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Antitumor and Immunostimulating Activities of *Elfvingia applanata* Hot Water Extract on Sarcoma 180 Tumor-bearing ICR Mice

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***Elfvingia applanata*, a medicinal mushroom belonging to Basidiomycota, has been used in the effort to cure cancers of the esophagus and stomach, and is also known to have inhibitory effects on hepatitis B virus infection. The hot water soluble fraction (as Fr. HW) was extracted from fruiting bodies of the mushroom. *In vitro* cytotoxicity tests showed that hot water extract was not cytotoxic against cancer cell lines such as Sarcoma 180, HT-29, HepG2, and TR at concentrations of 10~2,000 μg/mL. Intraperitoneal injection with Fr. HW resulted in a life prolongation effect of 45.2% in mice previously inoculated with Sarcoma 180. Treatment of Fr. HW resulted in a 2.53-fold increase in the numbers of murine spleen cells at a concentration of 50 μg/mL, compared with control. Incubation of murine spleen cells with Fr. HW at a concentration of 500 μg/mL resulted in improved immune-potwntiating activity of B lymphocytes through an 8.3-folds increase in alkaline phosphatase activity, compared with control. Fr. HW generated 12.5 μM of nitric oxide (NO) when cultured with RAW 264.7, a mouse macrophage cell line, at the concentration of 50 μg/mL, while lipopolysaccharide, a positive control, produced 15.2 μM of NO. Therefore, the results suggested that antitumor activities of Fr. HW from *E. applanata* might, in part, be due to host mediated immunostimulating activity.**

KEYWORDS : Anticancer activity, *Elfvingia applanata*, Hot water extract, Immunostimulating

Introduction

Cancer is one of the leading causes of death worldwide. Although medicines and technologies used in the effort to cure cancer have improved during the last three decades, the adverse side effects of chemotherapeutic treatment remain an unresolved problem. Therefore, strategies for development of new medicines have focused on effective natural products without side effects [1, 2]. Immunomodulatory and antitumor activities of polysaccharides and protein-bound polysaccharides extracted from a variety of higher fungi have been reported [3, 4]. Polysaccharides and protein-bound polysaccharides, such as schizophyllan, lentinan, and polysaccharide K (PSK), were isolated from *Schizophyllum commune*, *Lentinus edodes*, and *Trametes versicolor*, respectively. Effective inhibition of tumor cell growth and survival rate with low or without toxicity have been reported in Sarcoma 180 tumor-bearing mice treated with these compounds, which are used for immunopharmaceuticals for treatment of cancer related diseases in Asian countries [5-7]. In general, the antitumor activities are attributed to stimulation of the cell-mediated immune responses of the host without adverse effects [4].

Elfvingia applanata, mushroom, belonging to Polyporaceae of Basidiomycota, has long been used in Asian countries as a medicine for treatment of cancers of the esophagus and stomach, inflammation, rheumatism, and hepatitis B virus infection [8, 9].

In the present study, crude polysaccharides were extracted from fruiting bodies of *E. applanata* with hot water and antitumor and immuno-potentiating activities of the mushroom were investigated. The *in vivo* antitumor effect in Sarcoma 180 tumor-bearing mice and *in vitro* cytotoxic activities of 4 cancer cell lines were studied. In addition, for study of immunopotentiating activities, nitric oxide (NO) production, proliferation of splenocytes, and alkaline phosphatase (APase) activity in murine spleen cells were also investigated.

Meterials and Methods

Mushroom. Fresh fruiting bodies of *E. applanata* were collected in Seoul, Korea, in June, 2006. A pure culture was deposited in the Culture Collection and DNA Bank of Mushroom (CCDBM), Division of Life Sciences, University of Incheon, Korea, with acquired accession No.

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IUM-2378. After drying with hot air at 40°C for 48 hr, the fruiting bodies were pulverized.

Animals. Five-wk-old inbred male ICR mice (20~25 g) were purchased from Central Lab. Animal Inc., (Seoul, Korea). All mice were acclimated to the animal house for a period of 1 wk. Mice were housed under normal laboratory conditions (23 ± 2°C under 12 hr dark-light cycle (17:00~05:00) and a relative humidity of 50~60%. During the experimental period, mice received the standard basal diet, purchased from Central Lab Animal Inc., and water *ad libitum*.

Cell lines. Mouse Sarcoma 180, colon cancer (HT-29), and human hepatocellular liver carcinoma cell (HepG2) lines were purchased from Korean Cell Line Bank of Seoul National University, Seoul, Korea, and the thermoresistant radiation-induced fibrosarcoma cell line (TR) was obtained from the Central Laboratory of Inha University Hospital, Incheon, Korea. The HT-29 and HepG2 cell lines were cultured in Dulbecco's modified Eagle's medium and the TR cell line was cultured in Roswell Park Memorial Institution (RPMI-1640) medium supplemented with penicillin (100 U/m), streptomycin (100 mg/mL), and 10% fetal bovine serum at 37°C with 5% atmospheric CO_2 in a humidified incubator. Sarcoma 180 cells were maintained in ascitic form by serial transplantation every 7 days in an ICR male mouse.

Preparation of hot-water extracts from the mushroom. Pulverized fruiting bodies of *E. applanata* (200 g) were suspended in distilled water (3,000 mL). The suspension was then heated in a boiling water bath for 3 hr, and centrifuged to give supernatant and residue. The residue was then treated two more times in the same manner. All supernatants obtained were combined and mixed with 4 volumes of ethanol and allowed to stand overnight at 4°C. The precipitate formed was collected by centrifugation, dissolved in distilled water, dialyzed for 48 hr at 4°C, and lyophilized. This fraction, referred to as the hot-water extract (Fr. HW), was preserved at −40°C for later use.

Cytotoxicity by MTT assay. Rapid colorimetric methods previously described by Mosmann [10] were used in evaluation of the MTT assay, a measurement of cell viability and proliferation. Briefly, for the MTT assay, 100 μL of cells of HT-29, HepG2, and TR (1×10^5 cells/well) were treated with different concentrations of the hot water extract (10, 100, 1,000, and 2,000 μg/mL) of *E. applanata* and cultured for 24 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. Thereafter, 10 μL of 5 mg/mL of 3-(4, 5-dimethyl-1-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution was added, followed by incubation at 37°C with 5% atmospheric CO_2 for 4 hr under dark conditions. Following removal of the supernatant, purple formazan crystals produced were dissolved in 100 μL of dimethylsulfoxide, and quantified by measurement of optical density (OD) at 570 nm using a microplate reader. For the MTT assay of Sarcoma 180, 50 μL of Sarcoma 180 cells (2×10^5 cells/well) were treated with different concentrations of the hot water extract (10, 100, 1,000, and 2,000 μg/mL) and cultured for 24 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. Then, 1 mg/mL of 2,3-bis(2-methoxyl-4nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) solution was mixed with 30 μL of 25 μM phenazine methosulfate, followed by incubation at 37°C with 5% atmospheric CO_2 for 2 hr under dark conditions. OD was then measured using a microplate reader at 450 nm. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells that served as control. All experiments were replicated three times and mean values are presented.

***In vivo* assay of antitumor activity.** Antitumor activity of hot water extract was assayed against mouse Sarcoma 180 cells (ascitic type, 5×10^5 cells) implanted in a 6-wk-old ICR mouse. The test sample was dissolved in phosphate buffered saline (PBS, pH 7.4; Gibco BRL., Gaitherburg, MD, USA) and filtered through a 0.22 μm of membrane filter (Millipore Co., Bedford, MA, USA), followed by intraperitoneal injection in mice for 10 consecutive days at a dose of 20 mg/kg, starting from 24 hr before and after tumor implantation. Antitumor activity of the hot water extract against Sarcoma 180 tumor-bearing ICR mice was evaluated according to the increase in life span (ILS). The method previously described by Geran *et al*. [11] was used for calculation of ILS. $ILS = [(T - C)/C)] \times 100(\%)$, where T is the mean of survival day (MSD) of the treated groups and C is the MSD of the control group. Survival of mice was evaluated every day after tumor implantation until death. Experiments with mice were conducted in accordance with procedures and policies approved by the Animal Care and Ethics Committee at the University of Incheon.

Proliferation of murine spleen cells. The WST-1 assay was performed to test for proliferation of murine spleen cells [12]. Six-wk-old ICR male mice were sacrificed by cervical dislocation, followed by aseptic removal of the spleen and grinding of the spleen using a 100-mesh sieve (Bellco Glass Inc., Vineland, NJ, USA). Two volumes of lymphocyte separation medium (PAA laboratory Gmbh, Pasching, Austria) were added to the extracted solution, which was then centrifuged for 20 min at 400 ×g. Monocyte cells of spleen were selectively separated and centrifuged 3 times for approximately 5 min at 300 ×g. The spleen cells (2×10^5 cells/mL) were then added to RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum, followed by treatment with different concentrations

of the hot water extract (50, 200, and 500 μg/mL) and incubated for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO_2 under dark conditions. In the same manner, lipopolysaccharide (LPS), a positive control, was incubated with different concentrations of 5 and 10 μg/mL. Thereafter, 10 μL of a 5 mg/mL concentration of WST-1 assay solution was added to each well, followed by incubation for 4 hr at 37°C with 5% CO_2 under dark conditions. OD was measured using a microplate reader at 440 nm.

APase activity in murine spleen cells. A method previously described by Ohno *et al*. [13] was used for measurement of APase activity of murine spleen cells. Six-wk-old ICR male mice were sacrificed by cervical dislocation and cell suspension of the spleen was prepared aseptically. Various concentrations of the hot water extract (50, 100, and 200 μg/mL) were applied to 100 μL of spleen cells (1×10^6 cells/well), followed by incubation for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. Differing concentrations of LPS (5 and 50 μ/mL) were applied to 100 μL of spleen cells (1×10^6 cells/well), followed by incubation for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. Cell suspensions were collected and freezed-thawed, followed by addition of 50 mM of sodium carbonate buffer (pH 9.8) containing *p*-nitrophenyl-phosphate (0.1 mg/ml) and $MgCl_2$ (1 mM) to 10 μL of the cell lysate. The reaction mixture was incubated for 1 hr at 37°C with 5% atmospheric CO_2 and was terminated by addition of 500 μL of 0.3 N ice cold NaOH. Absorbance was measured at 405 nm. APase activity of spleen cells was expressed as the stimulation index (AL.P SI). AL.P SI = mean OD in the treated group/mean OD in control group.

NO production by RAW 264.6 macrophages. The method described previously by Choi *et al*. [14] was used for assessment of NO production in the culture supernatants of RAW 264.7. Briefly, 100 μL of RAW 264.7 cells (1×10^5 cells/well) treated with various concentrations of the hot water extract (50, 100, and 200 μg/mL) were incubated for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. LPS, the positive control, was applied to 100 μL of RAW 264.7 cells (1×10^5 cells/well) at concentrations of 1, 10, and 50 μg/mL, followed by culture for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. Then, an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthlethylenediamine dihydrochloride in 2.5% phosphoric acid) was mixed with the culture and allowed to stand for 10 min. OD was measured using a microplate reader at 540 nm. Nitrite concentration was calculated from a standard curve prepared with known concentrations of sodium nitrite. Each experiment was replicated three times.

Results and Discussion

Cytotoxicity by MTT assay. For the MTT assay, cytotoxicity was expressed as the survival fraction compared with untreated control cells. For evaluation of cell viability and proliferation, 100 μL of cells of HT-29, HepG2, TR, and Sarcoma 180 cell lines (1×10^5 cells/well) were treated with different concentrations of Fr. HW (10, 100, 1,000, and 2,000 μg/mL) of *E. applanata*, followed by incubation for 48 hr in 96-well microplates. Viabilities of HT-29 and Sarcoma 180 cell lines cultured with 10 μg/mL of Fr. HW

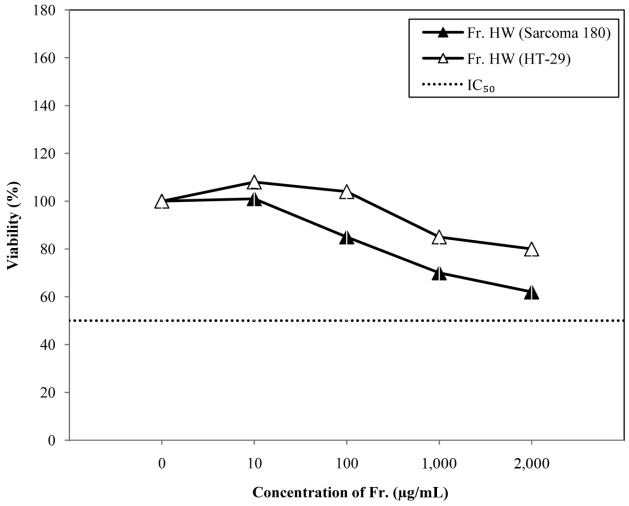

Fig. 1. *In vitro* cytotoxicity of hot water extract from fruiting bodies of *Elfvingia applanata* against Sarcoma 180 and HT-29. Cytotoxicity was measured after 48 hr of incubation. Concentration of cells was 1×10^5 cells/mL. Fr. HW, hot water soluble fraction.

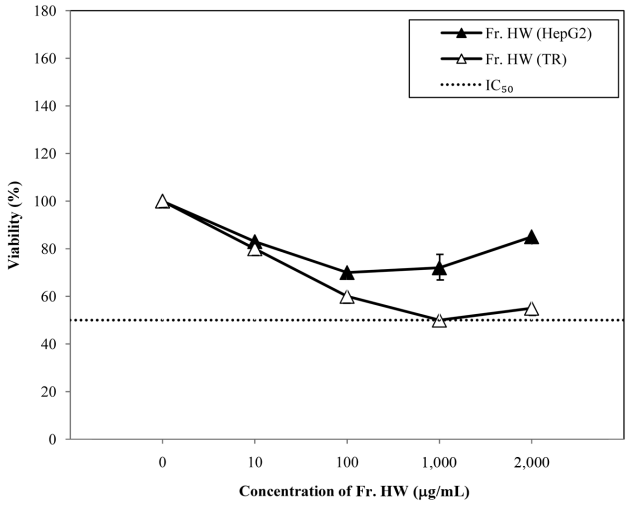

Fig. 2. *In vitro* cytotoxicity of hot water extract from fruiting bodies of *Elfvingia applanata* against HepG2 and TR. Cytotoxicity was measured after 48 hs of incubation. Concentration of cells was 1×10^5 cells/mL. Hot water was used for extraction of Fr. HW.

ranged from 100~110%, whereas those of the same cell lines cultured with 2,000 μg/mL were 62 and 83% (Fig. 1). Viabilities of TR and HepG2 cell lines cultured at 10 μg/mL of Fr. HW were 82% and 83%, whereas those of the same cell lines incubated at the concentration of 1,000 μg/mL were 50% and 75% (Fig. 2). Thus, the viabilities of Sarcoma 180 and HT-29 cell lines incubated for 48 hr at 10~2,000 μg/mL of concentration ranged from 61~110% and treatment with Fr. HW resulted in gradual inhibition of Sarcoma 180 and HT-29 cell lines in a dose-dependent manner. Viabilities of TR and HepG2 cell lines cultured in 10 μg/mL of Fr. HW ranged from 82~84%, while those of cell lines cultured in 1,000 μg/mL were 51 and 72%. Thus, the viabilities of Sarcoma 180 and HT-29 cell lines cultured for 48 hr at concentrations of 10~2,000 μg/mL of Fr. HW ranged from 61~110%. According to the results, Fr. HW of *E. applanata* had no significant cytotoxic effect on 4 cancer cell lines tested at concentrations of 10~2,000 μg/mL.

***In vivo* assay of antitumor activity.** Antitumor activity of hot water extract of fruiting bodies of *E. applanata* was tested against Sarcoma 180 tumor-bearing mice; the results are summarized in Table 1. The MSD for the control group was 15.5 days, while the MSD of the Fr. HW treated group was 22.5 days at a dose of 20 mg/kg. The life span of Sarcoma 180 tumor-bearing mice was increased by 45.2%, compared with the control group. Shim *et al*. [15] reported that treatment with methanol extract of *Paecilomyces sinclairii* resulted in inhibited growth of Sarcoma 180 tumor cells and prolongation of the life span of mice by 32.3%, compared with the control. Lee *et al*. [16] reported that the life span of Sarcoma 180 tumor-bearing ICR mice was increased by 66.7%, compared with control group, when injected with methanol extract isolated from fruiting bodies of *Tremella aurantialba*. In general, the criteria for judging the antitumor effect of any substances include prolongation of the life span by more than 25% [17]. According the results, the mean life span of the treated group with Fr. HW showed a significant increase and the fruiting bodies of *E. applanata* might contain effective antitumor substances against Sarcoma 180 tumor-bearing mice.

Table 1. The effects of hot water extract isolated from fruiting bodies of *Elfvingia applanata* on the life span of Sarcoma 180 tumor-bearing ICR mice (i.p.)

Group	Dose (mg/kg body weight)	Survival days	ILS (%)
Control	0	15.5 ± 0.00	-
Fr. HW	20	22.5 ± 2.35	45.2

Each experimental group consisted of 8 mice.
Survival days for each mouse in the experimental group were measured individually and the mean survival days (mean ± SE) were calculated for each group.
Hot water was used for extraction of Fr. HW.
ILS: increase of life span; i.p., intraperitoneal injection; Fr. Hw, hot water soluble fraction.

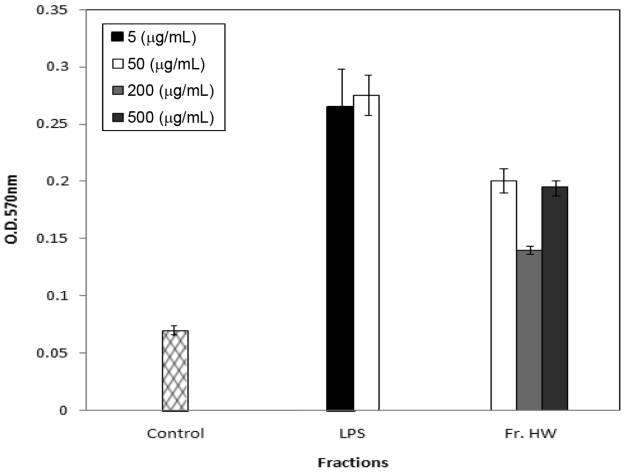

Fig. 3. Effect of hot water extract from fruiting bodies of *Elfvingia applanata* on proliferation of murine spleen cells. Concentration of spleen lymphocytes was 2×10^5 cells/mL. Proliferation of murine spleen cells was measured after 48 hr of incubation using the MTT method. Lipopolysaccharide (LPS) was purified from *Escherichia coli* 0111:B and was used as the positive control. Hot water was used for extraction of hot water soluble fraction (Fr. HW). OD, optical density.

Proliferation of murine spleen cells. Six-wk-old mice were sacrificed by cervical dislocation and cell suspension of the spleen was prepared aseptically from mice. Various concentration of the hot water extract (50, 100, and 200 μg/mL) were applied to 100 μL of spleen cells (1×10^6 cells/well), followed by incubation for 48 hr in 96-well microplates. The effects of Fr. HW on proliferation of murine spleen cells were evaluated. As shown in Fig. 3, treatment of murine spleen cells with LPS resulted in a significant, 3.2-fold, increase in cell numbers, and treatment with Fr. HW resulted in a 2.53-fold increase in cell numbers, compared with the control, at a concentration of 50 μg/mL. Li *et al*. [18] reported that proteoglycan extracted from crude liquid culture medium and mycelia of *Phellinus nigricans* stimulated proliferation of lymphocytes of spleen cells and also increased production of tumor necrosis factor-α. Murine spleen cells are the main residence of various immune cells and are also important for host immune response. According the results, it is concluded that treatment with Fr. HW can improve the immune response of the host via stimulating proliferation of immune-organ, murine spleen cells.

APase activity in murine spleen cell. Six-wk-old mice were sacrificed by cervical dislocation and cell suspension of

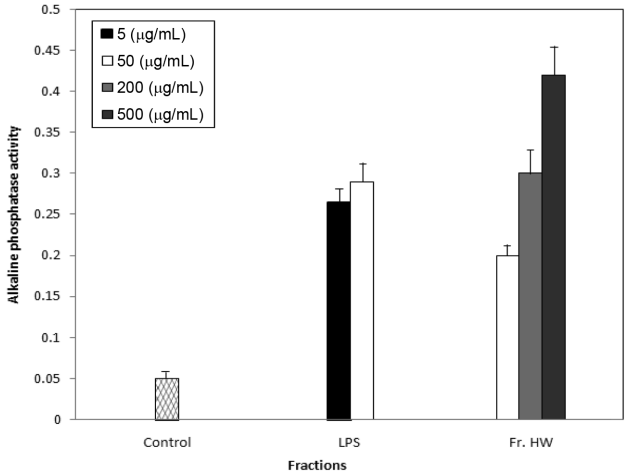

Fig. 4. Effect of hot water extract from fruiting bodies of *Elfvingia applanata* on alkaline phosphatase activity in murine spleen cells. Lipopolysaccharide (LPS) was purified from *Escherichia coli* 0111:B and was used as the positive control. Hot water was used for extraction of hot water soluble fraction (Fr. HW).

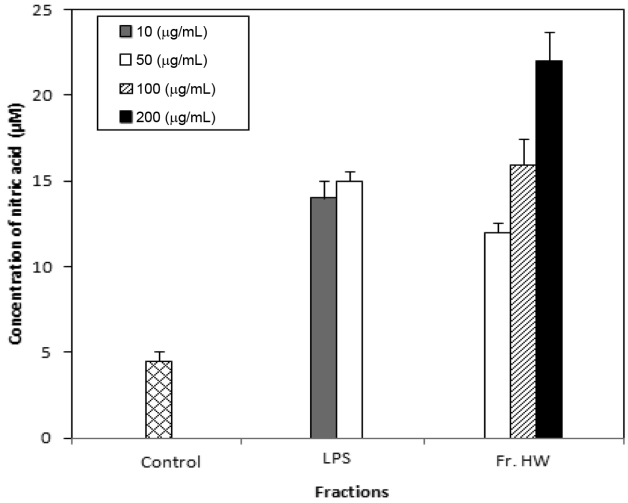

Fig. 5. Effect of hot water extract isolated from fruiting bodies of *Elfvingia applanata* on nitrite oxide production in RAW 264.7 macrophages. Concentration of RAW 264.7 cell was 1×10^6 cells/mL. Lipopolysaccharide (LPS) was purified from *Escherichia coli* 0111:B and was used as the positive control. Fr. HW, hot water soluble fraction.

the spleen was prepared aseptically. Various concentrations of Fr. HW (50, 100, and 200 μg/mL) were applied to 100 μL of spleen cells (1×10^6 cells/well), followed by incubation for 48 hr in 96-well microplates. Stimulation of splenic lymphocytes with LPS and Fr. HW at 50 μg/mL resulted in a 5.67- and 3.72-fold increase of APase activity, respectively, compared with control (Fig. 4). However, APase activity in splenocytes stimulated with 5 μg/mL of LPS was 1.42-folds higher than that of splenocytes stimulated with Fr. HW at 5 μg/mL. Murine spleen cells stimulated with LPS and Fr. HW showed a positive concentration-dependent increase in APase activity. Lee *et al.* [16] reported that APase activity were increased by 1.1~1.16-folds when stimulated with crude polysaccharides of *Tremella auratialba* at concentrations of 200~500 μg/mL. Therefore, it is concluded that treatment with Fr. HW could result in improved immunostimulating activity of the host via increasing alkaline phosphatase activity.

NO production by RAW 264.7 macrophages. NO production in culture supernatants of RAW 264.7 macrophage was evaluated using 100 μL of RAW 264.7 cells (1×10^5 cells/well) treated with various concentrations of Fr. HW (50, 100, and 200 μg/mL). In the control group, 4.5 μM of NO was released in RAW 264.7 macrophages, while 14.8 μM and 15.2 μM of NO were produced by treatment with LPS at concentrations of 10 and 50 μg/mL (Fig. 5). NO produced by Fr. HW were 12.5 μM, 16.3 μM, and 22.4 μM at concentrations of 50 μg/mL, 100 μg/mL, and 200 μg/mL, respectively. Thus, release of NO by RAW 264.7 macrophages activated by Fr. HW resulted in gradually increased production of NO with increasing concentrations of Fr. HW. Kim *et al.* [19] reported RAW 264.7 macrophages stimulated by polysaccharides extracted from *Phellinus linteus* increased the production of NO by dose-dependent manner. According to the results, treatment of RAW 264.7 macrophages with Fr. HW can result in increased production of NO and improvement of the immune response of mice. Ooi and Liu [1] reported that polysaccharides extracted from mushrooms exert anti-tumor effects through activation of different immune responses in the host rather than by direct killing of tumor cells. Our results also showed that hot water extract isolated from fruiting bodies of *E. applanata* had no significant direct cytotoxic effect against 4 cancer cell lines; rather, it has immunopotentiating activities. Therefore, further studies are needed for elucidation of major components of fruiting body of *E. applanata* involved in immunostimulation of mice.

Acknowledgements

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