

Original Article

## Anti-metastatic and Immunomodulating Activity of Water-Soluble Components from Five Mushroom Extracts

Hwa-Seung Yoo<sup>1</sup>, Sung-Soo Han<sup>1</sup>, Jungsun Kim<sup>1</sup>, Chong-Kwan Cho<sup>1</sup>, Yeon-Weol Lee<sup>1</sup>, Sang-Yong Park<sup>2</sup>, Soo-Jin Lee<sup>3</sup>

<sup>1</sup>Department of East-West Cancer Center, College of Oriental Medicine, Daejeon University, Daejeon, Korea

<sup>2</sup>R&D Lab. of Bulrolife Co., Ltd., Cheonan, Korea

<sup>3</sup>Department of Physiology, College of Oriental Medicine, Sangji University, Wonju, Korea

**Objectives** : This experiment was conducted to evaluate the inhibitory effects against lung metastasis and promotion of splenocytes by water-soluble components from five mushrooms extracts (WEFM): *Garnoderma frondosa*, *Coriouus versicolor*, *Codyceps militaris*, *Hericium erinaceus* and *Lentinula edodes*.

**Methods** : Colon 26-L5 carcinoma cells were injected through the tail vein to induce lung metastatic cancer. Changes in weight of lung were observed and cytokine level was analyzed to evaluate immunological changes.

**Results** : Oral administration of WEFM resulted in a significant inhibition of lung metastasis after intravenous injection of colon 26-L5 cells in a dose-dependent manner. There was also a significant increase in T cell and B cell mitogenic stimuli and production of IFN-g by splenocytes stimulated with Con A compared to untreated controls.

**Conclusion** : WEFM may have anti-tumor activities via Th1-type dominant immune responses.

**Key Words** : colon 26-L5 cell, lung metastasis, IFN- $\gamma$ , Th1 cytokine

### Introduction

Some chemotherapeutic agents have been used as adjuvants in surgical treatment for malignant tumors. Unfortunately, most anti-cancer agents are not sufficiently tumor-selective and sometimes cause severe side effects such as hematopoietic disorders and resistance to the chemotherapeutic regimen<sup>1</sup>. Some natural herbal medicines, including Traditional Oriental prescriptions, are known to show marked anti-tumor and anti-metastatic effects and possess immunomodulating properties while exhibiting low toxicity for normal tissues<sup>2-4</sup>.

Numerous studies have demonstrated that certain

components present in dietary mushrooms have been responsible for the modulation of cellular and physiological changes in the host. For this reason, mushrooms are often used as cancer therapeutic agents<sup>5-8</sup>.

The main purposes of this study were in investigating anti-tumor and immunomodulating effects of mushrooms. As preparatory tasks, XTT assay (colon 26-L5) and spleen proliferation assay *in vitro* utilizing several complex types of well-known traditional edible mushrooms were conducted to screen out a group of mushrooms with significant anti-tumor and immunomodulating effects.

In this study, the complex of water extracts from

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• Correspondence to : Hwa-Seung Yoo

Department of East-West Cancer Center, College of Oriental Medicine, Daejeon University,  
1136 Dunsan-dong, Daejeon 302-122, Korea

Tel : +82-42-470-9132, Fax : +82-42-470-9006, Email : altyhs@dju.ac.kr

those selected mushrooms (WEFM) (*Ganoderma frondosa*, *Coriarius versicolor*, *Codyceps militaris*, *Hericiium erinaceus*, *Lentinula edodes*) has been investigated for the effect of oral administration of thus prepared prescription on the experimental lung metastasis of murine colon 26-L5 cells and clarified its anti-metastatic mechanism with respect to its immunomodulating activities.

## Materials and methods

### 1. Prescriptions

Dried mushrooms, *Ganoderma frondosa* 300g, *Coriarius versicolor* 100g, *Codyceps militaris* 300g, *Hericiium erinaceus* 300g and *Lentinula edodes* 100g were obtained from Dunsan Oriental Hospital (Daejeon, Korea). One hundred grams of each mushroom were washed several times with distilled water, soaked in 1.5 L of pyrogen-free water for 2 h, and then boiled for 2 h. Solid particles and aggregates were removed by centrifugation at 3,000×g for 30 min and the supernatants were lyophilized. Finally, 26.35 g of the lyophilized water extract was obtained and used in this experiment. The general chemical composition of this prescription was analyzed in triplicate according to the methods of the Association of Official Analytical Chemists<sup>9)</sup>. For the high-performance thin-layer chromatography (HPTLC) analysis, beta glucan (10 mg/ml 50% MeOH. β-D-(1,3)-(1,6)-glucan), pachyman (100 mg/ml MeOH. β-D-(1,3)-glucan) and WEFM were dissolved in HPLC-grade methanol and applied to the pre-washed silica gel 60 F254 HPTLC plates (size 10×10 cm; thickness of the silica gel 0.2 mm; Merck, Darmstadt, Germany) with an automated applicator (Linomat IV, CAMAG, Merck KGaA, Germany). The samples were then separated (migration distance 75 mm) using HPLC-grade chloroform/methanol/water/formic acid (48:48:2:2). The migrated components were visualized at 254 nm using Reprostar 3 with a digital camera (CAMAG, Germany).

### 2. Cell cultures

The liver metastatic cell line of colon 26-L5 carcinoma (colon 26-L5) cells were maintained as monolayer cultures in RPMI-1640 medium (GIBCO BRL, Life Technologies Inc., NY, USA) supplemented with 10% fetal bovine serum (FBS; INC Biomedicals Inc., CA, USA). Colon 26-L5 cells were collected by brief treatment with EDTA, and then used for the experiments. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% O<sub>2</sub> air.

### 3. Animals

Six-week old, specific-pathogen-free female BALB/c mice were purchased from Daehan Biolinks (Korea). The mice were maintained under specific pathogen-free conditions and used according to institutional guidelines.

### 4. Assay for experimental lung metastasis of colon 26-L5 carcinoma cells

Log-phase cell cultures of colon 26-L5 cells were harvested with 0.05% EDTA in phosphate-buffered saline (PBS), washed three times with serum-free RPMI, and resuspended at appropriate concentrations in RPMI. In experimental lung metastasis assay, six BALB/c mice per group were given an intravenous injection of colon 26-L5 cells ( $2 \times 10^5$  cells/200-μL). WEFM was administered orally to the mice at dose of 50 mg/kg and 200 mg/kg for 9 days starting on day 3 after tumor inoculation. On the twelfth day, the mice were sacrificed and the lungs were fixed in Bouin's solution. The lung tumor colonies were investigated and the lung weight was measured.

### 5. Preparation of mouse splenocytes and peritoneal exudates cells

Splenocytes were obtained by passing pieces of spleen through a stainless mesh, treated with a hypotonic solution to lyse erythrocytes, and washed three times with PBS. The viability of the splenocyte was more than 95%, as assessed by the trypan

blue dye exclusion method. Whole splenocytes were suspended in RPMI-1640 medium supplemented with 10% FBS and then used for experiments.

#### 6. Splenocyte proliferation assay *in vitro*

For the splenocyte proliferation assay, WEFM was administered orally to BALB/c mice at appropriate doses for 2 weeks and splenocytes were obtained 1 day after the last administration as described above. Splenocytes ( $1 \times 10^5$  cells/100-mL) suspended in RPMI-1640 medium supplemented with 10% FBS were cultured in 96-well U-bottom culture plates with or without concanavalin A (Con A; Sigma) or lipopolysaccharide (LPS; Sigma) for 48 h at 37 °C. This assay was performed using triplicate cultures. XTT assay (Sigma) was performed to measure cell proliferation.

#### 7. FACS analysis

Isolated cells from spleen were stained with fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal anti-CD3, CD19, CD4, CD8, MAC-1, and NK1.1 antibodies (Becton-Dickinson, CA, USA) in PBS for 20 minutes at 4°C. Stained cells were analyzed on flow cytometry (FACS Calibur; Becton-Dickinson) using Cell Quest software. Differentiation of lymphocytes was determined using flow cytometric analysis of light scatter chamber characteristics relating size and granulation. Two-color flow cytometry was then performed to calculate the percentages of CD3, CD19, CD4, CD8, Mac-1, and NK1.1 positive cells in a subset of lymphocytes.

#### 8. Induction of cytokine production

Interferon (IFN)- $\gamma$  and interleukin (IL)-4 levels in the culture supernatant were evaluated using specific ELISA kits (BD Pharmingen, CA, USA) according to the manufacturer's instructions. Cell-free supernatant was prepared as follows: splenocytes ( $1 \times 10^6$  cells/well) were prepared as described above and then cultured in 24-well culture plates with or without various concentrations of Con A for 24 h at 37 °C.

The cell-free supernatant was collected from each well and stored at -80 °C until the ELISA assay.

#### 9. Statistical Analysis

Representative data from each experiment are presented as mean values SD, as described in the figure legends. The statistical differences between the groups were determined by applying the Student's two-tailed t-test. Dunnett's test was performed to decrease the multiplicity in comparisons of drug-treated groups with control group. Statistical significance was defined as a p value < 0.05.

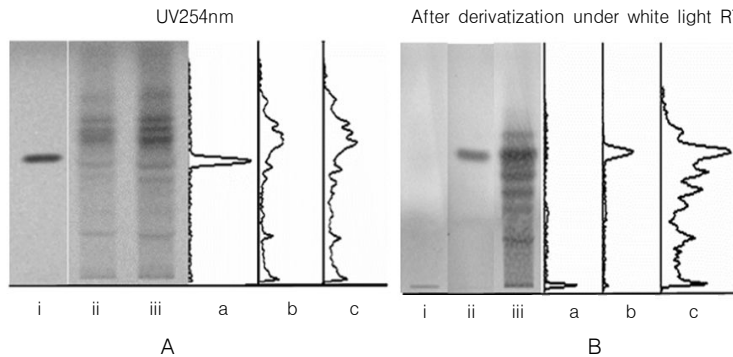
## Results

#### 1. HPTLC analysis

For quality control of the tested samples, high-performance-thin layer chromatography (HPTLC)-based fingerprinting was performed using the CAMAG Application System (Muttenez, Switzerland). WEFM,  $\beta$ -D-(1,3)-(1,6)-glucan and pachyman were dissolved in 90% HPLC-grade methanol and applied to a pre-washed silica gel 60 F254 HPTLC plate (10×10 cm, 0.2 mm thick silica gel, Merck, Darmstadt, Germany) with an automated applicator (Linomat IV, CAMAG, Muttenez, Switzerland). The samples were then separated (migration distance: 60 mm) using HPLC grade n-butanol/methanol/water (50:25:20). Thereafter, glucose-specific staining with aniline-diphenylamine-phosphoric acid and protein-specific staining with ninhydrin reagent was performed separately. The developed plate was visualized at 254 nm using a Reprostar 3 Digital Camera System (CAMAG; Figure 1A-1D). Analysis of carbohydrate using HPTLC showed that  $\beta$ -glucan and pachyman were one of the major components of WEFM (Figure 1A, B).

#### 2. Effect of WEFM on experimental lung metastasis

We first examined the effect of oral administration of WEFM on lung metastasis caused by intravenous



**Fig. 1.** Fingerprint of WEFM.

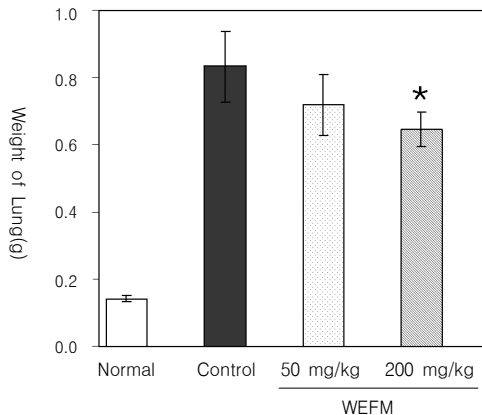
A. i (a): Beta glucan 10 µg, ii (b): WEFM 400 µg, iii (c): WEFM 800 µg

B. i (a): Beta glucan 10 µg, ii (b): Pachyman 500 µg, iii (c): WEFM 400 µg

WEHE, β-glucan and pachyman were dissolved in 90% HPLC-grade methanol and applied to a prewashed silica gel 60 F254 HPTLC plate. WEHE (2 or 4 mL of a 50 mg/mL solution) and 2 $\mu$ L β-D-(1,3)-(1,6)-glucan (10 mg/mL) were separated (migration distance 60 mm) using HPLC-grade n-butanol/methanol/water (50:25:20). Images were visualized at UV 254 nm (A) or under white light after staining with aniline-diphenylamine-phosphoric acid (B).

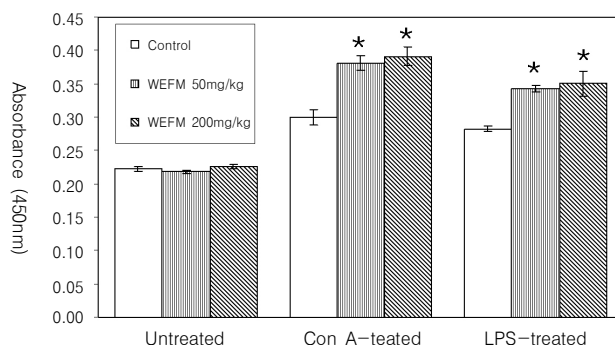
injection of colon 26-L5 carcinoma cells. Lung weight was measured on day 12 after tumor inoculation. Figure 2 shows that the oral administration of WEFM for 9 consecutive days from day 3 after tumor inoculation decreased the gain in lung weight in a dose-dependent manner in the range from 50 to

200 mg/kg/day. The administration of WEFM did not show any apparent side effects such as body weight loss (data not shown). These results indicate that WEFM is effective for preventing the experimental lung metastasis of colon 26-L5 cells.



**Fig. 2.** Effect of oral administration of WEFM on experimental lung metastasis produced by intravenous injection of colon 26-L5 carcinoma cells.

Female BALB/c mice (n=6) were intravenously infected with colon 26-L5 cells ( $2 \times 10^5$  cells/mice). WEFM prescription at the indicated doses was orally administered for 9 consecutive days from day 3 after tumor inoculation. 12 days after tumor inoculation, mice were sacrificed and lung weight was measured. The results are presented as mean S.D. \*,  $p < 0.05$  compared to the untreated control.



**Fig. 3.** Effect of oral administration of WEFM on the proliferation of mouse splenocytes in response to various mitogenic stimuli.

Female BALB/c mice were orally administered with WEFM for 2 weeks. One day after the last administration, mice were sacrificed and the splenocytes ( $1 \times 10^5$  cells/well) suspended in RPMI-1640 medium supplemented with 10% FBS were cultured with and without con A or LPS for 48 h. XTT assay was conducted to evaluate cell proliferation. The data is presented as the mean S.D. \*,  $p < 0.05$  compared to normal mice.

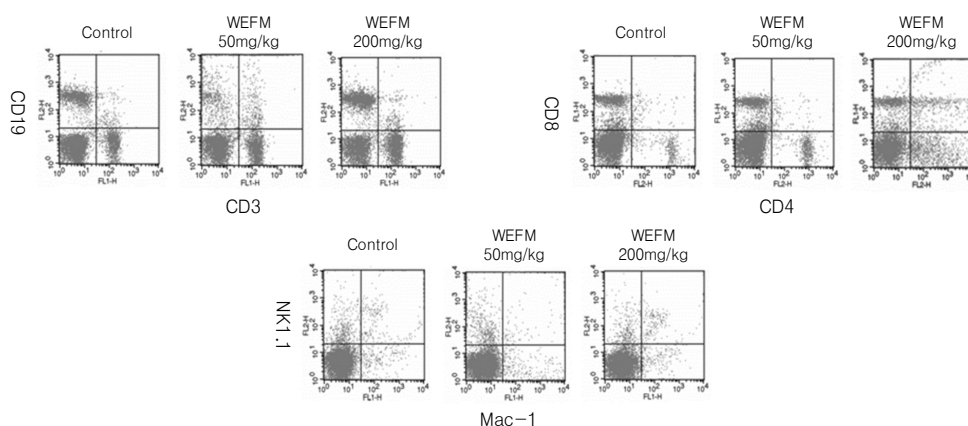
### 3. Effect of WEFM on the proliferation of splenocytes.

To clarify the biological properties of WEFM, mitogenic responses of mouse spleen cells after oral administration of WEFM was investigated. Splenocytes obtained from the control group or prescriptions-treated group were cultured with and without T cell mitogen (Con A) or B cell mitogen (LPS) for 48 h.

As shown in Figure 3, treatment with WEFM resulted in a significant increase in T cell and B cell mitogenic stimuli.

### 4. Phenotypic characterization of lymphocytes by WEFM

Next, we performed flow cytometric analysis to examine lymphocyte differentiation by WEFM. As



**Fig. 4.** Phenotypic characterization of lymphocytes by WEFM.

Female BALB/c mice were orally administered with WEFM for 2 weeks. One day after the last administration, mice were sacrificed and the splenocytes ( $1 \times 10^5$  cells/well) were stained with FITC- and PE-coniugated monoclonal anti-CD3, CD19 CD4, CD8, Mac-1, and NK1.1 antibodies. Stained cells were analyzed on flow cytometry using Cell Quest Software. A representative image of whole lymphocytes was sorted using the light scatters.

shown in Figure 4, the population of CD3, CD19, CD4, and CD8 positive cells increased in a dose-dependent manner in WEFM groups. However, we could not find any significant results from the population of Mac-1 and NK1.1 positive cells.

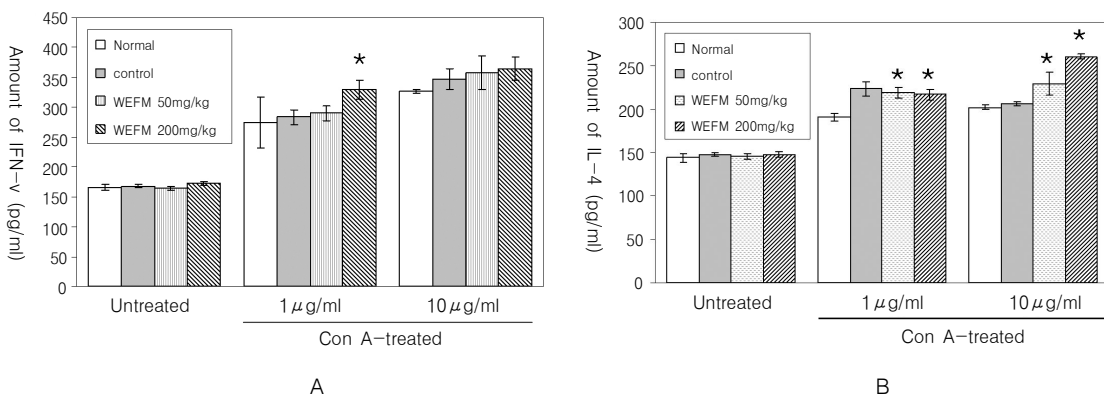
### 5. Effect of WEFM on the cytokine production

The next process examined whether or not the administration of WEFM can induce the production of Th1- and Th2-type cytokines by splenocytes of WEFM-treated mice. As shown in Figure 5, splenocytes from untreated control mice and WEFM-treated mice did not show any significant difference in cytokine production of both Th1 cytokine (IFN- $\gamma$ ) and Th2 cytokine (IL-4) without Con A stimulation. When splenocytes were incubated with Con A for 24 h, detectable changes of cytokine production were found in the cell-free supernatant. Oral administration of WEFM resulted in a significant enhancement of IFN- $\gamma$  production compared to the untreated control. Moreover, WEFM resulted in a significant decrease of IL-4 production. Thus, oral administration of WEFM may predominantly lead to the production of Th1 type cytokines.

## Discussion

Recent scientific research has validated the biochemical effects of many herbal prescriptions and thus, increased their application in clinical medicine. Among these herbal prescriptions, there has been a growing interest in the use of mushrooms and mushroom extracts as dietary and therapeutic supplements. Mushrooms have been used throughout Asia as traditional medicine to treat various human diseases. Recent findings, though they lack sufficient scientific data, have revealed immune system modulating effects of mushrooms and have attracted considerable attention to investigating various biochemical properties of mushrooms.

In Korea, complex mushroom extracts are commonly prescribed to treat cancer. In this study, the efficacy and mechanism of several types of well-known, edible mushrooms were investigated. First, we compiled a list of popular prescriptions of complex mushroom extracts and narrowed them down to six representative prescriptions. Then, XTT assay (colon 26-L5) was conducted and spleen proliferation assay *in vitro* to was investigated to find out its immunoenhancing and anti-tumor effects. Upon reviewing the results, it has been concluded



**Fig. 5.** Effect of oral administration of WEFM on production of IFN- $\gamma$  and IL-4 from splenocytes.

Female BALB/c mice were orally administered with WEFM for 2 weeks. One day after the last administration, mice were sacrificed and the splenocytes ( $1 \times 10^6$  cells/well) suspended in RPMI-1640 medium supplemented with 10% FBS were cultured with and without con A for 24 hr. Once cell culturing was completed, the cell-free supernatant was collected and the amount of IFN- $\gamma$  and IL-4 was measured by ELISA kits. The data is presented as the mean S.D. \*,  $p < 0.05$  compared to normal mice.

that water extracts from five mushrooms (WEFM), *Ganoderma frondosa*, *Coriarius versicolor*, *Codyceps militaris*, *Hericius erinaceus*, and *Lentinula edodes*, has a significant effect on T cell and B cell mitogenic stimulation and IFN- $\gamma$  by splenocytes production.

The water-soluble extract and the polysaccharides fraction of *Ganoderma lucidum* exhibited significant anti-tumor effects in several tumor-bearing animals by enhancing immune function. Alcohol-soluble extract and the triterpene fraction of *Ganoderma lucidum* possessed anti-tumor effects, which seems to be related to its cytotoxic activity against tumor cells<sup>10</sup>. PSK, extracted from *Coriolus versicolor*, may act by increasing leukocyte activation and inhibiting metalloproteinases and other enzymes involved in metastasis. Furthermore, it has been shown to have antioxidant ability, which not only enables it to defend the host from oxidative stress, but may also protect normal tissue from chemical and radio wave damage when used in combination with adjuvant or definitive chemotherapy and/or radiotherapy in the treatment of cancer<sup>11</sup>. The water-soluble extract of *Cordyceps militaris* fruiting bodies inhibited cancer cell proliferation by inducing cell apoptosis through the activation of caspase-3<sup>12</sup>. It also inhibits lung metastasis by the suppressing effects of basic-fibrous growth factor via anti-angiogenesis<sup>13</sup>. *Hericius erinaceus* induces IL-1 $\beta$  expression in macrophages at the transcriptional level by enhancing the activation of transcription factors, NF- $\kappa$ B, NF-IL6 and AP-1. It also induces iNOS gene expression followed by NO production in macrophages via enhancement of NF- $\kappa$ B activation<sup>14,15</sup>. The polysaccharide L-II isolated from the fruiting body of *Lentinus edodes* increases NO production and catalase activity in macrophages that stimulate with immune system responses. Anti-tumor activity of the polysaccharide L-II on mice-transplanted sarcoma 180 was mediated by modulation of the immune system to induce T-cells<sup>16</sup>. These previous findings have validated the immunoenhancing and anti-tumor effects of individual mushrooms and similar effects can be expected when used as a

complex mixture.

In the present study, oral administration of WEFM resulted in significant inhibition of lung metastasis after intravenous injection of colon 26-L5 cells in a dose-dependent manner (Figure 2) without causing any severe side effects such as body weight loss (data not shown). These outcomes indicate that WEFM is effective for early stage prevention of lung metastasis by colon 26-L5 cells. Additionally, oral administration of WEFM caused a marked augmentation of mitogen-stimulated splenocyte proliferation (Figure 3).

Th1/Th2 balance, controlled by Th1 or Th2 cells producing cytokines, plays an important role in anti-tumor and anti-metastatic immunity<sup>17,18</sup>. Several studies have shown that Th1-type cytokines increase the therapeutic efficacy of anti-tumor and anti-metastatic responses<sup>2,19</sup>.

Hence, to confirm these results, Th1 and Th2 cytokine from splenocytes were measured. Oral administration of WEFM resulted in the increased production of IFN- $\gamma$  and IL-4 by splenocytes stimulated with Con A compared with untreated controls (Figure 5). These findings indicate that oral administration of WEFM can lead to Th1-type and Th2-type immune responses. Therefore, WEFM may have anti-tumor activities via Th1, 2-type dominant immune responses.

These Th1, 2 type-dominant environments at the tumor site may differentiate many kinds of immune cells that induce systemic anti-tumor immunity. In light of the fact that natural products such as mushrooms and plants have been used as traditional medicines and now are potential sources of new drugs or nutraceuticals. Our study was to verify immunostimulating activity of WEFM; its action mechanism would contribute to the understanding of biological properties of WEFM and further consideration of the therapeutic applications.

In summary, anti-metastatic and immunomodulating activities of WEFM showed that the inhibitory effect of WEFM on lung metastasis is associated with its regulatory activities on the immune system.

As is so often the case, advanced-stage cancer patients almost always present complications of metastasis. In light of the results of this study, WEFM can be an effective prescription for preventing tumor metastasis, while having no apparent side effects. Henceforth, further investigation into the detailed mechanism and clinical studies of WEFM will be conducted to assess its potential as a metastasis-inhibitory agent.

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