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## The differential effect of *Eriobotrya japonica* hydrophilic leaf extract on cytokines production and modulation

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### Abstract

Stimulating or modulating the release of cytokines by immunomodulators or immunostimulating agents is an attractive mode for treating several diseases such as viral infections. For instance, patients with viral infections may be in need of increasing or inducing T helper 1 (Th1) or proinflammatory cytokines, which ultimately activate T cytotoxic and Natural killer lymphocytes to kill virally infected cells. Of these agents, we found that *Eriobotrya japonica* hydrophilic leaf extract (EJHE) can induce and modulate cytokines in dose-dependent manner. Twenty-four hour exposure of increasing concentrations of EJHE increased significantly ( $p < 0.001$ ) the production of IFN- $\gamma$  and TNF- $\alpha$ , from PHA+LPS-stimulated whole blood. However, the production of IFN- $\gamma$  and TNF- $\alpha$  plateaued at high EJHE concentrations (10–100  $\mu\text{g/ml}$ ). No significant changes in the production of IL-10 were seen. In addition, EJHE at 1 and 10  $\mu\text{g/ml}$  reversed significantly ( $p < 0.01$ ) the inhibitory effect of hydrocortisone on the IL-12 p70, IFN- $\gamma$  and TNF- $\alpha$  production from PHAS+LPS stimulated whole blood. Without PHA and LPS, EJHE was found to induce significantly ( $p < 0.001$ ) IFN- $\gamma$ , IL-12 p70, TNF- $\alpha$ , and IL-10 from whole blood culture in concentration dependent manner. The maximum induction of IFN- $\gamma$ , IL-12 p70, and TNF- $\alpha$  by EJHE was at 1 and 10  $\mu\text{g/ml}$ . On the other hand, IL-10 induction kept increasing even at the highest concentration used (100  $\mu\text{g/ml}$ ) of EJHE. Furthermore, intra-peritoneal injection of EJHE into mice increased significantly serum cytokines level mainly at 10 and 100  $\mu\text{g/ml}$ . Two-hour post i.p. injection, EJHE increased serum IFN- $\gamma$ , TNF- $\alpha$ , and IL-10 to  $\sim 750$ , 1000, and 250  $\text{pg/ml}$ , respectively. However, 24 h post i.p. injection, the levels of TNF- $\alpha$ , and IL-10 were similar to basal levels but IFN- $\gamma$  levels were 200  $\text{pg/ml}$ . These results indicate that EJHE induces proinflammatory and Th1 cytokines in concentration dependent manner and the effect of this induction should be studied further in viral models to check the efficacy of such cytokine induction.

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**Keywords:** *Eriobotrya japonica*; IL-12; IFN- $\gamma$ ; TNF- $\alpha$ ; IL-10

### 1. Introduction

Cytokines are the main regulators of the immune response. They induce the balance between inflammatory versus regulatory or antibody mediated reactions. For instance, interleukin (IL)-12, produced by activated monocytes/macrophages or other antigen presenting cells, is a major inducer of T helper (Th) 1 cytokines. Th1 cytokines such as IFN- $\gamma$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) plus TNF- $\alpha$  from monocytes/macrophages, stimulate the func-

tion of T cytotoxic cells, natural killer (NK) lymphocytes, and activated macrophages. The latter cytokines are considered proinflammatory cytokines because they stimulate the synthesis of nitric oxide and acute phase proteins, attract inflammatory cells, and upregulate the synthesis of secondary mediators and proinflammatory cytokines [1,2]. On the other hand, the anti-inflammatory cytokine, IL-10, derived from Th2 lymphocytes and monocytes, promotes humoral immunity and suppresses monocyte/macrophage and Th1 lymphocyte activation by inhibiting IL-12, TNF- $\alpha$  and IFN- $\gamma$  production [1,2].

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,

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allergy, autoimmune mediated diseases and cancer [3–7]. For instance, patients with viral infections or cancer may be in need of increasing or inducing Th1 or proinflammatory cytokines, which ultimately activate T cytotoxic, NK cells and macrophages to kill virally infected cells and tumor cells, and the enhancement of the host immune system would increase the ability to combat infection and thus reduce the problems of antibiotic resistance [3,5,7]. Furthermore, autoimmune diseases can be classified into proinflammatory (Th1) (e.g. rheumatoid arthritis) or antibody (Th2) (e.g. systemic lupus erythematosus) mediated diseases. In the latter cases, immunomodulating the immune cells and shifting the cytokine pattern would help in the treatment of the disease [3,4,6].

A good source of such agents is natural products. We started searching for such agents using hydrophilic extracts because these extracts contain polysaccharides, polar phenolic compounds that may induce and modulate cytokine productions [8–10]. Our preliminary data demonstrated that the hydrophilic extract from the leaves of *Eriobotrya japonica* (EJ) LINDL (Rosaceae) has immunostimulatory effects. We expanded our work on *E. japonica* hydrophilic extract (EJHE) and found it can induce and modulate cytokines in dose-dependent manner. We have tested the EJHE in (a) modulating the cytokine pattern towards proinflammatory or immunoregulatory cytokines in vitro from mitogen-stimulated human whole blood (b) inducing cytokines in vitro from peripheral blood of healthy volunteers, and (c) increasing different patterns of mouse serum cytokines following intra-peritoneal administration. The data demonstrated a valuable immunostimulatory/immunomodulatory agent that should be further studied in diseased models.

## 2. Materials and methods

### 2.1. Plant material

*Eriobotrya japonica* LINDL (Rosaceae) was collected from Marj Al Hamam area in Amman-Jordan (Jordan; 01/2002), washed thoroughly with tap water and then dried for one week at room temperature. The plant material was identified in comparison with authentic *E. japonica* obtained from the Herbarium of the Institut für pharmazeutische Biologie, Münster-Germany under PBMS78.

### 2.2. Extraction of the plant material

Powdered air-dried material (2 kg) was exhaustively extracted with boiled water (28 L). Briefly, boiled water was added to the air-dried material for five minutes followed by filtration. This step was repeated three times. Then, the combined extracts evaporated *in vacuo* to 2 L, concentrated and freeze dried to obtain 112 g of the *E. japonica* hydrophilic leaf extract (EJHE). Before use of EJHE in cell culture or in animals, EJHE was dissolved in endotoxin-free PBS, sterilized through filtration with

0.2 µm sterile filters and used fresh for each experiment. It has been noted that bacterial endotoxin levels from processed plants are variable but those plants that were cleaned have very low levels of endotoxins [11].

### 2.3. Reagents

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

### 2.4. Subjects

Twenty healthy volunteers (10 males and 10 females) with a mean age of 28.1 (±3.2) years old enrolled and signed an informed consent prior to their participation in the study. All females participated in the study 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, did any exercise, or ate before the blood sample was drawn. All blood samples were drawn in the morning between 8 and 9:30 a.m.

### 2.5. Human whole blood culture

Blood samples were drawn from twenty healthy volunteers into sterilized sodium heparin tubes (Vacutainer, Becton–Dickinson) and processed within 45 min. The blood was diluted with 1:10 with RPMI 1640, supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, without exogenous serum. To each well of the 6-well culture plates, 2.0 ml of the diluted blood was added [6,12,13]. To study cytokines induction, 0.001, 0.01, 0.1, 1, 10 or 100 µg of EJHE/ml was added to each well, followed by 48 h incubation at 37 °C and 5% CO<sub>2</sub>. To study cytokine modulation, 0.001, 0.01, 0.1, 1, 10 or 100 µg of EJHE/ml was added to each well, incubated for 24 h, followed by adding a mixture of PHA+LPS in 40 µl volume to give a final concentrations of 5 and 1 µg/ml for PHA and LPS, respectively, and incubated in 5% CO<sub>2</sub> at 37 °C for another 24 h. At the end of incubation, blood was collected from wells into sterilized tubes and each well was washed with 0.5 ml of RPMI to ensure removal of all well content. The supernatants were aliquoted and stored in sterilized tubes at –30 °C until assayed.

### 2.6. Mice

Forty three Balb/c mice weighing 20–30 g were used in the study and divided into groups. Mice were injected i.p. with 1 ml of endotoxin-free PBS alone, containing selected concentration of EJHE or with 5 µg/ml of LPS. Mice were

sacrificed either at 2 or 24 h later and blood was collected, left to clot and then centrifuged. Serum samples were kept frozen at  $-30^{\circ}\text{C}$  until date of analysis.

### 2.7. Cytokine analysis

Human cytokines (IL-12, IFN- $\gamma$ , IL-10, TNF- $\alpha$ ) and mouse cytokines (TNF- $\alpha$ , IL-10 and IFN- $\gamma$ ) were assayed using ELISAs adapting the procedures recommended by the manufacturer (mouse and human DuoSet, respectively, R&D Systems, UK). Briefly, captured antibodies for all cytokines were coated at  $4\ \mu\text{g/ml}$  in PBS pH 7.2–7.4, and anti-cytokine-biotinylated detector antibodies for IL-12 p70, IFN-g and TNF-a were used at  $175\ \mu\text{g/ml}$  and  $600\ \text{ng/ml}$  for IL-10. Standards for all assays were used in the range of 15.6–1000 pg/ml, except for IL-12 p70 (7.8–500 pg/ml) with 7 points of standard curve and the zero standard. Streptavidin–Horseradish peroxidase conjugate with  $\text{H}_2\text{O}_2$ –Tetramethylbenzidine (R&D, UK) substrate was used. Plates were read by SCO GmbH (Dingelstadt, Germany) ELISA plate reader and absorbance was transformed to cytokine concentrations (pg/ml) using a standard curve computed on Excel developed by the author. The sensitivities for all were between 4 and 6 pg/ml.

### 2.8. Statistical analysis

All data in the figures are presented as the mean concentration of each cytokine ( $\pm\text{SE}$ ). Comparisons between concentrations of EJHE were analyzed by one way ANOVA. Paired *t*-test was used to compare between two conditions (specific concentration with its control) when  $n > 10$ , whereas Wilcoxon-rank test was performed when  $n < 10$ .

## 3. Results

### 3.1. EJHE modulates cytokines production from Phytohemagglutinin (PHA)+Lipopolysaccharide (LPS) stimulated whole blood

Twenty-four hour exposure of increasing concentrations of EJHE enhanced significantly ( $p < 0.001$ ) the production of IFN- $\gamma$  and TNF- $\alpha$  more than PHA+LPS stimulated whole blood (Fig. 1). In addition, there was an apparent increase ( $p = 0.057$ ) in IL-12 p70 production at  $1\ \mu\text{g/ml}$  of EJHE. However, this apparent increase was not seen at high EJHE concentrations (10–100  $\mu\text{g/ml}$ ). IFN- $\gamma$  and TNF- $\alpha$  levels plateaued at higher concentrations too (10 and 100  $\mu\text{g/ml}$ ) but still were significantly higher ( $p < 0.001$ ) than PHA+LPS levels. No significant ( $p = 0.58$ ) changes in the production of IL-10 levels were seen.

### 3.2. EJHE reverses cortisol suppressive effect of cytokines production from PHA+LPS stimulated whole blood

We have shown earlier that increasing concentrations of hydrocortisone suppressed significantly the production of

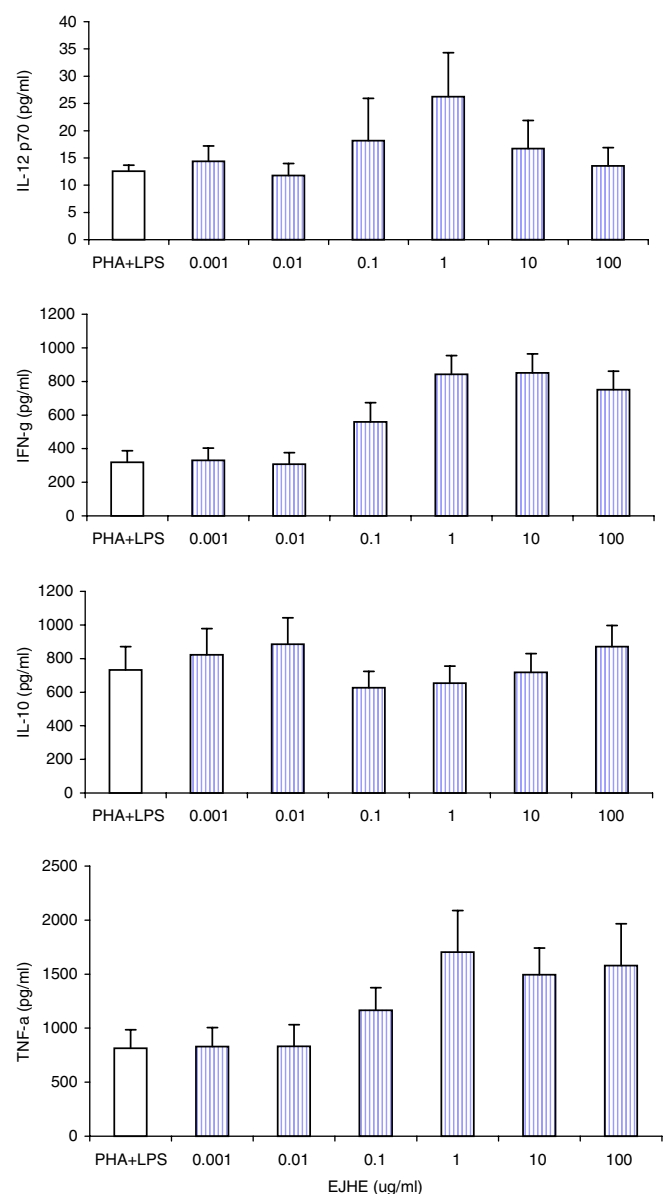


Fig. 1. The effect of EJHE on modulating cytokines production following PHA+LPS stimulation from 15 healthy volunteers is presented. EJHE increased significantly ( $p < 0.001$ ) the production of IFN- $\gamma$  and TNF- $\alpha$ . There was an apparent increase ( $p = 0.057$ ) of IL-12p70 at  $1\ \mu\text{g/ml}$  of EJHE. No significant changes were noticed in IL-10 levels.

IFN- $\gamma$ , IL-12, IL-10, TNF- $\alpha$  in PHA+LPS-stimulated whole blood [6]. In order to ensure the enhancement of EJHE on cytokines production could reverse hydrocortisone effect, hydrocortisone (100 nmol/l) was added simultaneously with 0.1, 1 or 10  $\mu\text{g/ml}$  EJHE for 24 h and then a mixture of PHA+LPS was added, left for another 24 h and the wells content was centrifuged and kept frozen until analysis. The results showed that at 1 and 10  $\mu\text{g/ml}$  of EJHE not only reversed significantly ( $p < 0.01$ ) the suppression of IL-12 p70, IFN- $\gamma$  and TNF- $\alpha$  by hydrocortisone but also increased significantly ( $p < 0.01$ ) the production of the cytokines more than the PHA+LPS stimulated blood (Fig. 2). For IL-10, no reversal effect was noticed at any EJHE concentration used.

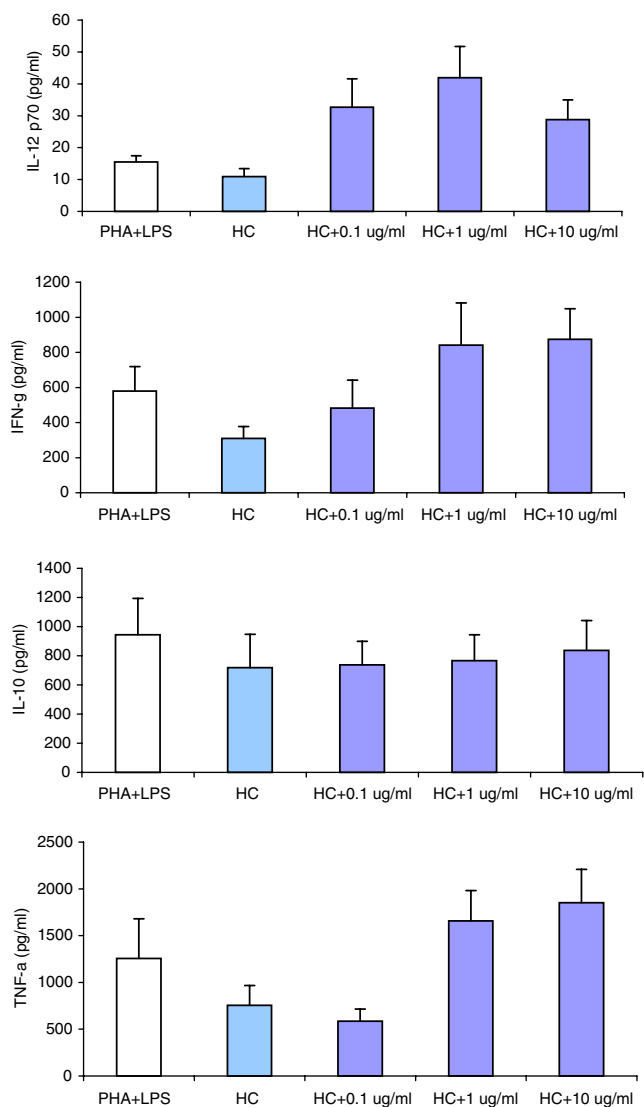


Fig. 2. EJHE reverses hydrocortisone suppressive effect on cytokines production in PHA+LPS-stimulated whole blood from five healthy volunteers (see text for more details). At 1 and 10  $\mu\text{g/ml}$  of EJHE the levels of IL-12 p70, IFN- $\gamma$  and TNF- $\alpha$  were significantly higher ( $p < 0.01$ ) than hydrocortisone suppressive levels and PHA+LPS-stimulated whole blood.

### 3.3. EJHE alone induced the production of IFN- $\gamma$ , IL-12p70, TNF- $\alpha$ and IL-10 in concentration dependent manner

To test if EJHE can induce cytokines production in humans, without PHA and LPS, it was cultured with whole blood drawn from healthy volunteers. Increasing concentrations of EJHE induced significantly ( $p < 0.001$ ) IFN- $\gamma$ , IL-12 p70, TNF- $\alpha$ , and IL-10 productions, however, in concentration dependent manner. The maximum induction of IFN- $\gamma$ , IL-12 p70, and TNF- $\alpha$  by EJHE was at 1 and 10  $\mu\text{g/ml}$  and this induction, however, declined at 100  $\mu\text{g/ml}$  (Fig. 3). IL-10 induction, on the other hand, kept increasing significantly even at the highest concentration used 100  $\mu\text{g/ml}$  of EJHE (Fig. 3).

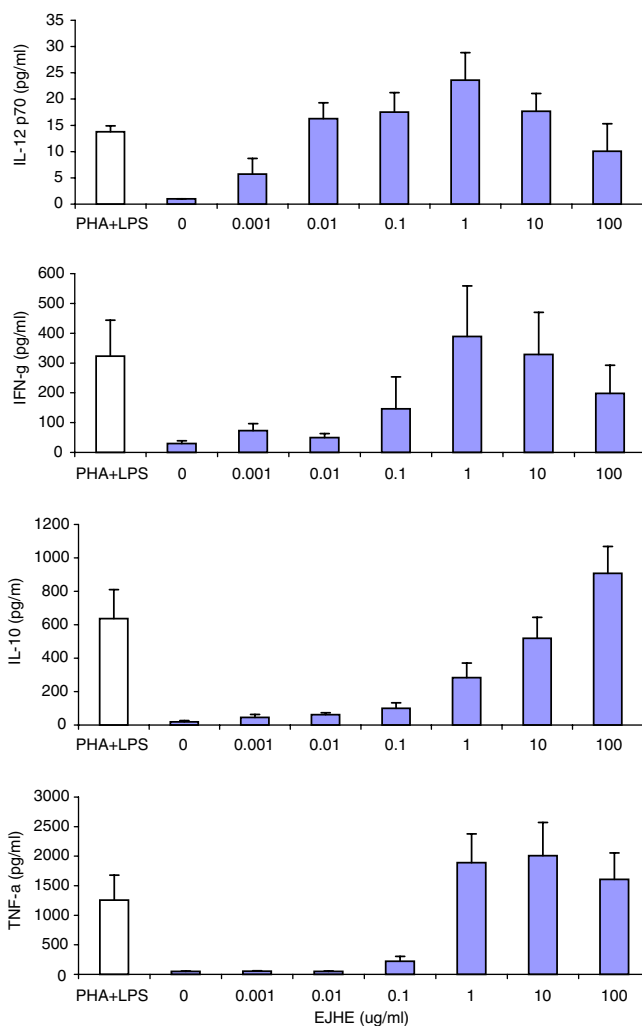


Fig. 3. EJHE induces cytokines production in vitro. This induction was significant ( $p < 0.001$ ). The data is presented from eleven for IFN- $\gamma$ , IL-10 and five healthy volunteers for IL-12p70, TNF- $\alpha$ .

### 3.4. Fractionated extracts from EJHE and cytokines production

The EJHE was further extracted with ethyl acetate–water (EJHE-EA) and butanol–water (EJHE-BU). The non-extracted material was referred to as water residue (EJHE-WR). The EJHE-EA did not induce IFN- $\gamma$  but induced a significant ( $p < 0.05$ ) amount (20%) of IL-10 when compared (wt/wt) to EJHE. EJHE-BU, however, neither induced IFN- $\gamma$  nor IL-10. On the other hand, the EJHE-WR induced IFN- $\gamma$  and IL-10 significantly ( $p < 0.01$ ) more than EJHE by 40 and 80%, respectively (data not shown).

### 3.5. EJHE induced the production of IFN- $\gamma$ , TNF- $\alpha$ and IL-10 in vivo

Intra-peritoneal injection of EJHE (1–1000  $\mu\text{g/ml}$ ) into mice ( $n = 4/\text{group/time}$ ) increased serum cytokines level. Two hours post i.p. injection of 10 or 100  $\mu\text{g/ml}$  EJHE

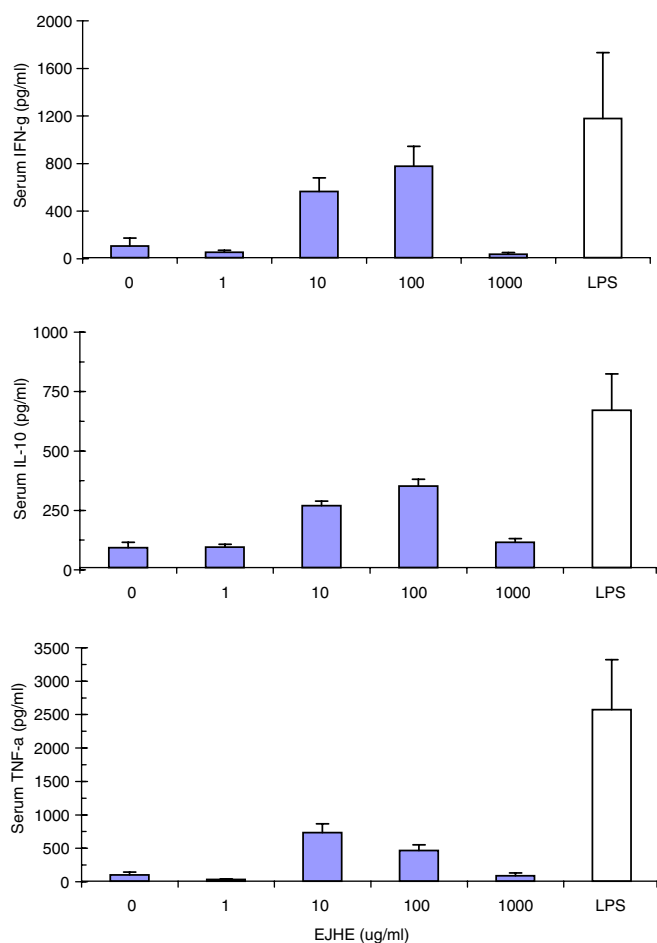


Fig. 4. EJHE induces cytokines in vivo. Two hours-post i.p. injection, EJHE increased significantly ( $p < 0.001$ ) serum levels of IFN- $\gamma$ , IL-10 and TNF- $\alpha$  (4 mice/time-point). However, levels of cytokines induced by 1 and 1000  $\mu\text{g/ml}$  of EJHE were significantly ( $p < 0.01$ ) less than those induced by 5  $\mu\text{g/ml}$  of LPS and were similar to PBS levels.

increased significantly ( $p < 0.001$ ) serum IFN- $\gamma$ , TNF- $\alpha$ , and IL-10 (Fig. 4). However, the levels of cytokines induced by 1 or 1000  $\mu\text{g/ml}$  of EJHE were significantly ( $p < 0.01$ ) lower than those induced by 5  $\mu\text{g/ml}$  of LPS and were similar to PBS levels. These results indicate that EJHE induces cytokines in dose dependent manner. Furthermore, 24 h post i.p. injection of EJHE, the levels of TNF- $\alpha$ , and IL-10 were similar to basal levels but IFN- $\gamma$  levels were  $\sim 200$  pg/ml.

#### 4. Discussion

The leaves of loquat, *E. japonica* has been found to contain triterpenes, sesquiterpenes, flavonoids, tannins and megastigmane glycosides and shown to have biological activities such as anti-inflammatory and anti-viral activities [14–16]. However, all of the above compounds were isolated from either methanol or acetone–water extraction. In the present work, we used water-extract because these extracts contain polysaccharides and polar phenolic compounds such as flavonoid glycosides, and other molecules

that may induce and modulate cytokine productions [8–10]. The present work demonstrates that EJHE induces proinflammatory cytokines, IL-12, IFN- $\gamma$ , TNF- $\alpha$  in concentration dependent manner i.e. at high concentrations the induction of proinflammatory cytokines was reduced. This reduction could be partially attributed to the increase in anti-inflammatory (regulatory cytokine) IL-10. In addition, 2 h after an i.p. administration of EJHE in mice increased significantly serum proinflammatory cytokines IFN- $\gamma$ , TNF- $\alpha$  and to a lesser extent IL-10. This increase was also observed only with IFN- $\gamma$  after 24 h of administration.

The present work also showed that 24 h exposure of hydrocortisone to immune cells suppressed the production of all cytokines with different degrees. This was clearly seen by the significant decrease in the IFN- $\gamma$ /IL-10 ratio. This latter decrease in IFN- $\gamma$ /IL-10 ratio was reversed by glucocorticoid receptor antagonists, prolactin or estradiol [6,12,17,18]. The data in the present study demonstrated a similar action of EJHE when was cultured with hydrocortisone, where EJHE restored cortisol-induced proinflammatory cytokines (IL-12, IFN- $\gamma$  and TNF- $\alpha$ ) suppression. Glucocorticoids inhibit NF- $\kappa\text{B}$  and AP-1 families of transcription and thus suppress the production of multiple cytokines [19]. In addition, glucocorticoids interact with the JAK-STAT signaling pathway which could have a positive or a negative impact on cytokine signaling [19–21]. For instance, Stoecklin et al. [20] have shown that glucocorticoid enhances JAK/STAT activation by hormones and that involves glucocorticoid receptor associating with and acting as a coactivator of STAT-5.

Although, LPS has been recognized to stimulate monocytes (without activating T cells) by producing IL-12, IL-10 and TNF- $\alpha$  following in vitro stimulation of human blood [6,12], it was found to stimulate T cells too following in vivo administration [17,22,23]. LPS activates monocytes via the expressed CD14 or Toll-like receptor 4 (TLR4) [24]. This involves the activation of NF- $\kappa\text{B}$ , NF- $\kappa\text{B}$  Map kinases and IRF-3. We propose that the contents of EJHE extract activate peripheral blood mononuclear cells via the family of TLRs which is a pattern of recognition receptors and involves the activation of NF- $\kappa\text{B}$  and Map kinases. The latter activation could be the reason of restoring cortisol-induced proinflammatory cytokines (IL-12, IFN- $\gamma$  and TNF- $\alpha$ ) suppression. Further studies are needed to confirm such binding and to explain the inhibitory effect of high concentrations of EJHE on IL-12, IFN- $\gamma$  and TNF- $\alpha$  production but not on IL-10 production.

In an inflammatory mouse model induced by 12-*O*-tetra decanoylphorbol-13-acetate, it was found that methanol extracts and ethyl acetate fractionation of *E. japonica* can inhibit such inflammation by  $>80\%$ . On the contrary, water or butanol fractions did not modulate such inflammation [14]. The results from the present study confirm the above observations by showing that EJHE induces proinflammatory cytokines, whereas ethyl acetate fraction induced anti-

inflammatory cytokine, IL-10, and butanol fraction did not induce any cytokines.

The differential effect of EJHE on cytokines induction i.e. favoring proinflammatory cytokines, opens several ideas for applications. It has been shown that deficiencies in IL-12 and Th1 cytokines do occur in patients with cancer and mainly at the tumor site. These low levels of such cytokines have been associated with tumor progression [25]. Therefore, polarizing towards Th1 cytokines from macrophages and dendritic cells at the cancer site basically by producing IL-12 may reduce tumor progression [3,25–27]. The present EHJE was found to induce IL-12 from blood monocytes, however, this has to be shown in a tumor model if it can reduce tumor progression. Similarly, therapies of viruses especially those that cause chronic type of diseases, such as hepatitis C (HCV) viruses, are not satisfactory [28]. The current therapies for HCV are based on interferon- $\alpha$  administration. Results of such therapy did not eradicate the viruses completely from the liver regardless of getting zero values of RNA copies for HCV from the polymerase chain reaction in the serum of the treated individuals [29,30]. So inducing proinflammatory cytokines in the liver of infected patients may help in eradicating such viruses when combined with the usual therapies. Finally, the use of immunomodulators, such as EHJE, may provide certain treatment advantages for the above diseases, however, extensive work are in need to evaluate such new tool.

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