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Inhibitory Effect of the Culture Broth Extract of *Aspergillus tamarii* on Nitric Oxide Production and Its Antioxidative Activity

Ki Man Lee, Geum Seon Lee, Hong Shim, Seung Hyun Kim, Sung Hee Nam[1], and Tae Jin Kang*

College of Pharmacy and Institute of Chronic Disease, Sahmyook University, Seoul 139-742, Korea

[1]Department of Agricultural Biology, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Korea

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Many studies have explored suppression of aflatoxin produced by *Aspergillus* Genus. On the other hand, this study examined the inhibitory effect of the culture broth extract (CE) of *A. tamarii* obtained from dead silkworm on nitric oxide (NO) production and its antioxidative activity. The culture broth was extracted with EtOAc, dried, and then used in this experiment. As a result, CE did not show cytotoxicity on RAW 264.7 cells at any concentration. Moreover, CE suppressed lipopolysaccharide (LPS)-induced NO production of RAW 264.7 cells in a dose-dependent manner. The total phenol content according to the Folin-Dennis method, the antioxidative activity by DPPH, and the nitrate radical scavenging capacity of CE were increased in a dose-dependent manner. Thus, many of the phenolic compounds were considered to represent the antioxidative activity.

Key words: *Aspergillus tamarii*, Nitric Oxide, Cytotoxicity, Antioxidative activity

Introduction

Entomopathogenic fungi called 'Dongchunghacho' in Korea usually refer to *Ascomycota* and *Cordyceps* among perfect fungi. However, in evolving research, approximately 800 species included in *Deuteromycotina* are well known (Samson *et al*., 1988; Sung *et al*., 1998; Nam *et al*., 1999). *C. militaris* and *Irasia tenuipes* (*Paecilomyces tenuipes*) were well studied species among a variety of entomopathogenic fungi because these two species have the multiple advantages in that their artificial cultivation is relatively simple and they are easily found from insects in nature (Lee *et al.,* 2008; Huang *et al*., 2006).

On the other hand, the *Aspergillus tamarii* used in this study was difficult to find in insects. In addition, most of the studies of *A. oryzae*, *A. tamarii*, *A. parisiticus*, and *A. flavus* have focused on suppression of aflatoxin produced by *Aspergillus* Genus (Kang *et al*., 2000). Especially, aflatoxin B1 was reported to be a possible carcinogen (Martins *et al*., 2008; Horn *et al*., 2009). However, in Korea, the fungi of *Aspergillus* called 'Nuruk-gompangi' are frequently used as ingredients of fermented foods such as soy sauce, soybean paste, and red pepper paste (Kim *et al*., 2011). In this study, in consideration of economic aspects, the culture broth of the by-product of *A. tamarii* isolated from dead silkworms was used. A variety of physiological effects of *A. tamarii* were measured.

Materials and Methods

Reagents and solvents

Potato dextrose broth (PDB) and potato dextrose agar (PDA) were obtained from Difco (Detroit, MI, USA). 1,1-diphenyl-2-picryhydrazyl (DPPH), Folin-Ciocalteau (FC) reagent, tannic acid, sulfanilamide, N-(1-naphtyl) ethylenediamine dihydrochloride (NED), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), acetic acid, ascorbic acid, lipopolysaccharide (LPS), and penicillin-streptomycin (P/S) were obtained from Sigma (St. Louis, MO, USA). Sodium nitrate ($NaNO_2$) and ethyl acetate (EtOAc) were obtained from Junsei (Tokyo, Japan). Sodium carbonate (Na_2CO_3) and phosphoric acid (H_3PO_4) were obtained from

*To whom the correspondence addressed
#403, The 3rd Science Hall, Sahmyook University, Gongneung 2-dong, Nowon-gu, Seoul 139-742, Korea
Tel: 82-2-3399-1608; Fax: 82-2-3399-1617
E-mail: kangtj@syu.ac.kr
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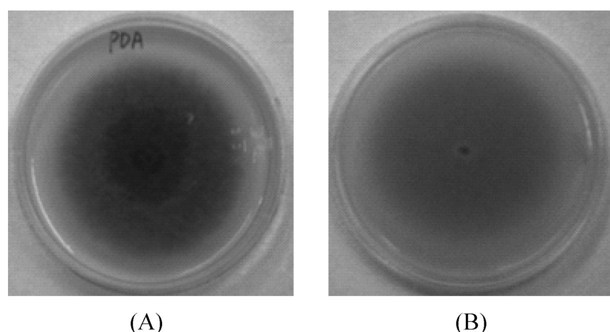

Fig. 1. Culture characteristics of *Aspergillus tamarii* on PDA medium. A. Colonies at 30°C for 7 days in the dark; B. reverse.

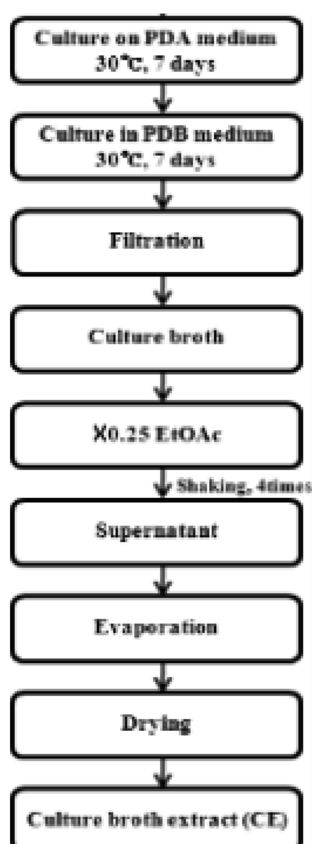

Fig. 2. The scheme for the preparation of culture broth extract (CE).

Samchun Chemical (Seoul, Korea). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Hyclone (Logan, UT, USA).

The strain and extraction

Aspergillus tamarii J64 was obtained from National Academy of Agricultural Science, Rural Development Administration (RDA) in Suwon, Korea (Nam *et al*., 2002). The strain was transferred on PDA medium and incubated at 30°C for 7 days in the dark (Fig. 1); then 250 milliliters of PDB media were sterilized in a 500 ml flask and inoculated with the inoculum. Each flask including *A. tamarii* J64 was incubated in the dark at 30°C in the shaking incubator. After one week, culture products were separated as mycelia and culture broth using a vacuum filtration system. The culture broth was shaken 4 times with EtOAc (1:0.25, v/v) in a separatory funnel. The supernatant was evaporated at 60°C, dried, and then used as the culture broth extract (CE) in the experiment (Fig. 2).

Cell viability

Murine macrophage RAW 264.7 cells were grown in DMEM with 10% FBS and 1% P/S. The cytotoxic effects of CE were determined using the MTT assay. 5×10^4 cells/well in 96 well plates were treated with CE and incubated for 24 hr. The supernatant was then removed and 100 µl of MTT solution (5 mg/ml in a PBS) was added to each well, and the plate was continuously cultured for 4 hr. After incubation, supernatant was replaced with 100 µl of DMSO and the absorbance was measured at 540 nm using a microplate reader.

Nitric oxide product

The RAW 264.7 cells were treated with CE and LPS. The supernatants were collected and used for Griess reaction. 50 µl of supernatants were mixed with 100 µl of Griess reagent (50 µl of 1% sulfanilamide in 5% H_3PO_4 and 50 µl of 0.1% NED). After 5 min, the absorbance was determined at 540 nm using an ELISA reader. The NO product was determined using the standard $NaNO_2$ calibration curve.

Total phenol content

Total phenol content was measured using the Folin-Dennis method. In brief, 50 µl of the samples were added to 50 µl of 1 N FC reagent in 96 well plates. After 3 min of shaking, 50 µl of 10% Na_2CO_3 reagent was added and the mixture was then allowed to stand for 1 hr in the dark. The absorbance was measured at 700 nm using an ELISA reader. Tannic acid was used as a standard for the total phenol content.

DPPH free radical scavenging capacity

Free radical scavenging activity was investigated as the degree of coloration using reduction of DPPH. DPPH solution (2×10^{-4} M) was prepared in 50% EtOH, and 180 µl of this solution was mixed with 20 µl of 10, 20, 50, 100, 200, 500 and 1000 µg/ml diluted CE in 96 well plates, respectively. The mixtures were then incubated at room temperature for 30 min in the dark and the absorbance of each sample was measured at 515 nm against 50% EtOH as blank. The capacity was calculated as the percentage of the absorbance value of the extract solution and control.

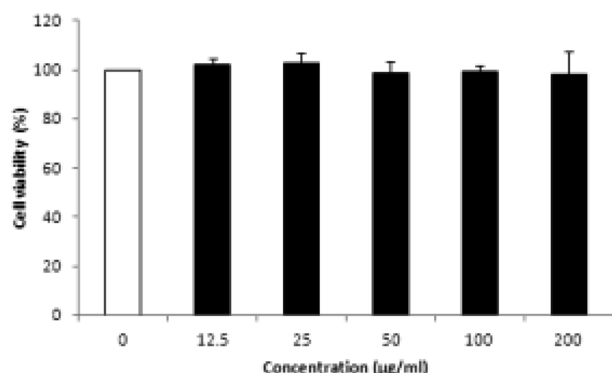

Fig. 3. Effect of CE on RAW 264.7 cell viability. RAW 264.7 cells were treated with 12.5 to 200 μg/ml of CE for 24 hr. Data are representative of at least two independent experiments in duplicate. Data are expressed as means ± SD of MTT assay.

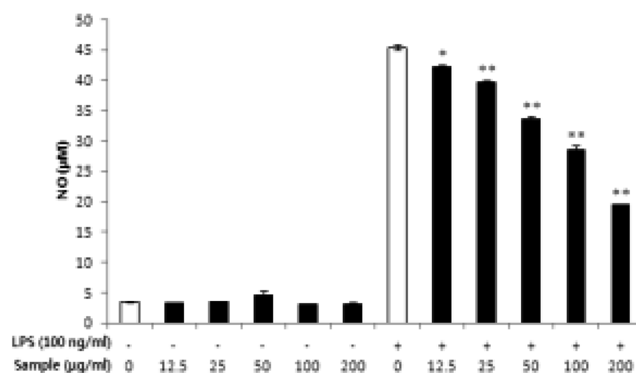

Fig. 4. Inhibitory effect of CE on NO production in RAW 264.7 cells. RAW 264.7 cells were treated with 12.5 to 200 μg/ml of CE. Data are representative of at least two independent experiments in duplicate. Data are expressed as means ± SD. **p<0.01, *p<0.05 versus LPS alone based on Student's t-test.

Nitrate scavenging capacity

100 μl of 1 mM $NaNO_2$ solution was mixed with 100 μl of diluted CE and then incubated at 37°C with 0.1 N HCl (pH 1.2, 800 μl). After 1 hr, 100 μl of this mixture was mixed with 2% acetic acid (500 μl) and Griess reagent (40 μl). Mixed solution of 200 μl into the 96 well plate and incubated at room temperature for 30 min in dark conditions. The absorbance of the sample was measured at 520 nm against the blank. The activity was calculated as the percentage of the absorbance of the extract solution and control.

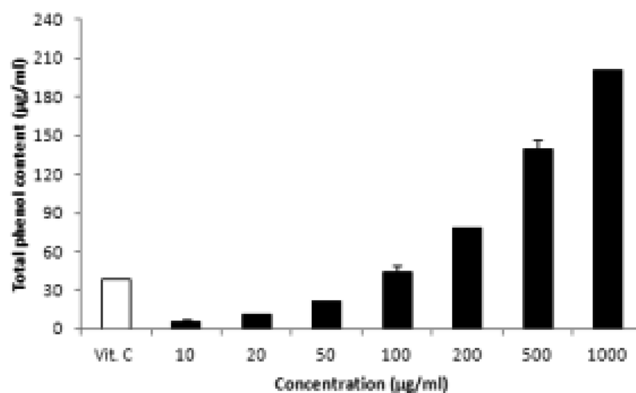

Fig. 5. Total phenol content of CE. Vitamin C (Vit. C, 10 μg/ml) was used as a control. Data are representative of at least two independent experiments in duplicate. Data are expressed as means ± SD.

Results and Discussion

Cytotoxicity

Cytotoxicity was measured by MTT assay (Hwang *et al.*, 2011). This method is relatively easy for measuring cell viability. The final concentrations of CE were adjusted to 12.5, 25, 50, 100, and 200 μg/ml and cytotoxicity was measured using RAW 264.7 cells. There was no cytotoxicity of CE on the cells even at high concentration (Fig. 3). Thus, the experiments involving NO production in RAW 264.7 cells were performed at high concentrations.

NO production in RAW 264.7 cell

When pathogen enters the human body, this event is first recognized by macrophages and dendritic cells, which are antigen-presenting cells (APCs). Also, various immune responses induce inflammatory responses. Inflammatory responses are controlled by cytokines and chemokines such as interleukin-1 (IL-1), IL-6, IL-8, IL-12, and tumor necrosis factor-α (TNF-α). IL-1 and IL-6 induce the production of proteins that lead to elevated body temperature. TNF-α induces an increase in the permeability of local vascular endothelial cells and enhances the movement of cells from the vasculature into the tissues (Kumar *et al.*, 2007; Doan *et al.*, 2008). The expression of inducible nitric oxide synthase (iNOS) induces NO production and inflammation (Chang *et al.*, 2009; Kindt *et al.*, 2007). Thus, the inhibitory effect of CE on nitric oxide production was measured to examine the role of CE as an anti-inflammatory material. CE dose-dependently suppressed NO production in RAW 264.7 cell activated with LPS. However, CE itself did not induce NO production (Fig. 4).

Total phenol content

The total phenol content was measured spectrophotometrically as in the Folin-Dennis method, Prussian blue method, and Vanillin HCl method (Whang *et al.*, 2001). The total phenol content of CE was determined using the Folin-Dennis method and the standard tannic acid cali-

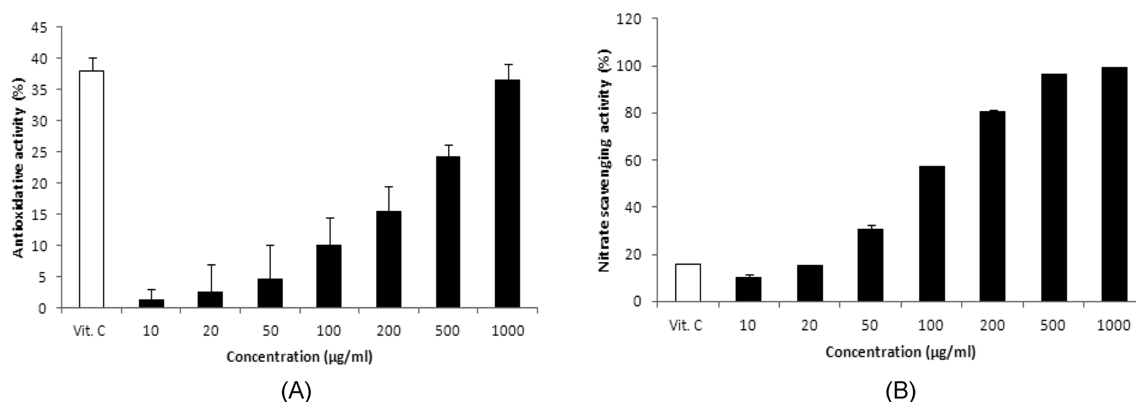

Fig. 6. Antioxidative activity of CE. Vitamin C (Vit. C, 10 μg/ml) was used as a control. Data are representative of at least two independent experiments in duplicate. Data are expressed as means ±SD. (A). DPPH free radical scavenging capacity; (B). Nitrate scavenging capacity.

bration curve. The results showed that the total phenol content of CE was increased in a dose-dependent manner (Fig. 5). The secondary metabolites of natural products contain flavonoid, terpenoid, alkaloid, phenolic compounds (Lee *et al*., 2000). Phenolic compounds comprise many of the biologically active components, and diverse research using these materials is required (Liu, 2011).

Antioxidative activity

The antioxidative activity was measured by DPPH and nitrate radical scavenging capacity. The results showed that the antioxidative activity of CE was increased in a dose-dependent manner and were consistent with the results for total phenol content (Fig. 6). It seems that the antioxidative capacity resulted from the content of phenolic compounds. Synthetic antioxidants have serious side effects and replacements are therefore required (Choe and Yang, 1982). The results of this study suggest that components of CE have antioxidative activity and their effects will be further analyzed.

Acknowledgements

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