Epicatechin suppresses IL-6, IL-8 and enhances IL-10 production with NF-κB nuclear translocation in whole blood stimulated system

Maysa Al-Hanbali, Dalia Ali, Mona Bustami, Suzan Abdel-Malek, Rami Al-Hanbali, Tawfiq Alhussainy, Fadi Qadan ◊, Khalid Z. Matalka*

Department of Pharmacology and Biomedical Sciences, Faculty of Pharmacy and Medical Sciences, Petra University, P. O. Box 961343, Amman, 11196, Jordan
◊ Department of Pharmaceutical Medicinal Chemistry and Pharmacognosy, Faculty of Pharmacy and Medical Sciences, Petra University, P.O. Box 961343, Amman, 11196, Jordan
* To whom correspondence should be addressed.

Correspondence to: TEL: +962-6-571-5546, FAX: +962-6-571-5570, EMAIL: kzm@uop.edu.jo

Submitted: 2008-11-24 *Accepted: 2008-12-03* *Published online: 2009-02-28*

Key words: **epicatechin; cytokine modulation; IL-8; IL-6; IL-10; NFkB**

Neuroendocrinol Lett 2009; **30**(1):131–138 **PMID:** 19300393 NEL300109A08 © 2008 Neuroendocrinology Letters • **www.nel.edu**

Abstract

OBJECTIVES: Cytokines induce the balance between inflammatory versus regulatory or antibody mediated reactions. So modulating the release of cytokines or inducing them by immunomodulating agents is an attractive mode for treating or help in treating several diseases such as autoimmune diseases. *Eriobotrya japonica* is a plant that is traditionally thought to have anti-inflammatory activities. Several compounds were isolated from the plant and showed distinctive biological effects. The purpose of this study was to determine the effects of epicatechin (EC) isolated from *Eriobotrya Japonica* on IL-6, IL-8, and IL-10 productions in whole blood stimulated with phytohemagglutinin (PHA) + lipopolysaccharide (LPS), and to examine if these cytokines are modulated through NFκB pathway. **METHODS**: Sixteen healthy males and females volunteered in the study. Blood samples were drawn, diluted, and cultured for 24h with different concentrations of EC and then PHA+LPS was added for another 24 h. The supernatant, then, was harvested and assayed for cytokines. In addition, mixing studies of EC and hydrocortisone were performed to examine the cytoplasmic and nuclear fractions of NFkB levels in association with cytokine production levels. **RESULTS**: Increasing concentrations of EC (1–100 μg/ml) in PHA+LPS stimulated whole blood cells culture suppressed significantly ($p< 0.001$) the production of IL-6 and IL-8. Moreover, increasing concentrations of EC modulated significantly the production of IL-10, as there was a significant increase in IL-10 level at 0.1, 1.0, and 10 μg/ml ($p= 0.058–0.004$), while a significant decrease at a concentration of 100 μg/ml EC ($p= 0.037$) was observed. In addition, an additive effect between EC and hydrocortisone (HC, 100 nmol/l) was seen in the production of IL-10, as there was a significant increase in IL-10 level (32%) compared with 27% for EC (10 μg/ml) and19% HC. Furthermore, a significant decrease in cytoplasmic fractions of NFκB p65 level was found in samples containing EC 1, 10 μg/ml, but not in 100 μg/ml, when compared with control ($p< 0.03$). These latter changes were accompanied with a 29%, 67%, and 98% increase, respectively, of NFκB p65 in nuclear fractions compared to 24% reduction of NFκB p65 level in HC culture ($p< 0.03$). **CONCLUSIONS:** These results indicate that EC suppresses the production of pro-inflammatory cytokines, IL6 and IL-8, enhances the production of anti-inflammatory cytokine, IL-10, and stimulates NFκB p65 translocation to nucleus in PHA+LPS stimulated whole blood culture.

1. INTRODUCTION

Cytokines are the main regulators of the immune response. They induce the balance between inflammatory versus regulatory or antibody mediated reactions (Elenkov & Chrousos, 1999). So, modulating the release of cytokines or inducing them by immunomodulating agents is an attractive mode for treating or help in treating several diseases such as infection, allergy, autoimmune mediated diseases and cancer (Elenkov & Chrousos, 1999; Tzianabos, 2000; Yu *et al.* 2006; Matalka, 2003a). One of the pro-inflammatory cytokines is IL-8. IL-8 is produced by macrophages, fibroblasts and epithelial cells (Kim, 2006). Locally, IL-8 is also produced by neutrophils, which may then attract other neutrophils and eosinophils (Jacobi *et al.* 1998). This cytokine is believed to play role in inflammation; pathogenesis of inflammatory airway diseases (Jacobi *et al.* 1998), psoriasis (Bruch-Gerharz *et al.* 1996), and coronary artery diseases (Boekholdt *et al.* 2004). On the other hand, IL-6 has both pro-inflammatory and anti-inflammatory properties depending on the inflammatory stimuli, cells that produce it, or the signaling pathway activated (Opal & De-Palo, 2000). IL-6 is produced by T-cells, macrophages, endothelial cells, osteoblasts and stromal cells (Heikkila *et al.* 2008). IL-6 can have direct effects on B lymphocytes as B cell differentiation factor and can mediate the effects of other cytokines. IL-6 is released in response to IL-1 and TNF-α, and produces its action after forming a complex with either its receptor (IL-6 R) or other membrane bound proteins through different signaling pathways as Janus Kinases (JAKs), STAT3 and transcription factor NFκB (Omoigui, 2007). Recently, it was reported that IL-6 is responsible for many inflammatory diseases as atherosclerosis, coronary artery diseases, rheumatoid arthritis, osteoporosis, and Alzheimer's disease and thus inhibiting signal transduction pathways of IL-6 using natural products derived from plants as polyphenols could be helpful in the management of such diseases (Omoigui, 2007). As anti-inflammatory cytokine, IL-10 regulates proliferation and differentiation of T cells, B cells, natural killer cells, and mast cells, blocks secretion of pro-inflammatory cytokines, terminates inflammatory (Opal & De-Palo, 2000), and enhances the production of anti-inflammatory mediators as IL-1Ra and soluble TNF- α receptors (Asadullah *et al.* 2003) IL-10 is produced mainly from T helper-2 (Th-2) cells, certain B cells subsets, dendritic cells, monocytes and mast cells. IL-10 inhibits synthesis of pro-inflammatory cytokines as IL-1α, IL-1β, IL-6, IL-8, IFN-γ, IL-2, IL-3, TNF-α, GM-CSF, reduces expression of class II major histocompatibility complex (MHC) following activation of monocytes with LPS (Opal, 2000), and suppresses the activity of macrophages, dendritic cells, neutrophils, eosinophils and Th-1 cells (Moore, 2001).

Eriobotrya japonica is a plant that is traditionally thought to have anti-inflammatory activities (Bown, 1995). Many constituents have been isolated from *Eriobotrya japonica* by different extraction methods giving compounds with different biological effects (Matalka el al. 2007; Banno *et al.* 2005; Tommasi *et al.* 1992; Ito *et al.* 2002). Recently, it has been shown that hydrophilic extract of *Eriobotrya japonica* induced the production of proinflammatory cytokines *in vitro* and *in vivo* (Matalka *et al.* 2007). On the other hand, methanolic and ethyl acetate extracts from *Eriobotrya japonica* showed anti-inflammatory effects (Banno *et al.* 2005; Matalka *et al.* 2007; Tommasi *et al.* 1992). The latter indicates that the method of extraction is the key regulator of natural product compounds that may modulate the immune function towards proinflammatory or anti-inflammatory pathways (Matalka *et al.* 2007).

One of the constituents of *Eriobotrya japonica* is epicatechin (EC) which is a member of polyphenols that are more widely known as flavonols or catechins. These latter compounds have been widely used in food, medicine, health products, as antioxidants (Luo *et al.* 1997), anti-inflammatory (Banno *et al.* 2005), anti-cancer (Nakachi *et al.* 2000), and decrease incidence of cardiovascular diseases (Luo *et al.* 1997; Nakachi *et al.* 2000). In addition, previous studies have shown that catechins may suppress production of some pro-inflammatory cytokines in concentration dependent manner (Kim *et al.* 2006). It was reported that catechins suppressed IL-8 production in microvascular endothelial cells (Kim *et al.* 2006), and EC (up to a concentration of 120 μM (35 μg/ml)) inhibited pro-inflammatory IL-1β in lipopolysaccharide (LPS) induced human whole blood (Mitjans *et al.* 2004).

In the present study, we are studying the effect of EC following its extraction, isolation and identification from *Eriobotrya japonica* LINDL (Rosaceae) through its effect on the production of IL-8, IL-6 and IL-10 in whole-blood stimulated with polyclonal activation. The advantage of using whole blood culture system is to provide cellular interactions and to keep natural microenvironment intact (Matalka, 2003a; Matalka, 2003b, Matalka & Ali, 2005). The polyclonal activation system relies on the use of phytohemeagglutinin (PHA) + LPS. Previous studies have shown that LPS do not activate human T cells in vitro (Matalka, 2003a; Elenkov *et al.* 1998) and PHA alone does not activate purified T cells without monocytes (Katzen *et al.* 1985; Gollob *et al.* 1996). In addition, using mixtures of PHA+LPS were found to stimulate both monocytes and T cells by producing higher levels of TNF-α and IL-10, but not IL-12, than LPS alone, and in such system LPS alone did not induce IFN-γ (Matalka, 2003a, Matalka, 2003b, Elenkov *et al.* 1998). In LPS-stimulated whole blood, monocytes/macrophages are the main inducers of IL-12, TNF-α and IL-10 (Matalka, 2003a, Matalka, 2003b, Elenkov *et al.* 1998). In addition, peripheral blood monocytes produced IL-8 more when were cultured with PHA+LPS than LPS alone (Leiber *et al.* 1994), indicating IL-8 is produced from two different pathways in PHA+LPS

Copyright © 2009 Neuroendocrinology Letters ISSN 0172–780X • www.nel.edu

whole blood stimulation (Leiber *et al*. 1994). It is known that LPS activates monocytes through TLR-4 which finally enhances nuclear translocations of transcriptional factors such as NFκB and MAPK (Liu *et al*. 1994). Similarly PHA can activate T cells through receptors mainly CD2/CD58/CD45 (Gollob *et al*. 1996), which finally activates NFκB and MAPK transcriptional factors (Mestas & Hughes, 2001). Therefore, studying the effect of EC on the production of cytokines in the above system give a better indication of what really happens in vivo.

2. MATERIALS AND METHODS

2.1 Plant material

Eriobotrya japonica LINDL (Rosaceae) leaves were collected from Tarek area in Amman-Jordan (06/2000) washed thoroughly with tap water and then dried for one week at room temperature. The plant material was identified in comparison with authentic *Eriobotrya japonica* obtained from the Botanical Institute, University Cologne (Germany). A voucher specimen is deposited at the Herbarium of the institute fur pharmazeutische Biologie, Munster-Germany under PBMS 18.

2.2 Extraction, isolation and identification of epicatechin

Air dried leaves (2 kg) were exhaustively extracted with acetone/water (7:3, 18 L) and the combined extracts evaporated in vacuo to 1.5 L, filtered to remove the precipitated chlorophyll, concentrated and defatted with petroleum benzene 30–50 °C. Successive extractions with ethyl acetate (7.5 L) followed by evaporation of solvent solid yield to 15.5 g ethyl acetate fraction (EAF). Ten grams of the ethyl acetate phase were fractioned on sephadex LH-20 (55 × 900mm) using ethanol (3L), ethanol : methanol 1:1 (6.5 L) and acetone : water 7:3 (2.5 L) to give 12 fractions (fr.). Fr.2 (1140–3720 ml, 117 mg) was subjected to chromatography on MCI-gel CHP 20P (25 × 250 mm) with a 10–80 % methanol linear gradient (17 ml/fr.) to afford epicatechin (EC) (subfr. 48–59, 60 mg).

^{1}H NMR spectra were recorded in $CDCL_3$ on a Varian Mercury 400 plus (400 MHz) relative to $CHCL_3$. ^{13}C-NMR were recorded at 100 150 MHz. CD data were obtained in MeOH on a Jasco J 600. MALDI-TOF mass spectrometer: LAZARUS II (home built), N2-laser (LSI VSL337ND) 337 nm, 3 ns puls width, focus diameter 0.1mm, 16 kV acceleration voltage, 1 m drift length, data logging with LeCroy 9450A, 2.5 ns sampling time and expected mass accuracy +/– 0.1 %.

Analytical TLC was carried out on aluminum sheets (Kieselgel 60 F_{254}, 0.2 mm, Merck) using ethylacetate-water-formic acid (90:5:5). EC was visualized by spraying with vanillin-HCL reagent and 1% ethanolic $FeCL_3$ solution.

2.3 Reagents

The following reagents: RPMI 1640, penicillin-streptomycin, L-glutamine, lipopolysaccharide (LPS, L-6143), phytohemeagglutinin (PHA-L, L-4144), endotoxin-free Dulbecco's phosphate buffer (without calcium and magnesium), hydrocortisone (HC) and bovine serum albumin (BSA) were purchased from Sigma. Plates (6-well culture and maxisorp 96-well flat bottom plates) were purchased from Nunc International, Denmark.

2.4 Subjects

Sixteen healthy volunteers (12 males and 4 females) with a mean age of 23.7 ± 0.9 years old enrolled in this study. Prior to their participation, which is only drawing a single 8 ml of a blood sample, each volunteer read and signed an informed consent. All females were in the early to mid follicular phase (days 3–9) of their menstrual cycle. None of the volunteers have taken any medication for at least a week before withdrawing blood samples, done any exercise, or eaten before the blood sample was drawn. All blood samples were drawn in the morning before 10:00 am.

2.5 Whole blood culture

Blood samples were withdrawn from healthy subjects (males and females) into sterile heparinized tubes (Vacutainer, Becton-Dickinson). To each well of the culture plates, diluted whole blood (1:9 with RPMI 1640, supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), without exogenous serum), was added. Epicatechin (83.3 mg) was dissolved in 50% ethanol, and filtered with 0.2 µm sterile filter under Laminar Flow hood. Different concentrations were prepared in sterilized phosphate buffer and added to each well in 0.2 ml volumes giving a 1:10 final dilution of the blood. The plates were then incubated in 5% CO_2 at 37 °C for 24 hours. After the first incubation, PHA+LPS in 40 µl volume were added to give a final concentration of 5 µg/ml and 1 µg/ml for PHA and LPS, respectively, and plates were further incubated in 5% CO_2 at 37 °C for 24 hours. After the second incubation, the blood was collected from wells into sterile tubes and each well was washed with 0.5 ml of sterile phosphate buffer saline to ensure removal of all content. Tubes were centrifuged and supernatant separated, aliquoted and stored in sterile tubes at –30 °C until assayed.

For NFκB assay and its relation to cytokines production, blood samples were diluted 1:2.8 with RPMI 1640, supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), but without exogenous serum. This dilution was used for yielding enough number of cells for NFκB assay. Epicatechin (24mg) was dissolved in 50% ethanol, filtered by 0.2 µm sterile filter, diluted to different concentrations, 1,10,100 µg/ml and then added to wells in 0.2ml volumes with sterile phosphate buffer, with or without HC (100 nmol/l), giving a 1:3 final dilution of the blood. The incubation

steps as described above were applied. After the second incubation, however, the blood was collected from wells into sterile tubes and each well was washed with 0.5ml of 1mM EDTA to ensure removal of the adherent cells. Tubes were centrifuged and supernatant separated, aliquoted and stored in sterile tubes at –30 °C for cytokine assay. The cell pellet was treated with 1ml cold RBC lysis buffer (1% NH_4CL, 0.125% $KHCO_3$, 5% EDTA) twice for 15min and the supernatant was discarded after centrifugation. The cell pellet was washed with 1ml cold PBS, centrifuged again and the supernatant was discarded. The cell pellet was then re-suspended in 500 µl of cold buffer (20mM Tris-HCL, PH 7.4, 10mM NaCL and 3mM $MgCL_2$) and allowed to swell on ice for 15 min. Cytosolic protein fraction was separated from nuclei by addition of 10% Igebal (25 µl) and centrifuged. The supernatant was separated and stored at –30 °C until assayed. Hypertonic buffer (10mM Tris-HCL, 100mM NaCL, 1mM EDTA, 1mM KF, 2mM Na3VO4, 1% Triton-X-100, 10% glycerol, 0.1% SDS, 0.5% tauro deoxycholic acid) in 50 µl volume was added to extract nuclear protein fraction from the remaining pellet, centrifuged for 30min at 4 °C. The nuclear proteins were separated and stored at –30 °C until assayed.

2.6 Cytokine assays

Measurements of IL-6, IL-8, and IL-10 were accomplished by ELISAs developed using the adapted procedures recommended by the manufacturer (Duoset R & D Systems, UK). Plates were read by SCO GmbH (Dingelstadt, Germany) ELISA plate reader and absorbance values were transformed to cytokine concentrations (pg/ml) using a standard curve computed on Excel. The sensitivities for all cytokine assays were between 4–6 pg/ml.

2.7 NFκB assay

A commercially available ELISA kit for NFKB p65 was purchased from Invitrogen, Carlsbad, California (Immunoassay kit, KHO0371), and the procedure used as the manufacturer's instruction. The sensitivity for NFκB P65 was <50 pg/ml. The measured NFkB concentrations (pg/ml) were transformed to pg/µg protein after measuring the cytoplasmic and nuclear protein concentration by Lowry method.

2.8 Cell viability

To ensure that EC at the concentrations used and ethanol (v/v) that was used to prepare the stock solutions of EC did not induce cell killing following the proper dilutions, cell viability using the trypan blue exclusion method was performed following cell culture (as described above). All percentages of ethanol (0.19% and lower) and EP concentrations (0.1–100 µg/ml) revealed no cytotoxic effect on leukocytes (i.e. the viability was similar to baseline levels) as previously reported (Matalka *et al.* 2007; Crouvezeir *et al.* 2001; Kim *et al.* 2004).

2.9 Statistical analysis

All data in the figures are presented as relative concentration of each cytokine (±SE). The relative concentration is a better indicator of a change upon stimulation by EC or HC at any given concentration (sample) compared to the control condition (own control) [Matalka, 2003a; Matalka 2004b, Matalka & Ali, 2005). Data were evaluated using two methods. First, data for cytokine production (IL-6, IL-8 and IL-10) by whole blood culture treated with different concentration of EC were assessed using ANOVA analysis for multiple comparisons, p value of less than 0.05 was considered significant. Second, paired Student's t-test was done for significant differences between different concentrations of EC when n > 10, whereas Wilcoxon-rank test was performed when n < 10.

3. RESULTS

3.1 Epicatechin does not induce IL- 6, IL- 8 or IL-10 from whole blood cultures

Increasing concentrations of EC without PHA+LPS did not induce IL-6, IL-8 or IL-10 more than the basal levels (18.5 ± 4.2, 36.2 ± 6.8, 48.7 ± 12 pg/ml for IL-6, IL-8, and IL-10, respectively). Moreover, the percentages of ethanol used as a vehicle did not modulate (p>0.05) the production of IL-6, IL-8, and IL-10 levels in PHA+LPS stimulated whole blood.

3.2 Modulation of IL- 6, IL- 8 and IL-10 levels by epicatechin in PHA+LPS stimulated whole blood cultures

Increasing concentrations of EC in 1:10 whole blood dilution suppressed significantly the production of IL-6 in LPS + PHA stimulated whole blood ($p< 0.001$, F (5,60) = 10.9) (Fig. 1a). This suppression was concentration dependent where the level of IL-6 was reduced by 17%, 29% and 56% at concentrations of 1, 10, and 100 µg/ml, respectively ($p= 0.03$, $p<0.001$, $p< 0.001$). However, there was no change in IL-6 level at low concentrations of EC (0.01, 0.1 µg/ml) ($p= 0.27$, $p= 0.31$ respectively) (Fig. 1a).

For IL-8, increasing concentrations of EC in 1:10 whole blood dilution suppressed significantly the production of IL-8 in LPS + PHA stimulated whole blood ($p< 0.001$, F (5, 60) = 4.6) (Fig. 1b). This suppression was concentration dependant where the level of IL-8 was reduced by 15%, 19%, and 26% at concentrations of 1, 10, and 100 µg/ml, respectively ($p= 0.004$, $p< 0.03$, & $p< 0.001$, respectively). However, there was no change in IL-8 level at low concentrations of EC (0.01 and 0.1 µg/ml) (Fig. 1b)

On the other hand, increasing concentrations of EC in 1:10 whole blood dilution modulated significantly the production of IL-10 in LPS + PHA stimulated whole blood (($p< 0.01$), F (5, 60) = 3.23) (Fig. 1c). There was a significant enhancement of IL-10 at a concentration of 0.1, 1, and 10 µg/ml EC ($p= 0.014$, $p= 0.004$, $p= 0.058$,

 Copyright © 2009 Neuroendocrinology Letters ISSN 0172–780X • www.nel.edu

respectively). On the other hand, there was a significant decrease in IL-10 levels at a concentration of 100 μg/ml ($p= 0.037$), and no significant change in IL-10 level at a concentration of 0.01 μg/ml of EC ($p> 0.2$) (Fig 1c).

3.3 *Effect of epicatechin and hydrocortisone on NF-κB activation and cytokines in LPS+PHA stimulated whole blood cultures*

Stimulation with PHA+LPS demonstrated a significant nuclear translocation of NFκB p65 from cytoplasmic fractions (CF) to nuclear fractions (NF) (18.1 pg/μg for NF versus 3.6 pg/μg; $p< 0.01$) (Fig. 2d). When whole blood was pre-incubated with HC (100 nmol/l), a significant decrease (40%) in NFκB p65 level was found in CF containing HC (100 nmol/l) (p = 0.03) (Fig. 2d). In addition, a 24% reduction of NFκB p65 was observed ($p= 0.05$) in NF (Fig. 2d). This slight but significant reduction may be due to the low concentration of HC (100 nmol/l)/mononuclear cells which was used in this culture system (1:3 dilution). These results indicate that HC reduces the production of NFκB p65 in the cytoplasm as well its translocation to the nucleus (Fig. 2d). In addition, the results of the cytokines in HC cultures showed a significant decrease in IL-6 levels (28%; p<0.03), a significant decrease in IL-10 (19%; $p< 0.03$) and no change in IL-8 levels (5%; p>0.05) (Fig. 2a, 2b, 2c). These results indicate that IL-6 production from whole blood stimulated by PHA+LPS is mediated by NFκB p65 and the reduction of NFκB by HC increased the production of IL-10 levels but did not cause a significant change in IL-8 production levels.

In CF, a significant decrease in NFκB p65 level was found in samples containing 1 and 10 μg/ml of EC, but not in 100 μg/ml, when compared with control ($p= 0.03$) (Fig. 2d). The latter modulations were accompanied with a 29%, 67%, and 98% increase, respectively, of NFκB p65 in NF ($p= 0.07$) (Fig. 2d). When NF/CF ratios of NFkB were analyzed, the ratios were significantly higher ($p< 0.03$) in EC 1 and 10 μg/ml cultures than those of control (Fig. 3). Also in the above cultures containing EC 1, 10 and 100 μg/ml, significant reduction (15%, 14% and 28% ; $p< 0.03$) in IL-6 levels were observed whereas significant increase in IL-10 levels (15%, 27% only for EC 1 and 10 μg/ml, respectively) were observed. These results suggest that EC (1, 10, and 100 μg/ml) stimulates NFκB p65 translocation to nucleus in LPS + PHA stimulated whole blood culture but with reduction in IL-6 and increase in IL-10 production levels.

When EC (10 μg/ml) was cultured with HC, IL-8 production levels were more suppressed (5% for EC and HC versus 17% for EC+HC; $p< 0.03$) (Fig. 2b), whereas IL-10 production levels apparently increased (15% for EC, 19% for HC versus 32% for EC+HC) (Fig. 2c). On the other hand, IL-6 production levels did not differ between HC versus EC+HC cultures (Fig. 2a). These changes in IL-8 and IL-10 production were associated with a 23% decrease in NFκB p65 nuclear fraction and a 3 fold decrease in the NF/CF ratio of NFκB from EC 10 μg/ml to EC+HC (Fig 2b, 2c, 2d & Fig. 3). Such reduction indicates the additive immunomodulatory effect of EC to HC.

4. DISCUSSION

Many constituents have been isolated from *Eriobotrya japonica* by different extraction methods giving compounds with different biological effects (Matalka el al. 2007; Banno *et al*. 2005; Tommasi *et al*. 1992; Ito *et al*. 2002). The results of the present study show that EC, which is isolated from ethyl acetate followed by methanol:ethanol fractions, suppresses pro-inflammatory cytokines IL-6, IL-8 and enhances the production of anti-inflammatory cytokine, IL-10. This indicates that ethyl acetate extracts from EC has anti-inflammatory action and coincide with literature with what is known traditionally or experimentally about *Eriobotrya japonica* as anti-inflammatory plant (Matalka *et al*. 2007; Banno *et al*. 2005; Tommasi *et al*. 1992; Ito *et al*. 2002). Furthermore, the data in present study show clearly that the effect of EC on production of cytokine in whole blood cultures is concentration dependent, i.e. increasing EC concentrations decreases the production of IL-6 and IL-8, in PHA + LPS stimulated whole blood. In addition, the present study demonstrates that EC increases production of IL-10 in PHA + LPS stimulated whole blood at 0.01–1 μg/ml of EC. This increase was not seen at high concentration of EC (100 μg/ml). The use of PHA+LPS in the present study revealed different results from the previously published regarding EC effect on cytokines production from LPS-stimulated whole blood or peripheral blood mononuclear cells (Mitjans *et al*. 2004; Crouvezier *et al*. 2001). The difference relies mainly on the effect of PHA on T lymphocytes as well on monocyte-T cell interactions, as discussed above. However, similar effects of EC on IL-8 were seen even though different type of effector cells were tested (Kim *et al*. 2006).

Previous studies on EC have shown its effect on cytokines and NFκB from LPS or IL-1-stimulated monocytes or macrophages or non-immune cells. Some of these latter studies have shown that EC suppressed NFκB nuclear translocation (Kim *et al*. 2004; Terra *et al*. 2007; Ahmed at al. 2004). In the present study, however, the authors used an ex vivo system that mimics in vivo by keeping the blood microenvironment intact and aimed to study the cumulative IL-6 and IL-10 production from both monocytes and T cells and IL-8 from monocytes following polyclonal activation (PHA+LPS). The data from the present study show that EC enhances NFκB nuclear translocation mainly from the activated T cells and/or PHA-activated monocytes. NFκB is transcriptional factor that plays a pivotal role in amplifying inflammatory responses. It is a dimmer protein composed of p50 and p65 and located in the cytoplasm in their in active form associated with inhib-

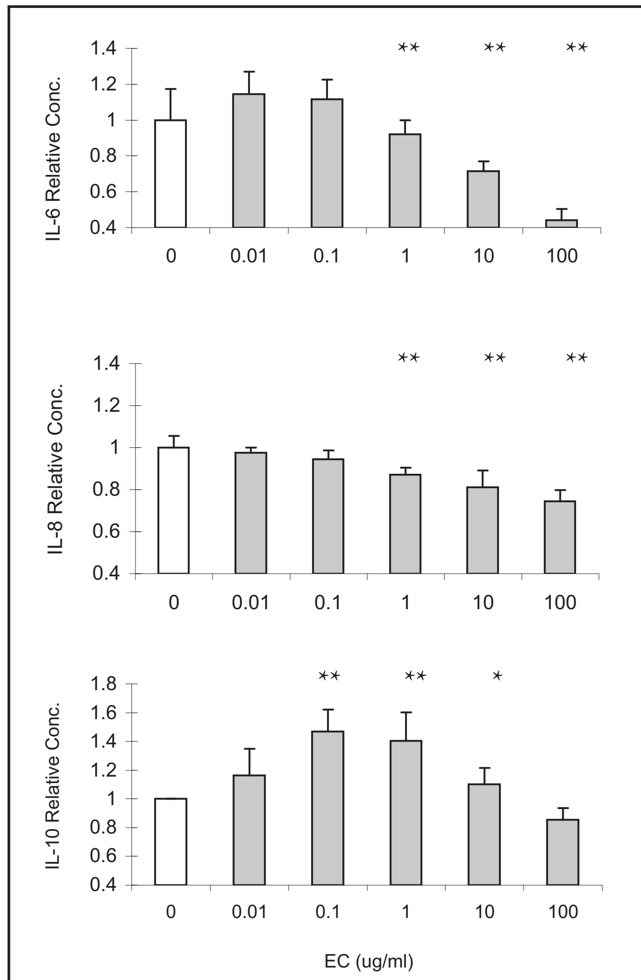

Fig. 1. Effect of EC on cytokines production in PHA + LPS stimulated whole blood diluted 1:10 from 11 normal volunteers (eight males and four females with a mean age of (22.8 ± 0.52 years). At EC concentration of 1, 10 and 100 µg/ml, IL-6 (1a) and IL-8 (1b) production levels decreased significantly (***p* = 0.03, *p*<0.001, *p*<0.001 for IL-6 and p< 0.004, *p*<0.03, *p*<0.001 for IL-8). On the other hand, EC concentration of 0.1, 1 and 10 µg/ml, increased significantly IL-10 (1c) production level (***p* =0.014, *p*=0.004, **p*=0.058). At EC concentration of 100 µg/ml, IL-10 production level decreased significantly (*p* = 0.037). The mean levels of IL-6, IL-8 and IL-10 in PHA + LPS were 2536 ± 410 pg/ml, 12315 ± 689 pg/ml, and 281 ± 53 pg/ml, respectively.

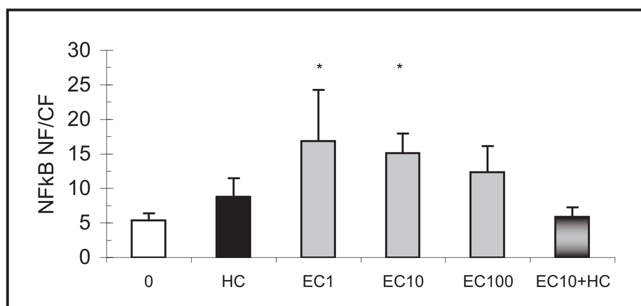

Fig. 3. Effect of EC, HC and EC+HC on NF/CF ratio of NFκB p65 in PHA+LPS stimulated whole blood from 4 normal volunteers (males) with a mean age of (26.3 ± 2.5 years). The highest ratio was observed at EC 1 and 10 µg/ml (**p*<0.03) which indicates a significant translocation of NFκB p65. When EC was cultured with HC, the ratio reduced to control level.

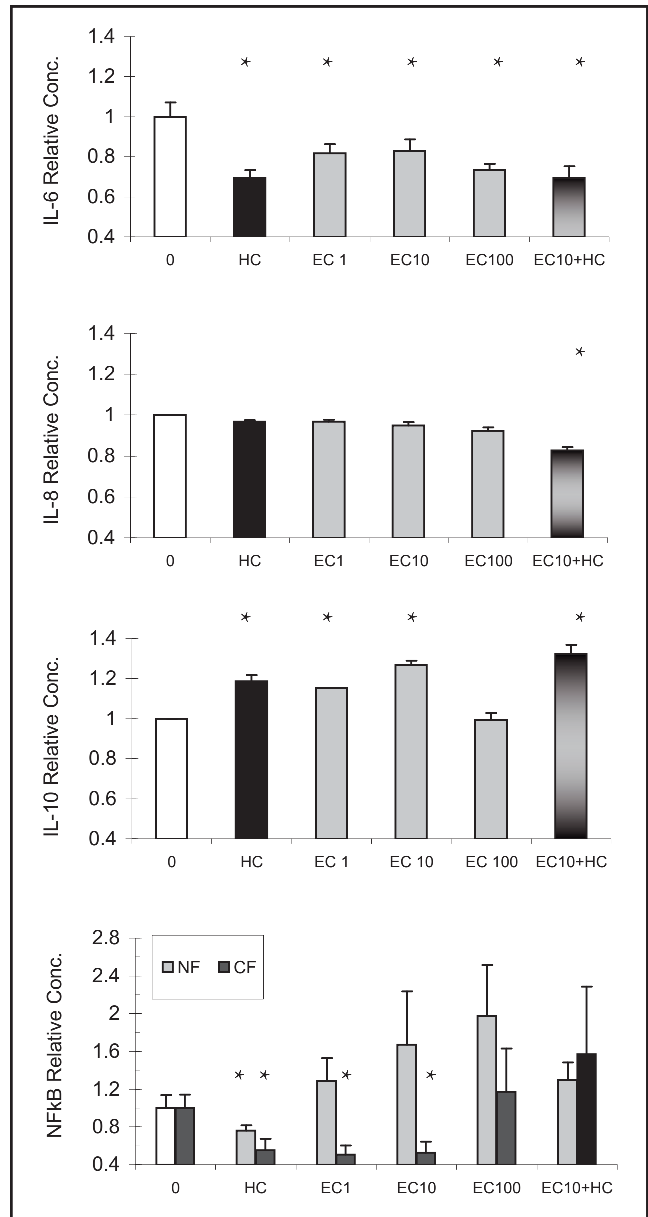

Fig. 2. Effect of EC and HC and EC+HC on cytokines production in PHA + LPS stimulated whole blood and NFκB p65 from 4 normal volunteers (males) with a mean age of (26.3 ± 2.5 years). The whole blood in these set of experiments was diluted 1:3.
Fig **2a** shows IL-6 production: at concentrations of 1, 10, 100 µg/ml of EC, and in cultures of HC with EC (10 µg/ml) or HC alone, IL-6 production levels decreased significantly (**p*< 0.03).
Fig **2b** shows IL-8 production: at 100 µg/ml of EC IL-8 production levels decreased significantly (**p*<0.03).
Fig **2c** shows IL-10 production: at concentrations of 1, 10 µg/ml of EC, and in cultures of HC with or without EC (10 µg/ml), IL-10 production levels increased significantly (*p*< 0.03).
Fig **2d** shows NFκB p65 cytoplasmic and nuclear fractions: at EC concentration of 1, 10 µg/ml, and HC 100 nmol/l NFκB p65 CF levels decreased significantly (**p*< 0.03). In NF, NFκB p65 levels reduced significantly in HC culture (*p*< 0.05), whereas at EC concentration of 100 µg/ml, EC10 µg/ml + HC (100 nmol/l), NFκB p65 levels apparently increased, *p* = 0.07. The mean levels of IL-6, IL-8, IL-8, NFκB p65 CF and NF levels in PHA + LPS were 3621 ± 254 pg/ml, 7208 ± 100 pg/ml, 588 ± 49 pg/ml, 3.6 ± 0.5 pg/µg, and 18.1 ± 2.5 pg/µg, respectively.

Copyright © 2009 Neuroendocrinology Letters ISSN 0172–780X • www.nel.edu

itory protein IκB. When monocytes and T lymphocytes are activated through LPS+PHA as in the present study, a cascade of kinase reactions occur through TLR4 and CD2/CD45/CD58 on monocytes and T lymphocytes, respectively that ultimately phosphrylayte IkB and leads to its proteolysis, freeing the p65 fraction and translocating it inside the nucleus to promote proinflammatory gene transcription. Another mechanism of inducing proinflammatory gene transcription is via involving the activation of MAPK subgroups such as p38 and JNK. The above mixture of mitogens also induces IL-10 transcription through MAPK (Liu *et al*. 1994) or through cAMP-response elements (CRE) (Rhen & Cidlowski, 2005). The latter suggests that EC induces its suppressive effect in the whole blood polyclonal activation system could be through either MAPK or through CRE.

Glucocorticoids induce their anti-inflammatory signaling through inhibiting NFκB and AP1 transcriptional activities (Brenner *et al*. 2003), activating MAPK phosphatase 1 which dephosphorylates and deactivates JNK, and other mechanisms. In the present work, HC (100 nmol/l) caused a significant decrease in NFκB cytoplasmic level and reduced NFκB nuclear translocation as well it suppressed the production of IL-6 and enhanced IL-10 production. When EC was cultured with HC, the induced IL-6 suppression did not change; IL-8 induced suppression became significant; and IL-10 induced production enhanced. The latter findings suggest that EC suppressive effect on IL-8 and IL-6 could be due enhancing MAPK phosphatase 1 and EC enhancement effect on IL-10 production could be through activating CRE. Further studies are needed to confirm the above EC effect.

In the present study, we predicted that EC may have an additive or synergistic effect on anti-inflammatory action of HC. When EC is added with HC, a significant suppression of IL-6 production, and a significant increase in IL-10 production are observed. However, the reduction of IL-6 level in EC (10 μg/ml) + HC (100 nmol/l) cultures is the same as in HC (100 nmol/l) culture, so no additive benefit of adding EC (10 μg/ml) to HC (100 nmol/l) to lower IL-6 production level. On the other hand, a higher increase in IL-10 production is observed when HC (100nmol/l) is cultured with EC (10 μg/ml) compared with HC alone. So an additive action is expected towards production of IL-10 compared with using HC alone. Moreover, HC (100 nmol/l) cultures show the same results of EC (1 μg/ml). So EC (1 μg/ml) could be used as replacer of HC (100 nmol/l) for enhancing IL-10 production level. In a study by Brunetti *et al* (1998), it was reported that IL-10 in combination with dexamethasone completely inhibited proliferation of T cells, but this inhibition was not observed by using IL-10 alone. Therefore, an additive effect could be demonstrated between EC and GC.

In patients with rheumatoid arthritis, it was found that there is an increase in pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α) levels (Paramalingam *et al.* 2007), more than the levels of the anti-inflammatory mediators as IL-10, IL-11, IL-1 receptor antagonist (IL-1 Ra) and soluble TNF or IL-1 receptors which were found in rheumatoid synovium (but in insufficient amounts). Therefore, many clinical studies which are still under investigation aim to decrease IL-6, IL-8 levels with the addition of recombinant IL-10 to treat RA (Asadullah *et al*. 2003). Moreover it has been shown an increase in IL-8 and decrease in IL-10 levels in patients with psoriasis mainly at psoriatic epidermal lesions, dermal fibroblasts, inflammatory cells, keratinocytes and in serum of these patients (Brush-Gerharz, 1996; Jacob *et al*. 2003). Therefore, many clinical studies which are still under investigation aim to decrease IL-6 and IL-8 levels to treat psoriasis [Brush-Gerharz *et al*. 1996; Jacob *et al*. 2003). On the other hand, low levels of IL-10 reduce the control over Th-1 cells activation. Therefore, the results of the present study suggest using EC in combination with usual therapies in treatment of RA and psoriasis. However, more studies must be performed to confirm the effect of EC in therapy of such inflammatory diseases.

The results of the present study open the possibility of using EC as a natural product with potential health benefits. Catechins, in general are well absorbed in humans and previous studies have shown that plasma concentration and AUC is dose dependent (Manach *et al*. 2005). The levels of 1–10 μg/ml (3–30 μM) could be reached following multiple dosing. Therefore, more comprehensive studies regarding bioavailability and anti-inflammatory effects of EC must be performed to characterize its efficacy in treating or help in treating certain autoimmune diseases.

ACKNOWLEDGMENTS

This work is supported by a grant # 4/5/2005 from the Deanship of Scientific Research at Petra University, Amman, Jordan. The authors wish to thank Dr. Mona Hassouneh for her helpful comments.

REFERENCES

1 Ahmed S, Wang N, Lalonde M, Goldberg VM, Haqqi TM (2004). Green tea polyphenol epigallocatechin-3-gallate (EGCG) differentially inhibits interleukin-1-β-induced expression of matrix metalloproteinase-1 and -13 in human chondrocytes. J Pharmacol Exp Therap **308**: 767–773.

2 Asadullah K, Döcke WD, Ebeling M, Friedrich M, Belbe G, Audring H, Volk HD, Sterry W (2003). Interleukin-10 therapy—review of a new approach. Pharmacol Rev **55**: 241–269.

3 Banno N, Askihisa T, Tokuda H, Yasukawa K, Taguchi Y, Akazawa H, Ukiya M, Kimura Y, Suzuki T, Nishino H (2005). Anti-inflammatory and anti-tumor-promoting effects of the triterpene acids from the leaves of Eriobotrya japonica. Biol Pharm Bull **28**: 1995–1999.

4 Boekholdt SM, Peters RJ, Hack CE, Day NE, Luben R, Bingham SA, Wareham NJ, Reitsma PH, Khaw KT (2004). IL-8 plasma concentrations and the risk of future coronary artery disease in apparently healthy men and women: the EPIC-Norfolk prospective population study**.** Arterioscler. Thromb. Vasc. Biol. **24**: 1503–1508.

5 Bown, D (1995)**.** Encyclopaedia of Herbs and their Uses. Dorling Kindersley, London. ISBN0-7513-020-31.
6 Brenner S, Prosch S, Schenke-Laylan K, Riese U, Gausmann U, Platzer C (2003). cAMP-induced interleukin-10 promoter activation depends on CCAAT/enhancer-binding protein expression and monocytic differentiation. J Biol Chem **278**: 5597–5604.
7 Bruch-Gerharz D, Fehsel K, Suschek C, Michel G, Ruzicka T, Kolb-Bachofen V (1996). A proinflammatory activity of interleukin 8 in human skin: expression of the inducible nitric oxide synthase in psoriatic lesions and cultured keratinocytes. J Exp Medicine **184**: 2007–2012.
8 Brunetti M, Colasante A, Mascetra, N, Piantelli M, Musiani P, Aiello FB (1998). IL-10 synergizes with dexamethasone in inhibiting human T cell proliferation. J Pharmacol Exp Ther **285**: 915–919.
9 Crouvezier S, Powell B, Van de Water J, Keen CL, Schmitz HH, Gershwin ME (2001). The effects of phenolic components of tea on the production of pro-and anti-inflammatory cytokines by human leukocytes in vitro. Cytokine **13**: 280–286.
10 Elenkov IJ, Chrousos GP (1999). Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. Trends Endocrinol Metab **10**: 359–368.
11 Elenkov IJ, Webster E, Papanicolaou DA, Fleisher TA, Chrousos GP, Wilder RL (1998). Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. J Immunol **161**: 2586–2593.
12 Gollob JA, LI J, Kawasaki H, Daley JF, Groves C, Reinherz EL, Ritz J (1996). Molecular interaction between CD58 and CD2 counter-receptors mediates the ability of monocytes to augment T cell activation by IL-12. J Immunol **157**: 1886–1893.
13 Heikkilä K, Ebrahim S, Lawlor DA (2008). Systematic review of the association between circulating interleukin-6 (IL-6) and cancer. Eur J Cancer **44**: 937– 945.
14 Ito H, Kobayashi E, Li SH, Hatano T, Sugita D, Kubo N, Shimura S, Itoh Y, Tokuda H, Nishino H, Yoshida T (2002). Antitumor activity of compounds isolated from leaves of *Eriobotrya japonica.* J Agr Food Chem **50**: 2400–2403.
15 Jacobi HH, Poulsen LK, Reimert CM, Skov PS, Ulfgren AK, Jones I, Elfman LB, Malling HJ, Mygind N (1998). IL-8 and the activation of eosinophils and neutrophils following nasal allergen challenge. Int Arch Aller Immunol **116**: 53–59.
16 Jacob SE, Nassiri M, Kerdel FA, Vincek V (2003). Simultaneous measurement of multiple Th1 and Th2 serum cytokines in psoriasis and correlation with disease severity. Mediators Inflamm **12**: 309–313.
17 Katzen D, Chu E, Terhost C, Leung DY, Gesner M, Miller RA, Geha RS (1985). Mechanisms of human T cell response to mitogens: IL-2 induces IL-2 receptor expression and proliferation but not IL-2 synthesis in PHA-stimulated T cells. J Immunol **135**: 1840–1845.
18 Kim IB, Kim DY, Lee SJ, Sun MJ, Lee MS, Li H, Cho JJ, Park CS (2006). Inhibition of IL-8 production by green tea polyphenols in human nasal fibroblasts and A549 epithelial cells. Biol Bull **29**: 1120–1125.
19 Kim M-J, Ryu GR, Kang J-H, Sim SS, Min DS, Rhie D-J, Yoon SH, Hahn SJ, Jeong I-K, Hong K-J, Kim M-S, Jo Y-H (2004). Inhibitory effects of epicatechin on interleukin-1β-induced inducible nitric oxide synthase expression in RINm5F cells and rat pancreatic islets by down-regulation of NFκB activation. Biochem Pharmacol **68**: 1775–1785.
20 Leiber JM, Kunkel SL, Burdick MD, Standiford TJ, Rolfe MW, Strieter RM (1994). Production of IL-8 and monocytes chemotactic peptide-1 by peripheral blood monocytes. Disparate responses to phytohemaagglutinin and lipopolysaccharide. J Immunol **152**: 241–249.
21 Liu MK, Herrera-Velit P, Brownsey RW, Reiner NE (1994). CD14-dependent activation of protein kinase C and mitogen-activated kinases (p42 and p44) in human monocytes treated with bacterial lipopolysaccharide. J Immunol **153**: 2642–2652.
22 Luo M, Kannar K, Wahlqvist ML, O'Brien RC (1997). Inhibition of LDL oxidation by green tea extract. Lancet **349**: 360–361.
23 Manach C, Williamson G, Morand C, Scalbert A, Remesy C (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailable studies. Am J Clin Nutr **81**(suppl): 230S–242S.
24 Matalka KZ (2003a). Prolactin enhances the production of interferon-γ, interleukin-12, interleukin-10, but not of tumor necrosis factor-α, in a stimulus specific manner. Cytokine **21**: 187–194.
25 Matalka K (2003b). The effect of estradiol, but not progesterone, on the production of cytokines in stimulated whole blood, is concentration-dependent. Neuroendocrinol Lett **24**: 185–191.
26 Matalka KZ, Ali D (2005). Stress induced versus preovulatory and pregnancy hormonal levels in modulating cytokine production following whole blood stimulation. Neuroimmunemodulation **12**: 366–374.
27 Matalka KZ, Ali D, El Khawad A, Qa'dan F (2007). The differential effect of *Eriobotrya japonica* hydrophilic leaf extract on cytokines production and modulation**.** Cytokine **40**: 235–240.
28 Mestas J, Hughes CCW (2001). Endothelial cell costimulation of T cell activation through CD58-CD2 interaction involves lipid raft aggregation. J Immunol **167**: 4378–4385.
29 Mitjans M, Martínez V, Campo J, Abajo C, Lozano C, Lluís Torres J, Vinardell MP (2004). Novel epicatechin derivatives with antioxidant activity modulate interleukin-1β release in lipopolysaccharide-stimulated human blood. Bioorganic Med Chem Lett **14**: 5031–5034.
30 Moore KW, Malefyt WR, Coffman RL, O'Garra A (2001). Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol **19**: 683–765.
31 Nakachi K, Matsuyama S, Miyake S, Suganuma M, Imai K (2000). Preventive effects of drinking green tea on cancer and cardiovascular disease: Epidemiological evidence for multiple targeting prevention. BioFactors **13**: 49–54.
32 Omoigui S (2007). The Interleukin-6 inflammation pathway from cholesterol to aging role of statins, bisphosphonates and plant polyphenols in aging and age-related diseases. Immun & Ageing **4**: 1–22.
33 Opal SM, De-Palo VA (2000). Anti-inflammatory cytokines. Chest **117**: 1162–1172.
34 Paramalingam SS, Thumboo J, Vasoo S, Thio ST, Tse C, Fong KY (2007). *In vivo* pro- and anti-inflammatory cytokines in normal and patients with rheumatoid arthritis. Annals Acad Med **36**: 96–99.
35 Rhen T, Cidlowski JA (2005). Antiinflammatory action of glucocorticoids-New mechanisms for old drugs. N Engl J Med **353**: 1711–1723.
36 Terra X, Valles J, Vitrac X, Merrillon JM, Arola L, Ardevol A, Blade C, Fernandez-Larrea J, Pujadas G, Salvado J, Blay M (2007). Grape-seed procyanidins act as anti-inflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. J Agric Food Chem **55**: 4357–4365.
37 Tommasi N, Simone F, Pizza C, Mahmood N, Moore P, Conti C, Orsi N, Stein M (1992). Constituents of *Eriobotrya japonica*. A study of their antiviral properties. J Nat Prod **55**: 1067–1073.
38 Tzianabos AO (2000). Polysaccharides immunomodulators as therapeutic agents: structural aspects and biologic function. Clin Microbiol Rev **13**: 523–533.
39 Yu J, Ren X, Cao S, Zhang W, Hao X (2006). Th1 polarization and apoptosis-inducing activity of CD4+ T-cells in cytokine-induced killers might favor the antitumor cytotoxicity of cytokine-induced killers in vivo. Cancer Biother Radiopharm **21**: 276–284.

Copyright © 2009 Neuroendocrinology Letters ISSN 0172–780X • **www.nel.edu**