

Flavonoid, Phenol and Polysaccharide Contents of *Echinacea Purpurea* L. and Its Immunostimulant Capacity *In Vitro*

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Abstract—*Echinacea purpurea* L. (EP) is a plant originally used by native Americans to treat respiratory infections and have long been used to aid in wound healing and to enhance the immune system. The purpose of this study was to demonstrate the ability of EP extracts to stimulate the production of nitric oxide (NO) and TNF- α as well as to evaluate the cell viability by the use of chicken peripheral blood mononuclear cells (PBMCs) and RAW 264.7 macrophages *in vitro*. The polysaccharides content of EP was 162.2 \pm 8.4 mg/g dry weight (DW). The EP extracted with a 55% ethanol at 55°C contained 22.3 \pm 1.0 mg gallic acid equivalent/g DW of total phenolic compounds and 86.0 \pm 4.6 mg quercetin equivalent/g of flavonoid content. The result of cell viability assay showed that 89% and 81% of chicken PBMCs and RAW 264.7 macrophages, respectively, were survived under the treatment of 3.2 mg/ml of EP extracts by microculture tetrazolium assays (MTAs). Moreover, the cell inhibitory activity did not show the 50% cyto-toxicity effect (IC₅₀). In addition, NO production by stimulated chicken PBMCs and RAW 264.7 macrophages exhibited individually the linear relations with the concentration of EP extracts, and they were approximately 70 and 60 μ M in 800 μ g/ml, respectively. The tendency with the release of TNF- α by RAW 264.7 macrophages also corresponded with EP extracts concentration. It is concluded, EP extracts act as an immunostimulant for both tested cells but have no serious effect on inhibiting chicken PBMCs viability.

Index Terms—*Echinacea purpurea* L., Flavonoid, Phenol, Cell viability, Immunostimulant.

I. INTRODUCTION

Chinese medicinal herb, also known as herbal medium or phytomedium or botanical medium, are generally refers to botanicals with medical effects and healthcare benefits. With proliferated knowledge and demand on natural health care, the World Health Organization (WHO), the US Food and

Drug Administration (FDA) and European Union administration have independently announced their management regulations and relevant measures on traditional medicine and Chinese medicinal herb.

Echinacea purpurea L. (EP) is one of the most important medical herbs and is a kind of Asteraceae natively perennial grown in North America, which is used pharmacologically and for aesthetic enjoyment. In 2005, *Echinacea* products ranked among the top botanical supplements sold in the United States. Its root and subterranean stem were used by North America in early period to treat trauma and alleviate symptoms of infection and inflammation. The EP have been proven to show good immunoregulation, antiinflammation and antioxidant capacity [1], [2] and with no hypersensitivity or other side effects during clinical trial stages [3]. Varieties of EP all contain similar main ingredients including caffeic acid derivatives, alkamides, flavonoids, essential oils, and polyacetylenes, and medical activities of which are yet to be exactly identified with corresponding diseases [4]. However, caffeic acid derivatives and alkamides have been proven to be active ingredients with immunoregulation effects [5]. Moreover, synergistic antioxidative effect of caffeic acid derivatives, alkamides and polysaccharide fractions was demonstrated by measuring their inhibition of *in vitro* Cu(II)-catalyzed oxidation of human low-density lipoprotein (LDL) [6].

For years, antibiotics have been popularly used in the animal industry such as broiler or chicken diet. However, the misuse or continuous use of antibiotics has led to the emergence of the antibiotic-residue and drug-resistance [7]. With mounting public concerns associated with antibiotic-residues and increasing rates of antibiotic resistance, antibiotics have been strictly banned in some areas of the world. Addition of medicine herb to feed is one of the alternatives to be used as a replacement for antibiotics. There is sufficient evidence to show that potential herbs are effective for the enhancement of immune system and increasing antioxidant activity for human. In current study, a medicinal plant, EP, with good potential to conduct assay on its cell viability and immune activation was investigated. The immune effect of EP active substances on the appraised items, and the influence of its extracts on cell viability (especially chicken PBMCs) were also studied in order to provide references for antibiotics substitution in chicken diet.

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II. MATERIALS & METHODS

A. Materials and extraction method

EP was cultivated in Department of Agronomy, National Chung Hsing University. The harvested whole plants were dried in a forced hot air dryer at 43°C for 4-7 day, and then ground to powder (ca. 1mm size). Extract of EP with 55% ethanol to distilled water (1:10, w/v) under 55°C for 3 h after filtering. Collect the extracts and stored under -20°C until analyzed. HPLC (Hitachi, Japan) consisting of a pump (L-7100), column oven (655A-52), UV-VIS detector (L-4200) (330 nm) and Mightysil RP-18 GP 5 µm 150 x 4.6 mm (Kanto, Tokyo, Japan) was used to determine the content of caftaric acid, chlorogenic acid, cichoric acid, cynarin, echinacoside and alkamide 8/9.

B. Polysaccharides, phenolic compounds and flavonoid contents

Polysaccharides, phenolic compounds and flavonoid content were presented in Table 1. Soluble polysaccharide assay was conducted with the phenol-sulfate method. Boil dried EP powder in 95°C for 2 h, with filtrate dialyzed under 4°C for 12 h. The obtained extracts were analyzed with results compared to the data shown on the glucose standard curve at 730 nm spectrophotometrically [8]. Total phenolic content was determined using Folin-Ciocalteu reagent according to the method reported by Kujala et al. [9]. The EP extracts were mixed with Folin-Ciocalteu reagent before adding the Na₂CO₃ solution and measured with a double beam Perkin Elmer UV/Visible spectrophotometer at 730 nm. Then determine the contents of phenolic compounds of extracts as microgram of the gallic acid equivalent (GAE) by using an equation that was obtained from standard gallic acid graph. Flavonoid content of EP extracts was determined by following colorimetric method [10]. Briefly, 0.5 mL of extracts in methanol were mixed with methanol, 10% aluminium chloride, 1 M potassium acetate and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at the concentrations from 12.5 to 100 µg/mL in methanol.

C. Macrophage RAW 264.7 cells culture

The murine peritoneal RAW 264.7 macrophage cell line were purchased from Food Industry Research and Development Institute in Taiwan (BCRC No. 60001) and routinely cultured in 75 cm² flasks DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% bovine calf serum (HyClone, Logan UT), 100 µg/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL amphotericin at 37 °C in a 5% CO₂ mixed with 95% air incubator.

D. Peripheral blood mononuclear cells (PBMCs) isolation and culture

PBMCs were isolated from blood of broilers and were added by 1% EDTA for anticoagulant treatment, before being processed by the density gradient centrifugation. The PBMCs were separated in histopaque®-1077 (Sigma, 10771), and then cultured in RPMI-1640 added with 100

µg/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL amphotericin at 37 °C in a 5% CO₂ mixed with 95% air incubator.

E. Microculture tetrazolium assays (MTAs)

The trypan blue exclusion assay for cell growth and survival rate were conducted based on Victoria et al. [11]. Suspensions of macrophage RAW 264.7 and chicken PBMCs cell lines at a density of 1×10⁶ cell/well were cultured at various concentrations in a 10 µL of suspension in 96-wells microplate. After 48 h, 20 µL of MTT (3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyl tetrazolium bromide) solution was added to each well, and the cells were incubated at 37°C for 5 h. Then, the medium was removed by aspiration, and formazan crystals were dissolved in DMSO. Each well was completely pipetted, and the absorption at 570 nm of formazan solution was measured using a micoplate reader. The absorbance of cell-free complete medium without EP extracts was subtracted from the value of the corresponding treatment groups.

F. Oxide assay

RAW 264.7 macrophage and chicken PBMCs cells at a density of 1×10⁶ cell/well and maintained at 37°C in an atmosphere of 5% CO₂. LPS (*Escherichia coli* O127:B8 Westphal type, 10 µg /mL), and EP extracts (50, 100, 200, 400 and 800 µg/mL) were prepared and incubated overnight. One hundred microliters from the surface of cultures was transferred into a new plate and the equivalent amount of Griess reagent was added (Stock-1: 0.2% naphylendia HCl, Stock-2: 2% sulfanilamide in 5% H₃PO₄). This plate was then incubated for 10 min at RT and measured by an ELISA reader at 540 nm. Standard calibration curves were prepared using sodium nitrite as a standard.

G. Tumor necrosis factor-α (TNF-α) quantification

RAW 264.7 macrophage at a density of 1×10⁶ cell/well was maintained at 37°C in an atmosphere of 5% CO₂. The LPS (10 and 20 µg/mL) and EP extracts (100, 800 and 1600 µg/mL) were prepared and incubated for 24 hr. Following incubation, TNF-α secretion was assessed with an OPTEIA™ Mouse TNF-α kit (Pharmingen, San Diego, CA, USA) according to manufacturer's protocol. Briefly, the sample and recombinant standards were added to antibody-coated plates and incubated for 2 h. TNF-α was detected via the addition of horseradish peroxidase-conjugated, streptavidin-labeled antibodies. Color was developed using tetramethylbenzidine (TMB) (BD Biosciences, Pharmingen, USA) for 30 min and the absorbance was recorded at 405 nm.

H. Statistical analysis

Results were the mean values of five times of the same sample, and data represented as mean ± standard deviation (Mean±SD).

III. RESULTS AND DISCUSSION

Soluble polysaccharides, phenolic, flavonoid contents and active ingredients

The use of herbal remedies or botanicals in human food/ animal diets has been proposed because of their natural stimulation of the immune system and/or enhancement of growth performance. Polysaccharide contents of the EP was 162.2 ± 8.4 mg/g DW determined by phenol-sulfuric method (Table 1). Major polysaccharides present in the plant come from the cell wall and its metabolites. In addition to providing needs for the biological metabolism and energy, polysaccharides also exhibit anti-viral, anti-bacterial, and anti-parasitic functions [2]. Some polysaccharides that exhibit bio-activities are composed of monosaccharides with molecular weights ranging from 10^4 to 10^7 . The complex and three-dimensional structures formed through the clustering of β -glycoside linkages [12]. As indicated by Tsiapali et al. [13], β -1-3-D-glucan and its derivatives not only have the immune-stimulating activity, but also possess various levels of free radical scavenging capacity. *Astragalus membranaceus* is a type of Chinese herb medicine, with the polysaccharide content at 101 mg/g. Although no significant positive effect on the growth performance was observed, it enabled the improvement in the immune stimulation and regulation in chicken broiler [14]. Polysaccharide contents are influenced by different cultivation conditions. The polysaccharide content of *Lentinus edodes* is 72.0 ± 4.0 mg/g using the general commercial cultivation media. However, the polysaccharide content can be increased to 410.0 ± 72.0 mg/g if switching to the permeate-based and whey culture media. Further, the polysaccharide content at 10 days will be twice as much than being cultivated at 20 days, indicating that polysaccharide contents were dependent on the metabolic processes and growth in plant [15].

Table 1. Analysis of the soluble polysaccharides, phenolic and flavonoid content of *Echinacea purpurea* L. extracts¹

	Soluble polysaccharides (mg/g DW)	Total phenolic (mg of GAE/g)	Flavonoid content (mg of quercetin equivalent/g)
<i>Echinacea purpurea</i> L.	162.2 ± 8.4	22.3 ± 1.0	86.0 ± 4.6

¹The value is expressed as mean \pm standard deviation (n=5).

The active ingredients of a medicinal plant are mainly its secondary metabolites, one of which is the phenolic compounds [16]. Currently, the active ingredients and in vitro nonspecific immunostimulatory properties as well as cell viability for chicken PBMCs and RAW 264.7 macrophage of EP was analyzed. The result shows total phenolic contents of EP extracts contained 22.3 ± 1.0 mg gallic acid equivalent (GAE)/g DW (Table 1). Phenolic compounds contents can be used as a critical index for determining the antioxidant capacity [17]. The antioxidant mechanism adopted by these phenol compounds is the direct reaction with free radicals, such as hydroxyl (\cdot OH) radicals, superoxide anion ($O_2^{\cdot-}$) radicals, and hydrogen peroxide (H_2O_2 , as oxygen in non-free radical state) for minimizing the damage to the cells and inhibiting lipid oxidation [18]. Moreover, the preventive effects are thus generated for cardiovascular diseases, aging, cancer and cranial nerve disorders [19]. To lower cellular damages caused by the free radicals, cells possess non-enzymatic (glutathione, ascorbic acid and α -tocopherol) and enzymatic (catalase, glutathione peroxidase and superoxide dismutase) antioxidant defense systems. Under a normal functional state, a dynamic balance will be achieved through the interactions between the organism's antioxidant system and the formation of active

oxygen products or free radicals. However, an imbalance in the oxygen-reducing defense system can be observed when aging continues and environmental fluctuations occur. Therefore, to enhance the comprehensive defense mechanisms, the increase in the body's antioxidant replenishments and glutathione peroxidase content, for instance, can thus sustain the balance [20]. The total amount of phenolic compounds contained in the Chinese herb medicine is not only affected by the species variations but also by the harvest season and cultivation conditions [21]. Hudac et al. [22] reported that *Echinacea* flower heads and leaves have higher phenolic contents, they suggested the result is the function of development stage. Prakash et al. [23] discovered that the total phenolic contents of the twenty-five Chinese herb medicines ranged from 2.8 mg GAE/g DW (*Withania somnifera*, root) to 107.8 mg GAE/g DW (*Cassia fistula*, fruit). Among these, the highest total phenol content was also found in the fruit (including the flowers), while the roots exhibited the lowest level.

The compounds such as flavonoids, which contain hydroxyl functional groups, are responsible for antioxidant effect in the plants, has been recognized on human nutrition and health [24]. Total flavonoid contents of EP extracts contained 86.0 ± 4.6 mg quercetin equivalent/g (Table 1), and the mechanisms of action of flavonoids are through scavenging or chelating process [25]. The effect of *Echinacea* on the immune system is also suggested by various substances such as derivatives of caffeic acid (cichoric acid), alkamids and polysaccharides. The result of the active ingredient of EP extracts was presented in Table 2. In the literature, the content of alkamides in *Echinacea* ranges from 0.2 to 1.41 mg/g DM [26]. The content of derivatives of caffeic acid in the plant shows a high variance in dependence of season and growth status [27], [28].

Table 2. Analysis of the active ingredient of *Echinacea purpurea* L. extracts¹

	Caftaric acid (mg/g DW)	Chlorogenic acid (mg/g DW)	Cichoric acid (mg/g DW)	Cynarin (mg/g DW)	Echinacoside (mg/g DW)	Alkamides (mg/g DW)
<i>Echinacea purpurea</i> L.	0.17 ± 0.04	1.55 ± 0.05	0.71 ± 0.06	0.09 ± 0.01	1.30 ± 0.11	2.30 ± 0.13

¹The value is expressed as mean \pm standard deviation (n=5).

Cell viability and immunostimulant assay

The result of cell viability assay was illustrated in Fig. 1. The MTAs method is currently mostly applied in the assessment of cell viability capacity. The results showed that the EP extracts give better viability for chicken PBMCs than RAW 264.7 macrophage at various concentrations. At a concentration of 3.2 mg/mL, the viabilities were 89% and 81% for chicken PBMCs and RAW 264.7 macrophage, respectively. Both values did not satisfy the cell viability inhibition standards defined by IC_{50} . The results showed that the negative effect of EP extracts on the chicken PBMCs was relatively less significant than on the RAW 264.7 macrophages. The evaluation of the potential inhibitory effect from the sweet potato extracts on the cell viability of the human leucocytes NB4 was conducted by Huang et al. [29]. However, it was less straightforward for investigating and comparing the survival rates of the normal animal cells.

For current study, the chicken PBMCs were the investigation candidates for enhancing the application feasibility on the organisms. In addition, nitric oxide (NO) production by stimulated chicken PBMCs and RAW 264.7 macrophages exhibited individually the linearity with its concentration of EP extracts, and they were approximately 70 and 60 μM in 800 $\mu\text{g}/\text{mL}$, respectively (Fig. 2). Moreover, the tendency with the release of TNF- α of RAW 264.7 macrophages also showed correspondingly with its dose (Fig. 3), and the released concentration ranged approximately from 900 to 1300 pg/mL correspondence with the EP extracts concentration for 100 to 1600 $\mu\text{g}/\text{mL}$, respectively. In summary, the results of this study showed that the medicinal plant EP extracts have good capability for immunostimulatory properties, but with no effect on inhibiting chicken PBMCs and RAW 264.7 macrophage cell viability at concentrations under investigation.

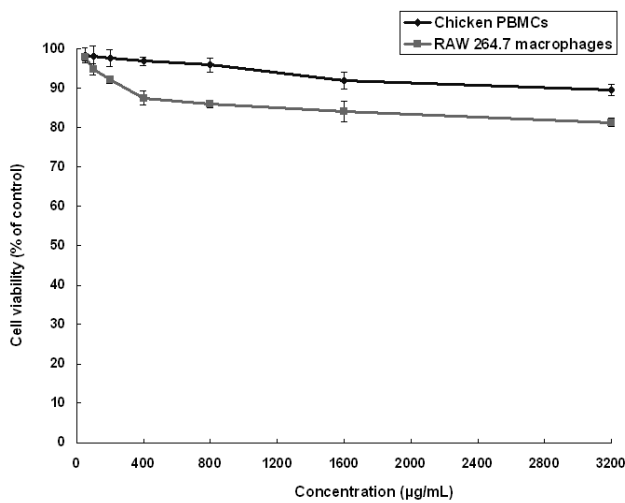


Fig 1. Effect of *Echinacea purpurea* L. extracts on cell viability of RAW 264.7 macrophages and chicken PBMCs for 24 h incubation.

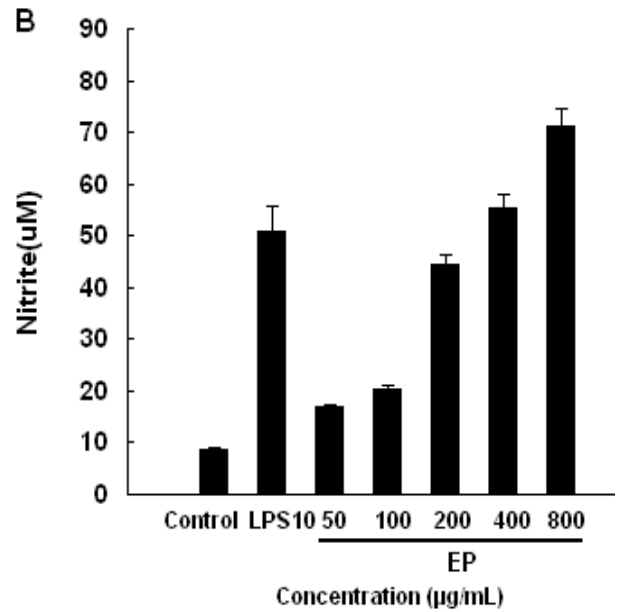
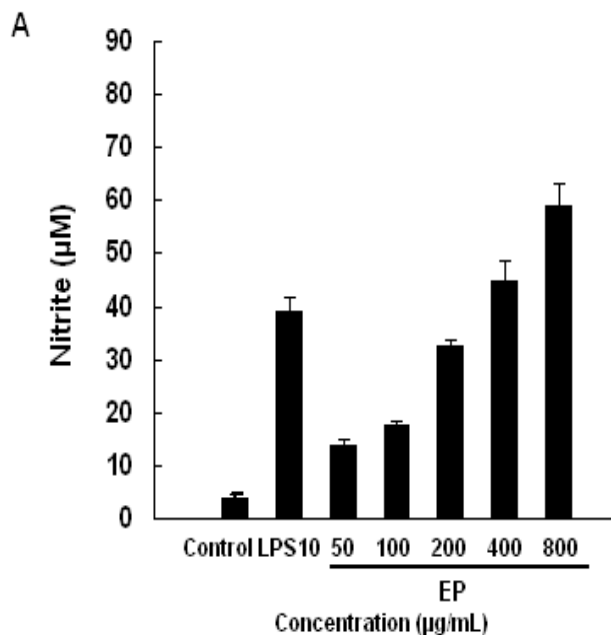


Fig 2. Effect of *Echinacea purpurea* L. extracts on NO production of RAW 264.7 macrophages (A) and chicken PBMCs (B) for 24 h incubation.

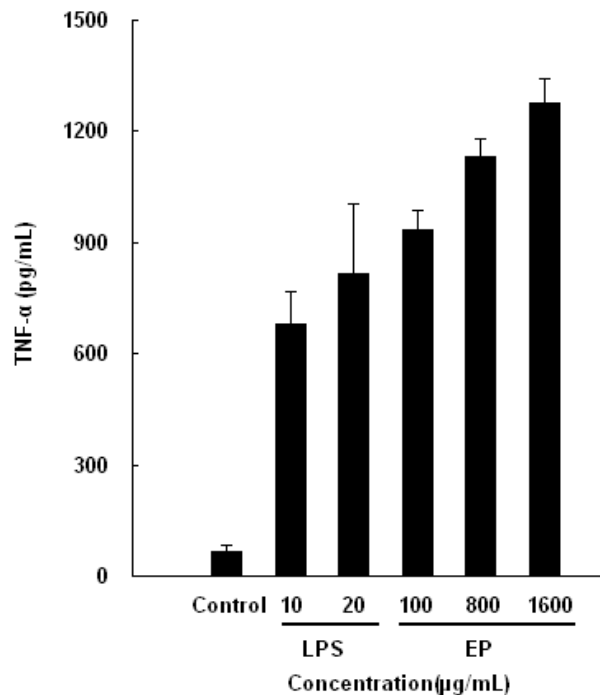


Fig 3. Effect of *Echinacea purpurea* L. extracts on TNF- α production after of RAW 264.7 macrophages for 24 h incubation.

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