, clinical studies are needed, which must take into consideration the pharmacokinetics and bioavailability of PGG.

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