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Commercial Application of *Anoectochilus formosanus*: Immunomodulating Activities

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Abstract: *Anoectochilus formosanus* is an important ethnomedicinal plant of Taiwan. We investigated the effect of oral administration of *A. formosanus* effective fraction (AFEF) on the innate immune response in mice. Male BALB/c mice were treated orally for 2 weeks with 500, 1000 and 1500 mg/kg of AFEF. Primary peritoneal macrophage harvest from mice that administered with AFEF (500 – 1500 mg/kg) was directed to activate phagocytosis. AFEF significantly increased interferon-γ production from lymph node cells by ConA stimulation for 48 hours in AFEF (1500 mg/kg) treated group. AFEF might be the active fraction in activation of innate immunity.

Keywords: *Anoectochilus formosanus*; immunomodulation; allergy.

1. Introduction

Anoectochilus formosanus (Orchidaceae) is an important ethnomedicinal plant of Taiwan. This herbal plant is also called "King medicine" because of its diverse pharmacological effects. In Taiwanese folk remedies, the whole plant of *A. formosanus*, fresh or dried, is boiled in water and taken orally in the treatment of chest and abdominal pains, diabetes, nephritis, fever, hypertension, impotence, liver and spleen disorder, and pleurodynia (Kan, 1986). In our previous works, we have confirmed the hepatoprotection of aqueous crude extract from *A. formosanus* (Shih et al., 2004, 2005). To develop the health food from *A. formosanus*, we have prepared an *A. formosanus* fraction (AFEF) which can reduce the liver injuries in rats induced by carbon tetrachloride (data unpublished). To improve the commercial application, we further investigated the immunomodulating activities of AFEF.

2. Materials and methods

2.1. Preparation of hebral drug extract

A. formosanus was purchased from YU-Jung Farm (Pu-Li, Taiwan), where they are cultivated. The plants were identified by the Institute of Chinese Pharmaceutical Sciences, China Medical University, where a plant specimen has been deposited.

Fresh whole plants of cultured *A. formosanus* were extracted with water and the filtrate was partitioned with organic solvent succes-

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sively. The aqueous fraction was further filtered and evaporated under reduced pressure yielding a purple residue (AFEF). The AFEF yield was approximately 3 %.

2.2. Animal treatment

BALB/c mice were housed under controlled conditions of temperature (22 ± 2^0C), humidity (45% - 65%) and artificial light (12 h light/12 h dark, lights on at 7:00 a.m.) with free access to rodent chow and water. Animals were housed and used in accordance with the guidelines of the committee on Care and Use of Laboratory Animal Resources of the China Medical University.

For the drug treatment, the animals were divided into four groups and given orally with AFEF (0.5, 1, 1.5 g/kg) for 2 weeks. The serum from individual mice was collected. All animals were sacrificed under deep ether anesthesia. Peritoneal macrophages were then obtained by lavage with a phosphate-buffered saline (PBS) solutions (pH=7.2 - 7.4) and both spleen and lymph node cells isolated.

2.3. Macrophage activity

The peritoneal lavage fluid was then individually collected and placed in plastic tubes 910 ml) kept in an ice bath; 90 μl of each tube suspension was then mixed with 10 μl of 0.1% Trypan blue to assess macrophage viability. The peritoneal cells were identified and counted using FACScan. The procedure yielded 2 × 10^6 peritoneal cells/ml, with more than 90% being macrophages. Only suspensions with 90% or more viability were used (Pinello et al., 2005).

Phagocytosis was initiated by adding 1.5 μl sample containing 1.05 × 10^5 FITC-labeled *E. coli* to each well. Plates were incubated for 25 minutes at room temperature in the dark. Following this, cells were washed with PBS for 3 times to remove any non-adherent bacteria, followed by the addition of 400 μl of quench FITC fluorescence of extracellular bacteria. Finally, cells were gently washed with PBS for 5 times. Macrophages were fixed with 96% methanol at -20^0C for 15 min and observed under a fluorescent microscope (Alsam et al., 2005).

2.4. ELISA for immunoglobulins

Immunoglobulin concentrations were measured using a sandwich ELISA technique as previously described (Liou et al., 2004). Briefly, a 96-well microtiter plate was pre-coated with 100 ng of rabbit anti-mouse IgG + IgM antibody (Zymed Laboratory, CA) per well and incubated at 40^0C overnight. The plate was blocked with PBS and 1% gelatin. Then, the diluted samples and standards were added (100 μl/well). The plates were incubated at 37^0C for 2 hours and then HRP-conjugated secondary antibody at 100 μl/well was added. After one hour incubation at 37^0C, the color developed. The absorbance in each well was read at 490 nm using an ELISA reader (EL311, Bio Tek, VT), and the data was analyzed using a log-logit model.

2.5. ELISA for cytokines

A cytokines ELISA system (R&D System, MN, USA) was used to quantify cytokines as previously described (Liou et al., 2004). The capture antibody was a rat monoclonal antibody against mouse IL-2, IL-4or IFN-γ, respectively. The detection antibody was a biotinylated goat anti- IL-2, IL-4or IFN-γ polyclonal antibody, respectively. After the antigen-antibody reaction was completed, a HRP-conjugated streptavidin (Zymed, CA, USA) was added and the plate incubated at 37^0C for 1 hour. The color was finally developed by a substrate solution containing hydrogen peroxide (0.02%) and 0.4 mg/ml tetramethylbenzidine (TMB) (kirkegaard & Perry Laboratories, Inc., MD, USA). Plates were incubated in the dark for 30 min at room

temperature and the reaction stopped by adding 100 μof 2 N sulfuric acid per well. The absorbance at 450 nm was read with an ELISA reader (EL311, BioTek VT, USA). The data was analyzed using a log-logit model.

2.6. Flow cytometric assay for T-cell population analysis

The effects of AFEF on the expression of surface markers in T-lymphocytes were estimated by flow cytometry (FASCCalibur. Becton Dickinson, San Jose, CA). CD3, CD4 and CD8 expression were monitored using a Cychrome conjugated hamster anti-mouse CD3 antibody, phycoerythrin (PE)-conjugated hamster anti-mouse CD4 antibody, or fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD8 antibody (Becton Dickinson, San Jose, CA), respectively. Briefly, the non-adherent lymph node cells were washed three times with sterile cold PBS and the concentration of cells adjusted to 1×10^6 cells/ml. Each sample was mixed with 1 μl of indicated fluorescence-conjugated antibody and incubated at room temperature for 15 min. After incubation, the cells were washed with cold PBS and centrifuged at 200× g for 10 min. The pellet was dispersed and mixed with 500 μl of 1.0% paradormaldehyde. The percentage of $CD3^+$ cells in the lymph node cells and the percentage of $CD4^+8^-$ and $CD4^-8^+$ cells among the CD3+ cell population were analyzed by flow cytometry.

3. Results and discussion

The present study showed that the AFEF, when administered by gavage, during 2 weeks, in mice, can stimulate the activity of peritoneal macrophage. The same treatment also increased IFN-γ (Th2-type) production.

Fig. 1 shows that mice treated with AFEF (500 – 1500 mg/kg) presented an increase in the phagocytosis of the peritoneal macrophage of mice. The ability of the AFEF to modulate phagocyte functions might offer obvious therapeutic benefits for bacterial infections, since phagocytes play an essential role in the host's defense against infection by ingesting invading microorganisms and by mediating inflammation process.

The amount of immunoglobulins (IgG and IgM) in sera were measured using ELISA. The results indicated that the AFEF treatment did not effect the serum levels of IgG and IgM.

The lymph node cells isolated from AFEF-treated mice, the concentrations of IFN-γ (Th1-type), but not that of IL-4 (Th2-type), was increased significantly in comparison to the control group (Fig. 2). IFN-γ is known to be potent a macrophage activator as well as immunomodulating agent. It was, therefore, possible that the AFEF activated macrophage by upregulating the synthesis and production of this cytokine Corradin et al., 1991).

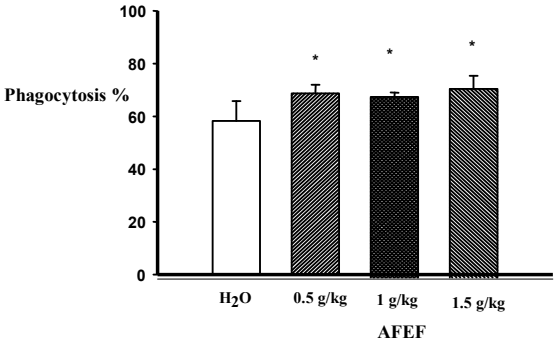

Figure 1. Effect of the treatment with AFEF for 2 weeks on the phagocytosis of peritoneal macrophage. Each value presents the mean ± SD (n=10). *P<0.05 compared with H_2O group.

A series of immunofluorescence assays were performed to monitor the variation in the T-cell sub-population after treating the mice with AFEF. Mice treated with AFEF had no change in the percentage of T-lymphocyte

sub-populations. Thus, our study on the immunomodulatory activity of AFEF has yielded significant results.

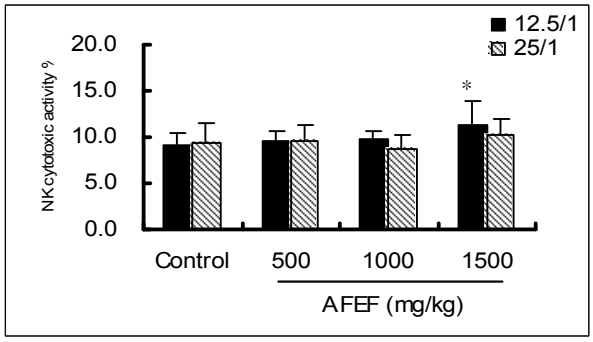

Figure 2. Effect of the treatment with AFEF for 2 weeks on the cytotoxic activity of NK cells. Each value presents the mean ± SD (n=10). *P<0.05 compared with H_2O group.

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