Investigation of immunomodulatory and anti-inflammatory effects of eriodictyol through its cellular anti-oxidant activity

Imen Mokdad-Bzeouich^{1,2} · Nadia Mustapha^{1,2} · Aicha Sassi^{1,2} · Ahmed Bedoui^{1,2} · Mohamed Ghoul³ · Kamel Ghedira² · Leila Chekir-Ghedira^{1,2}

Received: 17 February 2016 / Revised: 4 May 2016 / Accepted: 13 May 2016 / Published online: 1 June 2016
© Cell Stress Society International 2016

Abstract Many studies have been performed to assess the potential utility of natural products as immunomodulatory agents to enhance host responses against infection or to ameliorate immune-based pathologies. To determine whether eriodictyol has immunomodulatory effects and clarify which types of immune effector cells are stimulated *in vitro*, we investigated the stimulatory effect of eriodictyol on spleen cells isolated from BALB/c mice. Eriodictyol significantly stimulated splenocyte proliferation. However, only B lymphocytes (not T lymphocytes) could be stimulated by eriodictyol in a dose-related manner. Studies assessing potential effect of eriodictyol on innate immunity reported that eriodictyol enhanced significantly the killing activity of natural killer (NK) cells, T lymphocytes, and macrophages. We also demonstrated that eriodictyol inhibited nitric oxide (NO) production and lysosomal enzyme activity in murine peritoneal macrophages cultured *ex-vivo*, suggesting a potential anti-inflammatory effect *in situ*. Eriodictyol revealed also a cellular anti-oxidant activity in splenocytes and macrophages. Furthermore, eriodictyol increased catalase activity in spleen cells. From this data, it can be concluded that eriodictyol exhibited an

immunomodulatory effect that could be ascribed in part to a cytoprotective effect related to its anti-oxidant activity.

Keywords Eriodictyol · Immunomodulation · Cellular anti-oxidant activity

Introduction

The innate immune system constitutes the first line of host defense during infection and so plays a crucial role in early recognition and subsequent triggering of inflammatory responses to pathogens. The adaptive immune system is responsible for eliminating pathogens primarily through generation of immunological memory. A healthy state is believed based on a fine-tuning of various humoral and cellular factors that help regulate immunoregulatory mechanisms. Inappropriate immunity is a common etiology in an ever-growing array of pathological processes including cancers, allergies, and a variety of autoimmune disorders (Cherng et al. 2007). Control of these diseases by immunotherapies has two objectives: development of immunity by stimulation of the host immune system (immunostimulation) and suppression of undesired immune reactions. While pharmacies are replete with both immunostimulants and immunosuppressants, nevertheless, the search for better agents exerting these activities with few side effects remains a major interest of investigators around the world.

Medicinal plants are rich sources of substances that are claimed to impact immunity and impart minimal side effects (Sharififar et al. 2009). Flavonoids are polyphenolic compounds that impart significant anti-inflammatory, anti-tumor, and anti-oxidant effects (Hodek et al. 2002). Eriodictyol, a tetra-hydroxy-flavanone, has anti-oxidant and anti-inflammatory activities (Clavin et al. 2007; Rossato et al.

✉ Leila Chekir-Ghedira
leila.chekir@laposte.net

¹ Laboratory of Cellular and Molecular Biology, Faculty of Dental Medicine, University of Monastir, Avicenna Street, 5000 Monastir, Tunisia

² Unit of Bioactive and Natural Substances and Biotechnology UR12ES12, Faculty of Pharmacy, University of Monastir, Avicenna Street, 5000 Monastir, Tunisia

³ Laboratory of Biomolecular Engineering, National School of Agronomy and Food Industries, National Polytechnics Institute of Lorraine ENSAIA-INPL, Vandoeuvre les, 54505 Nancy, France

2011). Eriodictyol is also capable to protect human adult pigment epithelial (ARPE-19) cells against oxidative stress (Johnson et al. 2009). However, few studies have investigated the immunomodulatory effect of eriodictyol. The aim of the study is to examine the immunomodulatory, anti-inflammatory, and the anti-oxidant activities of eriodictyol in splenocytes and macrophages.

Material and methods

Chemicals

Eriodictyol was purchased from Sigma-Aldrich (Canada Ltd). Penicillin, streptomycin, RPMI-1640 medium, phosphate-buffered saline (PBS, pH 7.4), and fetal bovine serum were purchased from Gibco by Life Technology, France. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was procured from Euromedex (Mundolsheim, France). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Lipopolysaccharide (LPS, rough strain, *E. coli* EH100) was purchased from Sigma (Hamburg, Germany), and lectin (from *Phytolacca americana*, pokeweed) was from Sigma-Aldrich (St. Louis, MO, USA). Triton X-100 was purchased from Biomatik Corporation (Cambridge, UK). p-Nitrophenyl phosphate (p-NPP) was from AppliChem (GmbH, Darmstadt, Germany). 2,7-dichlorofluoresceindiacetate (DCFH-DA) was procured from Fluka (Steinheim, Germany). 2,2'-Azobis(2-amidinopropane) dihydrochloride (ABAP) was from Sigma-Aldrich (Steinheim, Germany).

Animals

Male BALB/c mice between 6 and 8 weeks old (weight, 20.0 $\pm$ 2.0 g) were purchased from the Pasteur Institute, Tunis, Tunisia. Animals were housed in a dedicated room in individually ventilated cages where temperature and relative humidity were set at 22 $\pm$ 6 °C and 40 $\pm$ 10 %, respectively. Artificial lighting provided a 24-h cycle of 12-h light/12-h dark, and food and water were available ad libitum. All experiments were performed in accordance with the guidelines for the care and use of laboratory animals as published by the US National Institute of Health. All experiments received the explicit approval of the Ethics Animal Committee in Tunisia.

Cell proliferation assay

Assays of lymphocyte proliferation were performed according to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method outlined by Mosmann (1983). Briefly, mice were euthanized and their spleens were aseptically isolated. Each spleen was minced with sterile forceps

and the liberated splenocytes then centrifuged (1500 rpm, 10 min). To remove any red blood cells present, the cells were resuspended in lysing buffer (144 mM NH₄Cl in 1.7 mM Tris base, pH 7.4) and placed on ice for 10 min. The cells were then washed twice with phosphate-buffered saline (PBS, pH 7.4) and then resuspended in complete RPMI (RPMI-1640 containing 10 % FBS and 10 μ g ml⁻¹ penicillin-streptomycin).

For this assay, splenocytes in complete RPMI (5 $\times$ 10⁶ cells/ml; 100 μ l aliquot/well) were pre-incubated (37 °C, 5 % CO₂) in 96-well plates for 24 h before addition (20 μ l/well) of LPS as mitogen or lectin as weak mitogen, each to achieve final concentration in well of 5 μ g/ml and the tested compound. For use, LPS, lectin, or the tested compound were all diluted from stocks in complete RPMI. Cells were then incubated for an additional 48 h before the plates were centrifuged at 1500 rpm for 10 min. The cell pellet in each well was resuspended in 40 μ l MTT (5 mg/ml RPMI) solution, and the plate was incubated for an additional 2 h at 37 °C. The plate was then centrifuged again, the MTT in each well was removed, and 100 μ l dimethyl sulfoxide (98 % DMSO; Sigma-Aldrich, St. Quentin Fallavier, France) was added. The absorbance in each well was measured at 570 nm in a microplate reader (Thermo Scientific, Vantaa, Finland) and the percentage of proliferation ultimately calculated using the following: proliferation (%) = 100 $\times$ (OD sample – OD control) / OD control.

Activity of NK cells

Natural killer (NK) cell activity was measured as previously described (Sarangi et al. 2006). In brief, the spleens above were used as the source of effector cells; isolated splenocytes were seeded into 96-well plates at 5 $\times$ 10⁶ cells/ml. The cells were then stimulated with different concentrations of eriodictyol for 24 h. To eliminate direct effects of eriodictyol on target cells, the spleens were washed once with RPMI-1640 and then target K562 cells (5 $\times$ 10⁴ cells/ml; yielding a 100:1 expected effector-target ratio) were added to each well in 100 μ l aliquots. The plates were then incubated for 4 h at 37 °C. An aliquot (40 μ l) of MTT solution (5 mg/ml) was then added to each well and the plate incubated a further 2 h. Absorbance in each well was measured at 570 nm in the microplate reader. Three kinds of control measurements were taken: target cell control, blank control, and effector cell control. NK cell activity was calculated as follows: NK activity (%) = 100 % $\times$ (ODT – (ODS – ODE)) / ODT, where ODT = optical density value of target cell control, ODS = optical density value of test samples, and ODE = optical density value of effector cell control.

Assay of CTL activity

Cell-mediated cytotoxicity was performed using MTT assay. Splenocytes in complete RPMI-1640 (5 $\times$ 10⁶ effector cells/

ml; 100 μ l aliquot/well) were pre-incubated in 96-well plates for 24 h and then treated with different concentrations of eriodictyol for 24 h. Thereafter, target B16-F10 melanoma cells (5×10^4 target cells/ml; yielding a 100:1 expected effector-target ratio) were added to each well in 100 μ l aliquots. The plates were then incubated for 24 h at 37 °C prior to addition of MTT. Three kinds of control measurements were performed: target cell control, blank control, and effector cell control. Cytotoxic T lymphocyte (CTL) cell activity was calculated as follows: % activity = $100 \% \times (\text{ODT} - (\text{ODS} - \text{ODE})) / \text{ODT}$, where ODT = optical density value of target cell control, ODS = value of test sample-treated cells, and ODE = optical density value of effector cell control.

Nitrite release determination

Peritoneal macrophages were obtained from the peritoneal cavity of other mice after sacrifice. Peritoneal cells were obtained after intraperitoneal injection of 4 ml sterile PBS, massaging of the peritoneum, and drawing back of the fluid (≈ 4 ml) into the syringe. The obtained cells were washed twice and resuspended in complete RPMI-1640 medium.

The amount of nitric oxide (NO) released by macrophages was measured by determining the amounts of accumulated nitrite (NO_2^-) in cell-free supernatants via the Griess reaction (Green et al. 1982). In brief, cells were incubated for 48 h at 37 °C in the presence of increasing doses of eriodictyol. Nitrite was then measured by adding 100 μ l of Griess reagent (1 % sulfanilamide and 0.1 % naphthylenediamine in 5 % phosphoric acid) to 100 μ l of harvested culture supernatant. The optical density at 570 nm (OD570) was then measured in the microplate reader. NO concentrations were calculated by comparison with the OD570 of a standard solution of sodium nitrite diluted in culture medium and placed in parallel wells in the assay plates.

Assessment of lysosomal activity

Lysosomal enzyme activity (reflected by acid phosphatase [AP] activity) in the macrophages was measured as previously described by Manosroi et al. (2005). Macrophages (100 μ l aliquot of 3×10^6 cells/ml) were seeded into flat-bottom 96-well plates, treated with different doses of eriodictyol, and incubated at 37 °C for 48 h. The medium was then removed, and 20 μ l of 0.1 % Triton X-100 (Sigma, St. Louis, MO), 10 μ l of 100 mM p-NPP solution (Sigma), and 50 μ l of 0.1 M citrate buffer (pH 5.0) were added to each well. The plate was then incubated for 30 min at 37 °C before 150 μ l 0.2 M borate buffer (pH 9.8) was added to each well. The absorbance in each well was then measured at 405 nm in the plate reader. The percentage lysosomal enzyme activity in treated culture cells relative to that in control cells was

calculated as lysosomal enzyme activity (%) = $100 \times (\text{OD sample} - \text{OD control}) / \text{OD control}$.

In vitro MMC assay

Macrophage-mediated cytotoxicity (MMC) was performed using the MTT assay. The macrophages (1.5×10^5 cells/well) were pre-incubated in 96-well plate for 2 h at 37 °C and then treated with different concentrations of eriodictyol for 24 h at 37 °C. Thereafter, target B16-F10 melanoma cells (10^4 cells/well; yielding a 15:1 expected effector-target ratio) were added to each well in 100 μ l aliquots. Cells were then incubated at 37 °C for an additional 24 h. Three kinds of control measurements were taken: target cell control, blank control, and effector cell control. Macrophage-mediated cytotoxicity (MMC) activity was calculated as follows: MMC (%) = $100 \% \times (\text{ODT} - (\text{ODS} - \text{ODE})) / \text{ODT}$, where ODT = optical density value of target cell control, ODS = optical density value of test sample-treated cells, and ODE = optical density value of effector cell control.

CAA assay

A cellular anti-oxidant activity (CAA) assay (Wolfe and Liu 2007) was used to measure anti-oxidant potentials of the tested molecule, eriodictyol. Splenocytes and macrophages were seeded (in 100 μ l PBS) separately at a density of 5×10^5 and 6×10^4 cells/well, respectively, in 96-well microplates. Triplicate wells were then treated with 10 μ l of eriodictyol (6.25, 12.5, 25, and 50 μ M) along with 5 μ l of 25 μ M 2',7'-dichlorofluoresceindiacetate (DCFH-DA; Fluka, Steinheim, Germany). After 1-h incubation at 37 °C, 100 μ l of 1.2 mM 2,2'-azobis(2-amidinopropane)dihydrochloride (ABAP; Sigma-Aldrich, Steinheim, Germany) in PBS was provided to each well. Here, DCFH-DA is taken up by cells and deacetylated to 2', 7'-dichlorofluorescein (DCFH). Peroxyl radicals generated from ABAP lead to oxidation of DCFH to fluorescent dichlorofluorescein (DCF). Accordingly, cells treated with a compound that has any anti-oxidant activity should have lower fluorescence compared to untreated cells. Fluorescence in each well was evaluated at 5-min intervals over 1 h using a BioTek fluorescence microplate reader (Winooski, VT) with 538-nm emission and 485-nm excitation filters. Each plate included triplicate control and blank wells: control wells contained cells treated with DCFH-DA and ABAP; blank wells contained cells with PBS and without DCFH-DA and ABAP. Fluorescence values for blank samples and initial fluorescence values were subtracted from sample values. The area under fluorescence vs. time curve was integrated at each time point to calculate the CAA units using CAA unit = $100 - ([\text{SA}] / [\text{CA}] \times 100)$, where $[\text{SA}]$ is the integrated area under the sample fluorescence versus time curve and $[\text{CA}]$ is the integrated area from the control curve.

Determination of catalase activity

Catalase activity in the spleen cells was measured by spectrophotometry at 240 nm, according to Kang et al. (2006). Spleen cells were treated with various concentrations of eriodictyol for 1 h. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and then lysed on ice by sonication twice for 15 s. Triton X-100 (1 %) was then added to the lysates and was incubated for 10 min on ice. The lysates were centrifuged at 5000g for 30 min at 4 °C to remove the cellular debris. Fifty microliters of lysates was added in the quartz cuvette containing 50 mM phosphate buffer (pH = 7) and 100 mM (v/v) H₂O₂. The activity of the catalase was calculated using the molar extinction coefficient (0.04 mM⁻¹/cm). Results were expressed as micromoles of H₂O₂ per minute per milligram of protein.

Statistical analysis

All data were expressed as mean (±SD) and compared using a Student's *t* test. Statistical significance was assigned at *p* value <0.05.

Results

Effects of eriodictyol on splenocyte proliferation

To test whether eriodictyol promoted or inhibited cell proliferation, splenocytes were isolated and cultured with tested compound + the mitogen LPS or with tested compound + lectin (a weak mitogen). Analysis of lymphocyte proliferation in response to eriodictyol exposure showed a dose-dependent increase of lymphocyte proliferation incubated both with eriodictyol and lipopolysaccharide (LPS). Whereas, when

incubated with lectin and eriodictyol, lymphocyte proliferation is not affected (Fig. 1a, b).

Effects on NK cell activity

Measures of the cytotoxic activity of splenic NK cells against NK-sensitive tumor cells (i.e., K562 cell line) revealed that, as compared with the control (medium) vehicle, eriodictyol at different concentrations (5, 10, and 20 μM) significantly enhanced NK cell activity. Eriodictyol increases the activity of these cells from 20 % (untreated cells) to 62 % (in presence of 20 μM of eriodictyol) (Fig. 2).

Effects on CTL activity

The data indicate that splenocyte CTL activity increased remarkably when cells were treated with eriodictyol in a dose-related manner. The cytotoxicity of lymphocytes toward B16-F10 melanoma cells reached 55 % when incubated with 20 μM of eriodictyol (Fig. 3).

Effects on macrophage NO production

The effect of eriodictyol on macrophage NO release is reported in Fig. 4. LPS (5 μg/ml) resulted in the production of ≥56.3 μM NO. This induction was inhibited by eriodictyol treatment in a dose-related manner. In fact, NO production was reduced to 31.8 μM by treatment with 20 μM eriodictyol.

Effects on macrophage lysosomal activity

As shown in Fig. 5, eriodictyol decreased markedly macrophage lysosomal activity.

Fig. 1 Eriodictyol upregulates mitogen-induced spleen cell proliferation ex vivo. Cells were incubated with lectin (5 μg/ml) or LPS (5 μg/ml) and with tested concentrations of eriodictyol for 48 h at 37 °C and 5 % CO₂. Values represent the mean ±SD of three separate experiments. ▲ Data

significantly different from untreated cells. *Data significantly different from cells treated with LPS at *P* < 0.05. ***Data significantly different from cells treated with LPS at *P* < 0.001

Fig. 2 Effect of eriodictyol at various concentrations, on mice natural killer (NK) cells. Splenocytes were cultured with different concentrations of eriodictyol for 24 h at 37 °C and 5 % CO₂ before K562 target cells were added (at expected 100:1 E/T ratio). NK activity was then measured using an MTT assay. Values represent the mean ± SD of three separate experiments. ***Data significantly different from untreated cells (control) at $P < 0.001$. **Data significantly different from untreated cells at $P < 0.01$

Effects on MMC

Macrophages treated with eriodictyol reduced the proliferation of B16-F10 melanoma cells more strongly than untreated macrophages. Therefore, eriodictyol treatment stimulated anti-melanoma activity in macrophages in a dose-related manner (Fig. 6).

Eriodictyol exhibited anti-oxidant activity

The CAA assay was used here to quantitatively evaluate the anti-oxidant activity of eriodictyol in splenocytes and macrophages. The results clearly indicated that the eriodictyol should be absorbed into the cells and exert anti-oxidant activity. Production of fluorescence by ABAP-mediated oxidation was reduced in a dose-related manner (relative to values for

Fig. 3 Effect of eriodictyol on cytotoxic T lymphocyte (CTL) activity. Splenocytes obtained from mice were treated with different concentrations of eriodictyol before being incubated with B16-F10 cells for 24 h. CTL activity was then measured using an MTT assay. Values represent the mean ± SD of three separate experiments. *Data significantly different from untreated cells (control) at $P < 0.05$. ***Data significantly different from untreated cells at $P < 0.001$

Fig. 4 Production of the nitrite by mouse peritoneal macrophages in response to eriodictyol. Macrophages were incubated in RPMI-1640 medium in the absence or in the presence of increasing concentrations of eriodictyol for 48 h at 37 °C and 5 % CO₂. Cells treated with 5 μg/ml LPS alone were used as positive control. Values represent the mean ± SD of three separate experiments. ▲ Value significantly different compared to untreated cells. ***Value significantly different compared with LPS-stimulated cells $P < 0.001$

untreated control cells) by eriodictyol over the test range of 6.25 to 50 μM (Fig. 7). The mean effective concentrations (EC₅₀) of eriodictyol were 13 and 14 μM in splenocytes and macrophages, respectively (Fig. 8).

Eriodictyol stimulated catalase activity

Catalase enzyme is an endogenous anti-oxidant that can protect cells against adverse effects of reactive oxygen species (ROS). Level of catalase can indicate the magnitude of oxidative stress that occurs. Our results demonstrated that eriodictyol induced a marked increase in catalase activity. Catalase activity increased from 151 μmol/min/mg protein

Fig. 5 Modulation of mouse peritoneal macrophage lysosomal enzyme activity by eriodictyol. Macrophages were incubated in microtiter plate in the presence of increasing concentrations of eriodictyol for 48 h. Control (untreated cells) included cell incubated with RPMI-1640 medium. The data plotted represent the percentage of lysosomal enzyme activity ± SD of three independent experiments. ***Eriodictyol significantly ($P < 0.001$) reduced lysosomal enzyme activity of peritoneal macrophages when compared with untreated cells

Fig. 6 Effect of eriodictyol on macrophage-mediated cytotoxicity activity. Macrophages obtained from mice were treated with different concentrations of eriodictyol before being incubated with B16-F10 cells for 24 h at 37 °C and 5 % CO₂. Values represent the mean ± SD of three separate experiments. **Data significantly different from untreated cells (control) at $P < 0.01$. ***Data significantly different from untreated cells at $P < 0.001$

(untreated cells) to 620.8 μmol/min/mg (cells treated with 50 μM of eriodictyol) (Fig. 9).

Discussion

The immune system is the ultimate defense against infectious diseases, tumor, and cancer growth. Modulation of immune response through stimulation or suppression may help in maintaining a disease-free state. For the above reasons, we focused on how eriodictyol can modulate lymphocyte and macrophage responses as well as other inflammatory mediators. The effects of eriodictyol on both LPS- and lectin-stimulated cell proliferation were analyzed. Lectin stimulates T cells that are involved in cell-mediated immunity, while LPS acts on B cells that are responsible for humoral immune responses (Harizi et al. 2011). Eriodictyol upregulated only LPS-induced cell proliferation. This result suggested potential synergistic effect of eriodictyol upon LPS. By enhancing LPS-induced cell proliferation, eriodictyol might act as modulator

of B cell function and, consequently, of humoral immune response in situ. Whereas, since lectin-induced splenocyte proliferation was not affected by eriodictyol, we concluded that the cellular immune response was not involved.

Natural killer (NK) cells are a component of the innate immune system and have the ability to both lyse target cells and provide an early source of immunoregulatory cytokines (Caligiuri 2008). NK cells are able to recognize and to destroy targets that have lost expression of MHC class I molecules (Ljunggren and Karre 1990). Flavonoids can stimulate NK cytotoxic activity. Myricetin and quercetin enhanced the activity of NK in a dose-dependent manner (Lindqvist et al. 2014). Resveratrol increased the cytotoxic activity of NK cells in vivo (Lu and Chen 2010). Furthermore, NK cell-induced K562 target cell death was increased in the presence of luteolin (Ismail et al. 2012). The present study is the first to show that in vitro NK cell activity can be affected by eriodictyol.

Cytotoxic T lymphocytes (CTLs) play an important role in protecting against viral infections and cancer and rejection of organ transplants. CTLs constitute the major antitumor effector population and are recognized for their involvement in host resistance against tumor growth and dissemination (Whiteside and Herberman 1995). In this report, we have demonstrated that eriodictyol treatment increases the activity of ex vivo splenic CTL. The flavonoid, genistein, modulated the immune system and increased the activities of cytotoxic T and NK cells against B16-F10 cancer cells (Guo et al. 2001).

Macrophages are an important component in innate immunity. They function primarily as phagocytic cells and are capable of phagocytosing pathogenic organisms (Kovacs et al. 2009). In addition, activated macrophages generate large amount of NO from arginine and oxygen by various nitric oxide synthases (NOS) and by reduction of inorganic nitrate (Manosroi et al. 2005). NO is released by bacterial infection or inflammatory responses and is toxic to bacteria and other human pathogens. Excessive amounts of NO react with superoxide to produce peroxynitrite. Peroxynitrite is a damaging oxidant and nitrating agent, since it can damage a wide array

Fig. 7 Kinetics of inhibited oxidation of DCFH in splenocytes (a) and macrophages (b) using a cellular anti-oxidant activity assay in the presence of eriodictyol

Fig. 8 Dose-response curve for inhibition of oxidation of DCFH to DCF in splenocytes and macrophages using a cellular anti-oxidant activity assay in the presence of different concentrations of eriodictyol ($\pm$ SD, $n = 3$)

of molecules in cells, including DNA and proteins. Compared with LPS, a potent inducer of macrophage NO production (MacMicking et al. 1997), our experiment revealed that eriodictyol inhibited NO synthesis, by peritoneal macrophages. Similarly, eriodictyol downregulated NO formation (Huang et al. 2009). Moreover, eriodictyol reduced the NO production in LPS-stimulated Raw 264.7 macrophages (Lee 2011). The presence of OH groups at positions 5, 7, 3', and 4', together with the double bond in the B ring, is involved in the high anti-inflammatory effect of flavonoids (Comalada et al. 2006). The presence of two hydroxyl groups at the 3' and 4' positions of the B ring of eriodictyol could explain its anti-inflammatory activity.

Macrophages carry out their non-specific defense function at the elimination stage of the phagocytic process by activating the lysosomal phosphatase in the cytosolic vesicles (Nworu et al. 2010). The activity of lysosomal enzymes like alkaline phosphatase increased markedly during inflammation process and thus caused tissue damages (Chayen et al. 1970). The present study showed that eriodictyol decreased lysosomal phosphatase activity, revealing its anti-inflammatory potential.

Similarly, eriodictyol exhibited an anti-inflammatory effect in lipopolysaccharide (LPS)-induced acute lung injury in a mouse model, by regulating the Nrf2 pathway and inhibiting the expression of inflammatory cytokines (TNF- α , IL-6, IL-

1 β , and MIP-2) in macrophages (Zhu et al. 2015). Furthermore, eriodictyol decreased the oxidative stress level by reducing the accumulation of H₂O₂, OH $\cdot$, and MDA in the lung tissues. Another study conducted in vivo showed that eriodictyol attenuated cisplatin-induced kidney injury by inhibiting oxidative stress and inflammation. In fact, eriodictyol treatment reduced the production of TNF- α , and IL-1 β in kidney tissues induced by cisplatin and also upregulated the activities of superoxide dismutase, catalase, and glutathione peroxidase (Li et al. 2016). The enzymes cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and xanthine oxidase (XO) play key roles in the pathology of inflammatory diseases. Eriodictyol had an inhibitory effect on COX-1 and COX-2 and had a suppressive effect on XO activity, which confirm its anti-inflammatory and its anti-oxidant potencies (Zhang et al. 2006).

Macrophages can also be stimulated, both in vivo and in vitro, to exert cytotoxic activity against tumor cells. Their antitumor potential is often more efficient than other kinds of immunocompetent cells (Dumont et al. 1985). In fact, these cells play a complex role in tumor biology; their presence within a tumor can either correlate with tumor destruction or tumor growth. To become tumoricidal in vitro, macrophages must be stimulated in two ways. First, cytokines prime the macrophages, while secondary signals allow the macrophage to kill the tumor cell (Russell et al. 1977). Killing of target cells can be mediated through direct contact or through the production of soluble factors, or both, and the effectiveness of the mechanisms of killing employed by the macrophages is dependent on the target type and the pathway of macrophage activation (Keller et al. 1990). In this study, eriodictyol treatment stimulates macrophage-mediated cytotoxicity, in a dose-dependent manner. In fact, the flavanone, naringenin, enhanced macrophage-mediated cytotoxicity activity against Ehrlich ascite tumor cells (Orsolić and Basić 2005).

ROS are free radicals that contain an oxygen atom and are formed as natural by-products of the normal metabolism of oxygen and have important roles in cell signaling (Rada and Leto 2008). Over-production of ROS/inadequate anti-oxidant defenses are associated with the pathogenesis of chronic inflammatory diseases. Our results clearly show that eriodictyol

Fig. 9 Effect of eriodictyol on catalase enzyme activity in spleen cells. Values represent the mean $\pm$ SD of three separate experiments. ***Data significantly different from untreated cells (control) at $P < 0.001$

prevented ABAP-induced DCFH oxidation in splenocytes and macrophages in a dose-related manner and increased the activity of catalase, one of the most effective anti-oxidant enzymes that are produced naturally in the body to prevent free radical damage. In fact, eriodictyol contains numerous double bonds and hydroxyl groups that can donate electrons through resonance to stabilize the free radicals. Furthermore, eriodictyol was comparable with luteolin, quercetin, and taxifolin, as efficient anti-oxidants in protecting retinal cells against oxidative stress (Areias et al. 2001).

Oxidative stress plays a key role in the pathogenesis of various serious diseases such as cardiovascular diseases. Eriodictyol upregulated oxygenase 1 (HO-1), a phase II detoxifying enzyme, in endothelial cells (HUVECs) through extracellular-regulated kinase (ERK)/nuclear factor erythroid 2-related factor 2 (Nrf2)/anti-oxidant response element (ARE) signaling pathways (Lee et al. 2015). Furthermore, eriodictyol treatment protected these cells against hydrogen peroxide-induced cell death.

Diverse anti-inflammatory drugs have a radical-scavenging mechanism as part of their activity. Free radicals propagate inflammation by causing release of inflammatory cytokines, such as interleukin-1, tumor necrosis factor- α , and interferon- γ , that stimulate recruitment of additional neutrophils and macrophages. Neutralization of free radicals by anti-oxidant agents can attenuate inflammation (Delaporte et al. 2002). Thus, as eriodictyol exhibited a highly anti-oxidant potential, we believe that it may be responsible at least partly for its anti-inflammatory and immunomodulatory potentials.

Based on the results presented here, we conclude that eriodictyol has anti-oxidant properties as well as immunomodulating effects upon splenocytes, NK cells, and macrophages. Further studies on the specific mechanism of eriodictyol, in order to establish its therapeutic potential for the prevention of immune diseases, are required.

Acknowledgments The authors acknowledge the “Ministère Tunisien de l’enseignement supérieur et de la recherche scientifique” for financial support of this study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

- Areias FM, Rego AC, Oliveira CR, Seabra RM (2001) Anti-oxidant effect of flavonoids after ascorbate/Fe²⁺-induced oxidative stress in cultured retinal cells. *Biochem Pharmacol* 62:111–118
- Caligiuri MA (2008) Human natural killer cells. *Blood* 112:461–469
- Chayen J, Bitensky L, Butcher RG, Poulter LW, Ubhi GS (1970) Methods for direct measurement of anti-inflammatory action on human tissue maintained in vitro. *Br J Dermatol* 82:62–66
- Cherng JM, Chiang W, Chiang LC (2007) Immunomodulatory activities of common vegetables and spices of *Umbelliferae* and related coumarins and flavonoids. *Food Chem* 106:944–950
- Clavin M, Gorzalczany S, Macho A, Muñoz E, Ferraro G, Acevedo C, Martino V (2007) Anti-inflammatory activity of flavonoids from *Eupatorium arnottianum*. *J Ethnopharmacol* 112:585–589
- Comalada M, Ballester I, Bailón E et al (2006) Inhibition of pro-inflammatory markers in primary bone marrow derived marrow derived mouse macrophages by naturally-occurring flavonoids: analysis of the structure-activity relationship. *Biochem Pharmacol* 72:1010–1021
- Delaporte RH, Sánchez GM, Cuellar AC, Giuliani A, Palazzo de Mello JC (2002) Anti-inflammatory activity and lipid peroxidation inhibition of iridoid lamiide isolated from *Bouchea fluminensis* (Vell.) Mold (Verbenaceae). *J Ethnopharmacol* 82:127–130
- Dumont S, Hartmann D, Poindron P, Oberling F, Faradji A, Bartholeyns J (1985) Control of antitumoral activity of human macrophages produced in large amounts in view of adoptive transfer. *Eur J Cancer Clin On* 24:1691
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [¹⁵N] nitrate in biological fluids. *Anal Biochem* 126:131–138
- Guo TL, Mccay JA, Zhang LX, Brown RD, You L, Karrow NA, Germolec DR, White KLJR (2001) Genistein modulates immune responses and increases host resistance to B16-F10 tumor in adult female B6C3F1 mice. *J Nutr* 131:3251–3258
- Harizi H, Chaabane F, Ghedira K, Chekir-Ghedira L (2011) Inhibition of proinflammatory macrophage responses and lymphocyte proliferation in vitro by ethyl acetate leaf extract from *Daphne gnidium*. *Cell Immunol* 267:94–101
- Hodek P, Trefil P, Stiborova M (2002) Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. *Chem Biol Interact* 139:1–21
- Huang DW, Chung CP, Kuo YH, Lin YL, Chiang W (2009) Identification of compounds in adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) seed hull extracts that inhibit lipopolysaccharide-induced inflammation in Raw 264.7 macrophages. *J Agr Food Chem* 57:10651–10657
- Ismail N, Abdullah H, Seidel V, Rotondo D (2012) Human natural killer (NK) cell activation by luteolin from *Brucea javanica* leaves. *J. Cancer Res. Exp. Oncol.* ISSN: 2141–2243
- Johnson J, Maher P, Hanneken A (2009) The flavonoid, eriodictyol, induces long term protection in ARPE-19 cells through its effects on Nrf2 activation and phase 2 gene expression. *Invest Ophthalmol Vis Sci* 50:2398–2406
- Kang KA, Lee KH, Chae S, Zhang R, Jung MS, Ham YM, Baik JS, Lee NH, Hyun JW (2006) Cytoprotective effect of phloroglucinol on oxidative stress induced cell damage via catalase activation. *J Cell Biochem* 97:609–20
- Keller R, Kelst R, Wechsler A, Leist TP, Van Der Meide PH (1990) Mechanisms of macrophage-mediated tumor-cell killing: a comparative analysis of the roles of reactive nitrogen intermediates and tumor necrosis factor. *Int J Cancer* 46:682–686
- Kovacs EJ, Palmer JL, Fortin CF, Fülöp T Jr, Goldstein DR, Linton PJ (2009) Aging and innate immunity in the mouse: impact of intrinsic and extrinsic factors. *Trends Immunol* 30:319–324
- Lee JK (2011) Anti-inflammatory Effects of Eriodictyol in Lipopolysaccharide stimulated raw 264.7 murine macrophages. *Arch Pharm Res* 34:671–679
- Lee SE, Yang H, Son GW, Park HR, Park CS, Jin YH, Park YS (2015) Eriodictyol Protects endothelial cells against oxidative stress-induced cell death through modulating ERK/Nrf2/ARE-dependent heme oxygenase-1 expression. *Int J Mol Sci* 16:14526–14539

- Li CZ, Jin HH, Sun HX, Zhang ZZ, Zheng JX, Li SH, Han SH (2016) Eriodictyol attenuates cisplatin-induced kidney injury by inhibiting oxidative stress and inflammation. *Eur J Pharmacol* 772:124–30
- Lindqvist C, Bobrowska-Hägerstrand M, Mrówczyńska L, Engblom C, Hägerstrand H (2014) Potentiation of natural killer cell activity with myricetin. *Anticancer Res* 34:3975–9
- Ljunggren HG, Karre K (1990) In search of the “missing self”: MHC molecules and NK cell recognition. *Immunol Today* 11:237–244
- Lu CC, Chen JK (2010) Resveratrol enhances perforin expression and NK cell cytotoxicity through NKG2D-dependent pathways. *J Cell Physiol* 223:343–51
- MacMicking J, Xie QW, Nathan C (1997) Nitric oxide and macrophage function. *Annu Rev Immunol* 15:323–350
- Manosroi A, Saraphanchotiwithaya A, Manosroi J (2005) In vitro immunomodulatory effect of *Pouteria cambodiana* (Pierre ex Dubard) Baehni extract. *J Ethnopharmacol* 101:90–94
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63
- Nworu CS, Temchura V, Okoye FB, Akah PA, Esimone CO, Uberla K (2010) Activation of murine lymphocytes and modulation of macrophage functions by fractions of *Alchornea cordifolia* (Euphorbiaceae) leaf extract. *Immunopharmacol Immunotoxicol* 32:28–36
- Orsolić N, Basić I (2005) Water-soluble derivative of propolis and its polyphenolic compounds enhance tumoricidal activity of macrophages. *J Ethnopharmacol* 102:37–35
- Rada B, Leto TL (2008) Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. *Contrib Microbiol* 15:164–187
- Rossato MF, Trevisan G, Walker CI, Klafke JZ, de Oliveira AP, Villarinho JG, Zanon RB, Royes LF, Athayde ML, Gomez MV, Ferreira J (2011) Eriodictyol: a flavonoid antagonist of the TRPV1 receptor with antioxidant activity. *Biochem Pharmacol* 81:544–551
- Russell SW, Doe WF, McIntosh AT (1977) Functional characterization of a stable nonlytic stage of macrophage activation in tumors. *J Exp Med* 146:1511
- Sarangi I, Ghosh D, Bhutia SK, Mallick SK, Maiti TK (2006) Anti-tumor and immunomodulating effects of *Pleurotus ostreatus* mycelia-derived proteoglycans. *Int Immunopharmacol* 6:1287–1297
- Sharififar F, Pourmohammadi SH, Arabnezhad M, Rastegarianzadeh R, Ranjbaran O, Pourhemati A (2009) Immunomodulatory activity of aqueous extract of *Heracleum persicum* Desf. *Mice Iran J Pharm Res* 8:287–292
- Whiteside TL, Herberman RB (1995) The role of natural killer cells in immune surveillance of cancer. *Curr Opin Immunol* 7:704–710
- Wolfe KL, Liu RH (2007) Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *J Agric Food Chem* 55:8896–8907
- Zhang X, Hung TM, Phuong PT, Ngoc TM, Min BS, Song KS, Seong YH, Bae K (2006) Anti-inflammatory activity of flavonoids from *Populus davidiana*. *Arch Pharm Res* 29:1102–8
- Zhu GF, Guo HJ, Huang Y, Wu CT, Zhang XF (2015) Eriodictyol, a plant flavonoid, attenuates LPS-induced acute lung injury through its antioxidative and anti-inflammatory activity. *Exp Ther Med* 10:2259–2266