Food Sci. Biotechnol. Vol. 18, No. 6, pp. 1316 ~ 1321 (2009)

Food Science and Biotechnology

Immunomodulatory Effects of a Methanol Extract from *Opuntia ficus indica* on Murine Splenocytes

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Abstract Multiple beneficial properties of *Opuntia ficus indica* (OPF) are well established. In the present study, we have investigated the immunological role of OPF extract (OPFE) on murine splenocytes. OPFE dose- and time-dependently enhanced the proliferation of splenocytes without cytotoxicity. Our results also showed that the number of $CD4^+$ helper T cells and $CD45R/B220^+$ pan B cells increased markedly, but not $CD8^+$ cytotoxic T cells or $CD11b^+$ granulocytes/macrophages. In addition, OPFE significantly decreased the production levels of T helper (Th) 1 type cytokines, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, although had no significantly differences in those of interleukin (IL)-4, a Th2 type cytokine in concanavalin A (Con A)-stimulated blastogenic cells. Furthermore, OPFE alone strongly increased IL-4 production and decreased TNF-α production even in the absence of Con A. On the basis of these results, this study suggests that OPFE enhances immunity by regulating the pro- and anti-inflammatory response, indicating that this extract exerts a marked immunomodulatory effect, confirming its usefulness as therapy for immune-related diseases.

Keywords: *Opuntia ficus indica*, splenocyte, Th1 cytokine, Th2 cytokine, immunomodulatory effect

Introduction

The cactus pear fruit, *Opuntia ficus indica* (OPF), a member of the Cactaceae family, is a plant originating from the tropical and subtropical zones of South America. Currently, it is cultivated in Jeju Island in South Korea for the production of health foods such as tea, jam, and juice. All parts of OPF are considered to be good sources of various bioactive substances. The pulp of OPF is rich in glucose, fructose, pectin, and polyphenols. Even the seeds contain cellulose and notable amounts of proteins and lipids (1). OPF contains betalains which are nitrogenous pigments, mainly a mixture of red betacyanins and the yellow betaxanthins. These pigments can also be used as natural food colorants as well as potential free radical scavengers (2,3). Multiple beneficial and therapeutic properties of OPF extract are also well established. In oriental medicine, OPF has been used to treat burns, wounds, edema, bronchial asthma, diabetes, hypertension, and indigestion (4). OPF of the fruit and stems exhibit hypoglycemic (5), antiulcer (6), antiallergic (7), wound healing (8), and antioxidative activity (4,9). Additionally, recent studies have shown that OPF has an anti-inflammatory action that provides a protective effect against gastric lesions *in vivo* (10) and contains an active anti-inflammatory principle, β-sitosterol (11). However, the detailed knowledge about the immunological mechanism of OPF is still lacking.

During the last decade, an upsurge of information has surfaced concerning the immunological role of plant-derived materials used in transitional medicine. The antiviral properties of green tea (*Camellia sinensis*) polyphenols have been documented (12), and an extract of *Glycyrrhiza glabra*, i.e., licorice root, aloe (*Aloe vera*), *Elaeocarpus sylvestris* var. *ellipticus*, and *Ecklonia cava* have antiulcer, anti-inflammatory, immunomodulatory, and antiallergic functions (13-16). The fruit of OPF is a fleshy berry whose nutritional importance is attributed to the presence of potential anti-inflammatory compounds like amino acids, fiber, and ascorbic acid (17,18). However, evidence for the immunological activity of OPF on the cellular level is insufficient compared to that available for other plants. Therefore, it was thought worthwhile to investigate the immunological role of OPFE *in vitro* using cells of the immune system such as splenocytes.

In this study, we have elucidated the influence of the methanol extract from OPF (OPFE) on the immune response by assessing the splenocyte viability, proliferative ability, and cytokine production during the immune response.

Materials and Methods

Extraction from OPF The prickly pear fruit, OPF was obtained from the cactus-growing village of Hallim on Jeju Island in South Korea. Cleaned samples of the berries were then lyophilized and ground into a fine powder as a source of the extract. This OPF was prepared using 10 g of fine cactus powder macerated for 24 hr at 15°C in a 1 L solution prepared with a methanol. The resultant mixture was filtered through Whatman No. 1 paper. The filtrate was placed in a vacuum to evaporate a methanol. Finally, the dried OPFE was dissolved in RPMI-1640 medium (Gibco, New York, NY, USA) for the following assays. The components of OPFE were analyzed by the spectrophotometric assay. Betalains (the predominant pigments of cactus pear fruit) are water-soluble nitrogenous pigments, mainly a mixture of red betacyanins and yellow betaxanthins. Previous studies have reported the maximum

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Received February 16, 2009; Revised September 10, 2009;
Accepted September 10, 2009

absorbances for betaxanthin and betacyanin observed at wavelengths 484 and 535 nm were due to indicaxanthin and betacyanin respectively (19). The absorbance of OPFE was measured at 2 wavelengths and OPFE showed higher absorbance at 535 nm, a wavelength for betacyanin, than at 484 nm for betaxanthin. These results suggest that OPFE has betalains enriched by betacyanins.

Preparation of splenocytes ICR mice purchased from SLC (Shizuoka, Japan), the animals were housed in conventional animal facilities with NIH-07 diet and water *ad libitum* under constant temperature (23±1°C) according to the guideline of animal care and use committee of Jeju National University. Spleens from mice were removed aseptically from animals and their splenocytes were isolated as described by SaiRam *et al*. (20). Single splenocytes were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) with antibiotics (Gibco). Briefly, the cells were centrifuged at 890×g, and erythrocytes were lysed by adding ACK lysis buffer including 0.84% ammonium chloride. Later, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) (Gibco), and viability was determined by trypan blue dye exclusion. The splenocytes (viability>90%) were then suspended in RPMI-1640 medium supplemented with 10% FBS with antibiotics, and the cell concentration was adjusted to 10^6 cells/mL.

Assessment of cytotoxicity by MTT assay The viability of splenocytes was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the splenocytes (5×10^4 cells/mL) were incubated with OPFE (from 1 to 1,000 μg/mL) in 96-well culture plates (Nunc, Copenhagen, Denmark) for 48 hr at 37°C. After 48 hr, MTT (Sigma-Aldrich) stock solution (50 μL, 2 mg/mL) was applied to each well and then incubated for 4 hr at 37°C. The plates were centrifuged for 20 min at 890×g, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μL of dimethylsulfoxide (DMSO) (Sigma-Aldrich), and the absorbance was measured at a wavelength of 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The data is expressed as mean percentages of the viable cells versus the respective control. The assays were performed in triplicate.

Assessment of cell viability The viability of splenocytes was determined by trypan blue dye exclusion test. Cells treated with OPFE at 100 μg/mL were incubated at 37°C for 24 and 48 hr. Cells were loaded with trypan blue dye on a hematocytometer slide at the ratio 1:19 (v/v), and the number of viable cells was evaluated under a field microscope. The cells in 4 chambers of a hematocytometer were counted per each sample, and assays were performed in triplicate.

Assessment of proliferative responses The proliferation assay identifies the extent of target cell division based on the principle that the thymidine base of DNA sequence in a cell is replaced by the radioactive reagent (^{3}H)-thymidine. Proliferative responses of splenocytes were assayed in 96-well culture plates by uptake of (^{3}H)-thymidine (specific activity 42 Ci/mmol; Amersham Pharmacia Biotech, Tokyo, Japan). The splenocytes (4×10^5 cells/mL) were cultured with the indicated concentration of OPFE (50 and 100 μg/mL) for 72 hr, and for the last 18 hr in the presence of 1 μCi (^{3}H)-thymidine. These cells were harvested on glass-fiber filters (Amersham Pharmacia Biotech), and the label uptake was determined using standard liquid scintillation techniques.

Flow cytometric analysis To quantify the splenocyte phenotypes, single cell suspensions (4×10^6 cells/mL) were cultured for 48 hr in the presence or absence of OPFE (100 μg/mL). The cells were harvested and then washed twice with DPBS. The cells were blocked with an anti-mouse immunoglobulin G (IgG) solution in DPBS and incubated with fluorescently labeled antibodies (Abs) for 15 min at room temperature in darkness. All Abs were directly labeled with fluorescent tags, i.e., fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)- for CD4 (H129.19), CD8a (53-6.7), CD11b (M1/70), and CD45R/B220 (RA3-6B2). The tags used in these experiments were CD4-FITC as a specific marker for T helper lymphocytes, CD8a-PE as a specific marker for cytolytic T lymphocytes, CD11b-FITC as a specific marker for granulocytes and macrophages, and CD45R/B220-FITC as a specific marker for pan B lymphocytes. Appropriate isotype controls were always included. The cells were washed with 1 mL of wash-buffer (DPBS containing 1% FBS) and centrifuged at 890×g for 5 min. Supernatants were carefully decanted, and the cells were resuspended in 500 mL of 1% paraformaldehyde in DPBS. All samples were analyzed using a BD FACSCalibur™ flow cytometer and CellQuest software (BD Bioscience). This analysis was performed on 10,000 events.

Enzyme linked immunosorbent assay (ELISA) for cytokines To establish whether the immunological function of OPFE stems from Th1 type or Th2 type cytokines, splenocytes (4×10^5 cells/mL) were cultured in a 96-well culture plate in the presence or absence of OPFE for 48 hr. In addition, the mitogen concanavalin A (Con A) (Sigma-Aldrich) at a concentration of 5 μg/mL was used to promote the production of cytokines by stimulating T cell proliferation. Culture supernatants were collected and used for measuring cytokines by currently available interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-4 ELISA kits (BioSource International, Camarillo, CA, USA). The amounts of IFN-γ, TNF-α, and IL-4 released were determined in a sandwich ELISA using pairs of specific capture and biotinylated detection antibodies and streptavidin-horseradish peroxidase (HRP) according to the manufacturer's instructions. Each sample was assayed in duplicate, and essentially the same results were obtained.

Statistical analysis The results are reported as mean± standard error (SE). All experiments were carried out on at least on 2 occasions, and the results were analyzed using Student's *t*-test; a difference of $p<0.05$ was considered significant. The difference of proliferative T cell response and amounts of cytokines between groups was analyzed for significance by Fisher's exact test and Mann-Whitney's U-test, respectively.

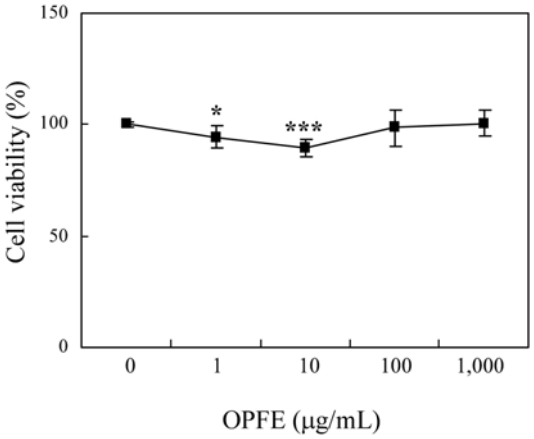

Fig. 1. Cytotoxicity of OPFE in splenocytes. Result is the mean±SE (within 5% of the mean values) from 3 independent experiments. *p<0.05 and ***p<0.005 significantly different from values in untreated controls by Student's t-test.

Results and Discussion

Cytotoxicity of OPFE on splenocytes To identify whether OPFE was cytotoxicity, the MTT assay was performed first. Splenocytes isolated from mice were treated with OPFE at concentrations from 1 to 1,000 μg/mL. After 48 hr, the surviving splenocytes were measured by MTT assay. As shown in Fig. 1, the cell viabilities induced by OPFE were similar to that of control cells at all concentrations from 1 to 1,000 μg/mL. In addition, the cell viabilities had a significance by treatment of OPFE (1 and 10 μg/mL) compared to that of control cells (p<0.05 and p<0.005, respectively). This finding suggests that OPFE has no cytotoxic effect even at high doses.

Effect of OPFE on cell viability and proliferative ability To elucidate the effect of OPFE on viability and proliferation of splenocytes, we performed the trypan blue dye exclusion test and (^{3}H)-thymidine incorporation assay (Fig. 2 and 3). In this experiment, the number of viable splenocytes was increased after treatment with OPFE (100 μg/mL). In addition, the number of splenocytes after 48 hr of OPFE treatment (open columns) was 3 fold higher than that after only 24 hr (closed columns) in the same concentration of OPFE (p<0.005) (Fig. 2).

To confirm these results with another technique, we measured the proliferative responses of splenocytes to OPFE by using the (^{3}H)-thymidine incorporation assay. As Fig. 3 illustrates, the proliferative responses of splenocytes increased in parallel to rising concentrations of OPFE. OPFE enhanced cell proliferation at concentrations from 50 to 100 μg/mL in a dosage-dependent manner (p<0.005). However, increasing OPFE concentration beyond that did not result in proportional benefit on cell proliferation. The cell proliferation at 1,000 μg/mL was similar to that at 100 μg/mL (data not shown). These results indicate that OPFE obtained from the prickly pear plant time-dependently strengthened the significant survival and proliferation of murine splenocytes without causing cytotoxicity.

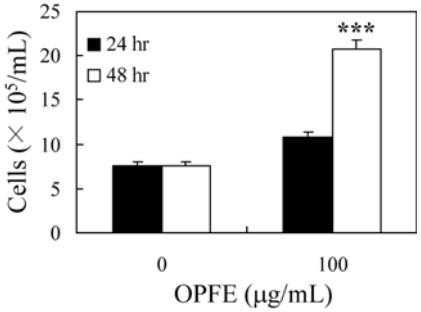

Fig. 2. Effect of OPFE on viability of splenocytes. Result is the mean±SE (within 5% of the mean values) from 3 independent experiments. ***Significantly different from values in untreated controls by Student's t-test (p<0.005).

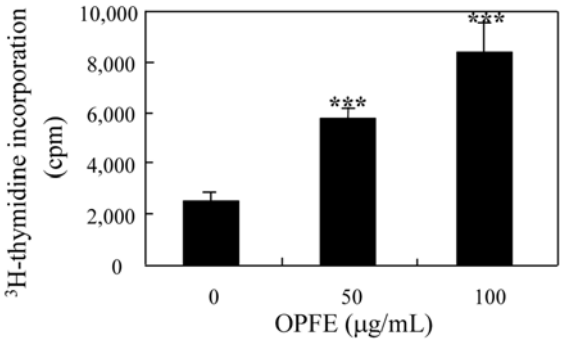

Fig. 3. Effect of OPFE on proliferation of splenocytes. Result is the mean±SE (within 5% of the mean values) from 3 independent experiments. ***Significantly different from values in untreated controls by Student's t-test (p<0.005).

Quantification of the increased splenocyte phenotypes by flow cytometry analysis Next, the specific cell phenotypes of these abundantly proliferating splenocytes (Fig. 3) by flow cytometry analysis using immune cell-specific phenotype markers were analyzed. Splenocytes treated with OPFE (100 μg/mL) for 48 hr were stained with fluorescently labeled Abs, i.e., CD4-FITC as a specific marker for T helper lymphocytes, CD8-PE as a specific marker for cytolytic T lymphocytes, CD11b as a specific marker for granulocytes/macrophages, and CD45R/B220-FITC as a specific marker for pan B lymphocytes. As the results in Fig. 4 portray, the number of $CD4^+$ cells and $CD45R/B220^+$ cells which are helper T lymphocytes and B lymphocytes was markedly increased from 55.3 to 60.0% and from 36.9 to 51.6%, respectively. In contrast, the number of $CD8^+$ cells, which are cytotoxic T lymphocytes, was not modified significantly by OPFE, and the same pattern was evident for $CD11b^+$ cells, which are granulocytes/macrophages (data not shown). Therefore, these splenocytes subsets are differentially activated by OPFE, with significant increases present only in B lymphocytes and T lymphocytes whose effect is to reinforce immune responses.

Cytokine analysis by ELISA To know more precisely the immunological role of OPFE on the cytokine level, the production of Th1 and Th2 cytokines was assessed by

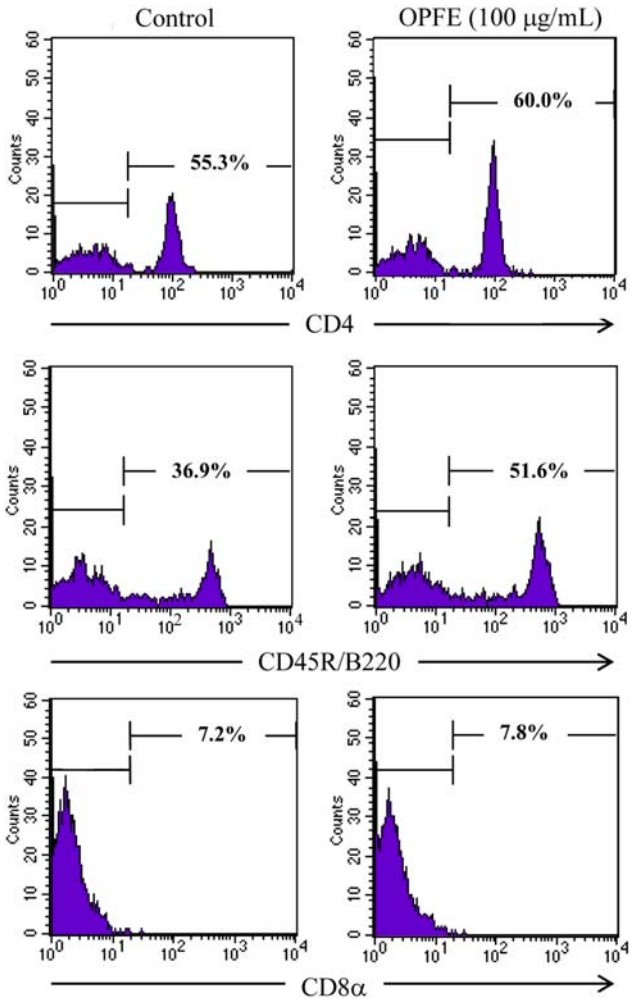

Fig. 4. Quantification of the increased splenic phenotypes by flow cytometry analysis. The effects of OPFE on the population of specific cell phenotypes were investigated by FACS.

using currently available ELISA kits. Splenocytes cultured as described previously and were treated with OPFE at concentrations of 100 and 1,000 μg/mL in the presence or absence of Con A for 48 hr. Con A was used to promote the production of cytokines, since in supernatants from the splenocytes tested, very low levels of cytokines were produced without specific mitogen stimulation. Culture supernatants were harvested and tested for the cytokines IFN-γ (Fig. 5A and 5B), TNF-α (Fig. 5C and 5D), and IL-4 (Fig. 5E and 5F) by ELISA. OPFE alone had no effect on IFN-γ levels (Fig. 5A) but potently suppressed TNF-α production by as much as 19 fold even without stimulation by Con A (Fig. 5C). Further, high concentration (1,000 μg/mL) of OPFE significantly increased IL-4 production (p<0.005) (Fig. 5E). Generally, the addition of Con A increased the levels of IFN-γ and TNF-α production (Fig. 5B and 5D), compared with those in the absence of Con A (Fig. 5A and 5C). In sharp contrast, OPFE (from 100 to 1,000 μg/mL) dose-dependently decreased the levels of IFN-γ and TNF-α production compared to those of only-Con A stimulated cells. Especially, 1,000 μg/mL of OPFE treatment combined with Con A stimulation significantly decreased production of IFN-γ by 13 to 14 fold and TNF-α by 2 fold below those in the treatment of Con A alone (p<0.005) (Fig. 5B and 5D). However, the production level of IL-4 was not significantly changed by the application of OPFE (Fig. 5F). These findings indicate that OPFE treatment of splenocytes induces down-regulation of the Th1 type cytokines and substantial up-regulation of the Th2 type cytokine. The function and efficiency of immune system are influenced by many exogenous and endogenous factors such as food and pharmaceuticals as well as physical and psychological stresses, resulting in either immunosuppression or immunostimulation (21). Apart from being specifically stimulatory or suppressive, certain agents that possess the ability to normalize or change immunological processes are called immunomodulatory agents (22). The growing interest in identifying, characterizing, and using natural compounds with immunomodulatory activity in modern medicine prompted our interest in testing OPF, one of several plants long used in traditional medicine systems for rejuvenation therapy and treatment of chronic ailments (23,24).

Previous studies have identified specific immune responses that are clearly regulated by distinctive cytokines released by Th1 and Th2 type cells. Normally, Th1 type cells stimulate immune functions via macrophage activation and lead the attack against intracellular pathogens such as viruses and bacterial antigens by producing pro-inflammatory cytokines such as IL-2, IFN-γ, and TNF-α. To the contrary, Th2 type cells suppress immune functions via inhibiting macrophage or Th1 type cell activation by processing anti-inflammatory cytokines as IL-4, IL-5, IL-9, IL-10, and IL-13 (25,26). Moreover, any factor that affects the balance of either Th1/Th2 cell cytokines or antibody isotypes may play a role in the development or resolution of autoimmune disease (26).

In this study, we documented that OPFE down-regulated production levels of the Th1 type cytokine, TNF-α with incremental increases of concentration but up-regulated the production levels of the Th2 type cytokine, IL-4 in the absence of Con A. Additionally, the production of IFN-γ was significantly suppressed by OPFE in Con A-stimulated cells although it had no significant difference in only OPFE-treated cells. These results indicate that OPFE specifically modulates the production of Th1 and Th2 type cytokines. Normally, immunomodulation leads to the reduction of inflammation response or the activation of immune response resulting in the production of Th1 and Th2 type cytokines (25,26). This suggests that the effects of OPFE on the increment of IL-4 production or the decrease of IFN-γ and TNF-α production in the presence or absence of Con A can be useful in suppressed the progression of several immunological diseases particularly of Th1 or Th2-mediated diseases and an important factor in regulating immune responses.

In the latter regard, researchers have stated that optimal immunotherapy should restore or maintain a well-balanced Th1 and Th2 response (25). Our results assert that OPFE has immunostimulatory characteristics, in that it activates T cells and B cells, and also ideal modulatory characteristics evident here in production of Th1 as well as Th2 type cytokines. OPFE also seems to be a potential agent for selectively modulating either Th1 or Th2 responses and, thereby, may provide means of achieving T cell homeostasis.

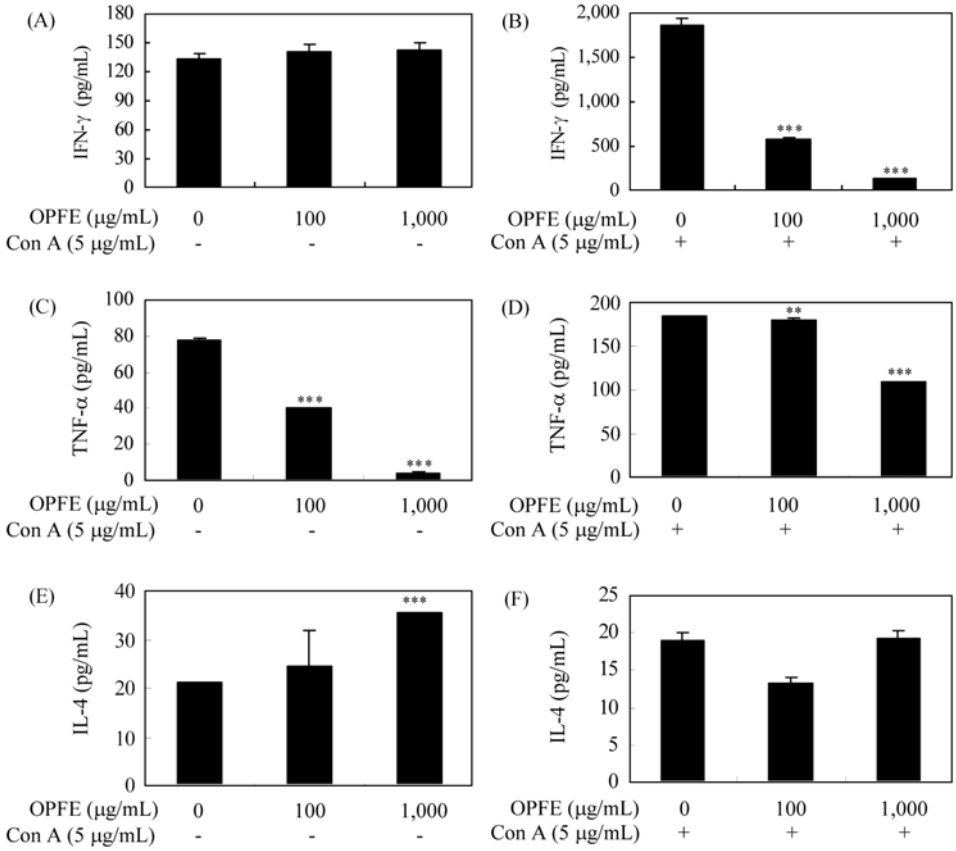

Fig. 5. Analysis by ELISA of the cytokines IFN-γ (A, B), TNF-α (C, D), and IL-4 (E, F). Significantly different by Student's *t*-test at $^{**}p<0.01$ and $^{***}p<0.005$.

In addition, as that previous study reported, polyphenolic compounds such as pigment rich in OPFE have anti-inflammatory benefits and such modulating effects as the activation of transcription factors and protein tyrosine kinase (27-29). Thus, the immunomodulatory effects of OPFE might be related to the function of polyphenolic compounds. In conclusion, results from the present study demonstrate that OPFE, with the advantage of being a natural product, may provide a present-day drug for treating immunologic diseases as it has in the past for many afflictions of humans.

Acknowledgments

This research was performed under the program of Basic Atomic Energy Research Institute (BAERI) which is a part of the Nuclear R&D Programs funded by the Ministry of Science & Technology (MOST) of Korea.

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