The antioxidant and chemopreventive potentialities of *Mosidae* (*Adenophora remotiflora*) leaves

Ae-Jung Kim¹§, Myung-Ryun Han¹, Myung-Hwan Kim², Myoungsook Lee³, Taek-Joon Yoon⁴ and Sang-Do Ha⁵

¹Department of Food and Nutrition, Hyejeon College, San 16, Namjang-ri, Hongsung-eup, Hongsung-gun, Choongnam 350-702, Korea
²Department of Food Engineering, Dankook University, Cheonan 330-714, Korea
³Department of Food and Nutrition, Sungshin Women’s University, Seoul 130-742, Korea
⁴Department of Food and Nutrition, Yuhan University, Bucheon 442-749, Korea
⁵Department of Food Science and Technology, Chung-Ang University, Anseong 456-756, Korea

Abstract

Our study focused on the antioxidant activities of Mosidae leaf ethanol extract (MLE) and included measurements of reducing power, total phenolic compounds, DPPH radical scavenging activity, and hydroxyl radical scavenging activity. In order to determine whether or not MLE evidences any chemopreventive activities, experimental lung metastasis was induced via the i.v. inoculation of colon26-M3.1 carcinoma cells into BALB/c mice. Additionally, we attempted to characterize any possible cytotoxic effects in murine normal splenocytes and tumor cells (B16-BL6 and colon26-M3.1). The total phenolic content and reducing capacity were measured at 39 mg/100 mL and 1.24, respectively, whereas the DPPH and hydroxyl radical scavenging activities of MLE were measured to be 88.89% and 22.10%, respectively. Prophylactic i.v. treatment with MLE resulted in a dose-dependent and significant inhibition of lung metastasis. Specifically, a MLE dose of 200 ug per mouse resulted in an 88.90% inhibition of lung metastasis. For the cytotoxicity assay, MLE doses up to 100 ug/mL were not shown to affect the growth of normal murine splenocytes. Additionally, the survival of normal cells was not affected at MLE doses below 500 ug/mL. However, MLE doses up to 500 ug/mL reduced the percentage of tumor cell growth for B16BL6 (67% alive) and colon26-M3.1 (62% alive) cells.

Key Words: Mosidae (*Adenophora remotiflora*) leaves, antioxidant activity, cytotoxicity assay, tumor metastasis

Introduction

Plants generate a broad variety of biologically active substances, some of which are associated with special flavors or tastes, and others that evidence antioxidant and/or antimicrobial activities. Antioxidants reduce damage to cells and biomolecules attributable to reactive oxygen/nitrogen species (ROS/RNS) [1]. In the past, both ROS and RNS have been implicated in the oxidative destruction of food products as well as in the pathogenesis of several human diseases including atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders including Alzheimer’s disease, and certain types of cancer [2,3]. Among the dietary antioxidants, phenolic compounds and secondary metabolites from plants are the most abundant [1].

A variety of natural products with biological properties that enhance the host defense system have been previously applied as cancer immunotherapies [4-7], where they are becoming increasingly viewed as alternatives to cytotoxic drugs [8]. This is relevant, considering that innate immune system activation may perform a critical role in defense against foreign antigens including tumors [9].

*Mosidae* (*Adenophora remotiflora*) is a perennial, wild plant of the *Campanulaceae* family, which inhabits the Korean peninsula. *Mosidae* has been traditionally utilized in Korea as a Complementary and Alternative Medicine (CAM) for the treatment of certain diseases including expectoration, chills, fever, poisoning, and phlegm discharge [10]. The principal constituents of Mosidae leaves (ML) are the organic acids, saponin and inulin, and unlike individual constituent crude drugs its efficacy and quality can be altered. Additionally Mosidae has also been reported to exert antioxidant effects [11] in a few *in vitro* and *in vivo* studies [12]. However, the anti-metastatic effects of Mosidae have yet to be clearly elucidated.

This study was supported by a grant from the Agricultural R & D Promotion Center (100605-03-1-HD120).

¹ Corresponding Author: Ae-Jung Kim, Tel. 82-41-630-5249, Fax. 82-41-630-5175, Email. aj5249@naver.com

Received: October 9, 2009, Revised: February 1, 2010, Accepted: February 5, 2010

©2010 The Korean Nutrition Society and the Korean Society of Community Nutrition

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
In this study, we evaluated the antioxidant activity of *Mosidae* leaves ethanol extract (MLE) which was extracted with 70% ethanol solution in water bath at 30°C for 1 hour, by assessing its reducing power, total phenolic compounds, DPPH radical scavenging activity, and hydroxyl radical scavenging activity. We also evaluated the anti-metastatic activity of MLE through the prophylactic inhibition of lung metastasis of colon26-M3.1 carcinoma cells. The cytotoxic effects of MLE were evaluated using an experimental metastasis model featuring murine splenocyte and tumor cells (B16BL6 and colon26-M3.1).

**Materials and Methods**

**Materials**

Two-month-old fresh (a) and vacuum freeze-dried (b) *Mosidae* (*Adenophora remotiflora*) leaves were donated by Eco-Sprout Co, Ltd. (Gyeonggido, Korea), an environmentally-friendly agricultural company (Fig. 1). The material was filtered through a 0.2 um micro filter and stored at 4°C until use.

The proximal compositions of fresh *Mosidae* leaves (ML), moisture, protein, fat and ash (%), were determined by following AOAC [13] procedures.

**Antioxidant activities**

**Total phenolic compound content**

Total phenolic content of the MLE was measured using Folin-Ciocalteau reagent [14,15]. Folin-Ciocalteau reagent was diluted by 2 times using deionized water. The 2.0 mL of MLE solution (25 mg/mL) was mixed with diluted reagent (0.4 mL) and 2.0 mL of 2% sodium carbonate solution. Then mixed solution was held at room temperature for 30 min. After the absorbance of the solution was determined at 750 nm by an UV/visible spectrophotometer (UV-1601, Shimadzu, Tokyo, Japan). Standard curve was drawn using a tannic acid and calculated from it.

**DPPH free radical scavenging activity**

The free radical scavenging activities of MLE were evaluated according to the method described by Huang *et al.* [16], albeit with slight modification. Briefly, 0.2 mL of MLE was mixed with 1.0 mL of 0.2 mM DPPH ethanol solution. After 30 min at room temperature, absorbance values were measured by a UV-visible spectrophotometer set at 517 nm. The percent inhibition of DPPH radical by the sample extract was calculated by the following formula: DPPH free radical scavenging activity (%) = (1- As/Ab)×100, where Ab is the blank absorbance and As is the absorbance of the extract sample [16]. α-Tocopherol and BHT were used as positive standards.

**Reducing power**

The reducing power was determined according to the method of Oyaizu [14]. The 1.0 mL of sample solution was mixed with 0.5 mL of 50 mM sodium phosphate buffer (pH 6.5) and 0.7 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After 1.0 mL of 10% trichloroacetic acid and 1.0 mL of distilled were added, the mixture was centrifuged at 3,000 rpm for 10 min. The 1.0 mL of upper layer was mixed with 0.1 mL of 0.1% of ferric chloride, and the absorbance was measured at 700 nm. The assays were carried out in triplicate and the results were expressed as mean values standard deviations. BHT and α-tocopherol were used as standards.

**Hydroxyl radical scavenging effect**

The effect of hydroxyl radical was assayed by using the 2-deoxyribose oxidation method [17]. The reaction mixture contained 1.0 mL of 0.1 M sodium phosphate buffer (pH 7.4), 0.20 mL of 10 mM 2-deoxyribose, 0.20 mL of 10 mM FeSO4-EDTA, 0.20 mL of 10 mM hydrogen peroxide and 0.20 mL of extract solution in a tube. After incubation at 37°C for 1 hour, the reaction was stopped by adding 1.0 mL of 1.0% trichloroacetic acid and 1.0 mL of 1.0% of thiobarbituric acid. The mixture was boiled for 10 min, cooled in ice and then measured at 532 nm. Hydroxyl radical-scavenging effect was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radical.

**Mice and cell cultures**

Specific pathogen-free female BALB/c mice (6 weeks old) were purchased from NARA Biotech. (Seoul, Korea) and housed in a specific pathogen free environment. All experiments were conducted in accordance with the guidelines established by the Animal Care and Use Committee of Yuhan University (2009E-001). Water and dietary pellets were supplied *ad libitum*. Highly lung metastatic sublines of colon 26 carcinoma (colon26-M3.1) and murine B16-BL6 melanoma cells were maintained as monolayer cultures in Eagle's minimal essential medium (EMEM) supplemented with 7.5% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids and L-glutamine, all...
Mosidae (Adenophora remotiflora) leaves

of which were purchased from Gibco BRL (Gibco: Carlsbad, CA, USA). Splenocytes from normal mice were cultured in RPMI-1640 (Gibco: Carlsbad, CA, USA) supplemented with 7.5% fetal bovine serum and glutamine [18].

Experimental lung metastasis

Experimental lung metastasis was induced via i.v. inoculation of colon26-M3.1 carcinoma cells (3×10⁴) into BALB/c mice [19]. To assess the anti-metastatic activity of MLE, groups of five mice each were i.v. treated with MLE (50 µg- 10 mg/kg mice) 2 days prior to tumor cell inoculation. The mice were sacrificed 14 days later and their lungs were fixed in Bouin's solution. Lung tumor cell colonies were counted under a microscope [18].

Cytotoxicity assay

For the cytotoxicity assay, various doses of MLE (0.1-500 µg/mL) in culture medium were added to each well of a 96-well plate containing either murine tumor cells (B16-BL6 cell and colon26-M3.1) (1×10⁵/well) or splenocytes (2×10⁵/well) from normal mice. After incubation for 72 hours, cytotoxicity was assayed by WST-1 based colorimetric assay (Wako Pure Chemicals Industrials, Ltd., Osaka, Japan) [19]. The absorbance of each well was monitored at 450 nm using a micro-plate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

The data were expressed as mean ± SD values using SAS (SAS Institute, Cary, NC, USA). Each experimental set was compared by one-way analysis of variance (ANOVA) and Duncan’s multiple-range test (P < 0.05) using the SAS program (SAS Institute, Cary, NC, USA).

Results

Proximate compositions

The proximate compositions of fresh ML are shown in Table 1. The fresh ML consisted of moisture (91.92%), protein (1.63%), fat (0.04%) and ash (0.6%).

Antioxidant activities

In this study, the antioxidant activity of MLE was evaluated by measuring reducing power, total phenolic compounds, DPPH radical scavenging, and hydroxyl radical scavenging. As shown in Table 2, the concentration of total phenolic compounds in MLE was 39 mg/100 mL. MLE evidenced a high reducing capacity (1.24), similar to that of α-Tocopherol (1.72). The DPPH scavenging activity of MLE was 88.89%, a value much lower than that of BHT (99.06%) and α-Tocopherol (95.45%). Nonetheless, we noted no significant differences between MLE and α-Tocopherol. The hydroxyl radical scavenging activity of MLE (22.10%) was significantly lower than that of BHT (29.37%), but did not differ significantly from that of α-Tocopherol (27.71%).

Inhibitory effect of MLE on lung metastasis

In order to determine whether MLE has any inhibitory effect on lung tumor metastasis, we assessed the effects of MLE on the experimental lung tumor metastasis of colon26-M3.1 cells. As shown in Table 3, prophylactic i.v. MLE treatment significantly inhibited lung metastasis in a dose-dependent manner. Specifically, a dose of 200 µg MLE per mouse strongly inhibited lung metastasis by 88.90%. Thus, MLE treatment within a certain range exerted no apparent side effects, such as a reduction in body weight (data not shown).

Cytotoxicity assay

The cytotoxic effects of MLE on murine splenocyte and tumor cells (B16BL6 and colon26-M3.1) were examined in vitro as shown in Fig 2. MLE doses up to 100 µg/mL had no effect on the growth of normal murine splenocytes. Additionally, the
survival of normal murine splenocytes was not shown to be influenced at MLE doses below 500 ug/mL. However, MLE doses up to 500 ug/mL resulted in decreased tumor cell growth in the B16BL6 (67% alive) and colon26-M3.1 (62% alive) cells. These results demonstrate that MLE had no effect on normal murine splenocytes, but demonstrated its cytotoxic effects against tumor cells.

**Discussion**

Mosidae (Adenophora remotiflora) has been traditionally used as an alternative medicine in Korea. However, there are currently no reports concerning its biological activity on the basis of the scientific evidence. Our study focused on the antioxidant activities of Mosidae leaf ethanol extract (MLE) by measuring reducing power, total phenolic compounds, DPPH radical scavenging, and hydroxyl radical scavenging. We also evaluated the anti-metastatic activity of MLE with regard specifically to its prophylactic lung metastasis inhibitory activity in colon26-M3.1 carcinoma cells. The cytotoxic effects of MLE were examined in an experimental metastasis model of murine splenocyte and tumor cells (B16BL6 and colon26-M3.1).

The proximate compositions of fresh ML are provided in Table 1. The moisture content of the fresh ML was 91.92%, the protein content of fresh ML was 1.63%, the fat content was 0.04%, and the ash content of fresh ML was 0.6%. An increasing amount of evidence suggests that the production of reactive oxygen species underlies a variety of disorders, and is also responsible for cellular damage [20]. The reducing capacity of cellular damage may be a significant indicator of any potential antioxidant activity [21]. Accordingly, MLE might contain a sizable amount of reductant, which may react with free radicals to stabilize and terminate free radical chain reactions. In this study, the reducing capacity of MLE was similar to that of vitamin E, but less profound than that of BHT. Phenolic compounds are thought to perform important functions as dietary antioxidants for the prevention of oxidative damage in living systems. Cho [22] reported that tannins extracted from fresh ML harbored high levels of total phenolic compounds as compared with spinach and aster, and moreover exhibited high DPPH radical scavenging activities. In this study, the total phenolic content of MLE was 39 mg/100 mL. The DPPH assay is known to provide reliable information concerning the antioxidant potential of a particular compound [23]. The results in Table 3 show that the DPPH radical scavenging activity of MLE is similar compared with α-Tocopherol and BHT. Kim et al. [10] did report the DPPH radical scavenging activity of fresh MLE (55.63%), which was lower than the value we reported (88.89%). However, such a difference could have been caused by the different cultivation conditions in our experiments. For instance, the samples utilized in this study were cultivated by the environmentally-friendly agricultural company, Eco-Sprout Co, Ltd.

The hydroxyl radical is considered a quick initiator of lipid peroxidation through the abstraction of hydrogen atoms from unsaturated fatty acids. Hence, the hydroxyl radical scavenging activity of MLE was evaluated and the results are shown in Table 2. In this study, the hydroxyl radical scavenging activity of MLE was 29.37%, a level similar to that of BHT and α-Tocopherol. Therefore, MLE appears likely to be an effective radical scavenger. Furthermore, the results of this study show that MLE
is a good scavenger of DPPH and hydroxyl radical. In an experimental tumor metastasis model, natural products commonly used in Oriental medicine were deemed useful in suppressing tumor growth and inhibiting tumor metastasis [18].

Unlike conventional chemotherapeutic agents, natural products have been shown to be relatively nontoxic to normal cells and to enhance host defense by stimulating immune-related cells [24]. In fact, many previous reports have demonstrated that some Oriental medicines inhibit the proliferation of macrophages, as well as metastasis in experimental tumor models [7,18,25]. In this study, prophylactic i.v. treatment with MLE inhibited the lung metastasis of colon26-M3.1 carcinoma cells in a dose-dependent manner. Specifically, a 200 μg dose of MLE per mouse strongly inhibited lung metastasis by 88.90%. Therefore, MLE treatment in this range exerted no apparent side effects, such as reduction in body weight or piloerection (data not shown).

Since the inception of the National Center for CAM, there has been an enormous increase in the number of basic science studies and therapy-based clinical trials designed to explore Complementary and Alternative Medicine (CAM). This subspecialty of immunology represents a particularly productive area with a large number of therapies shown to affect the immune system. Some recent studies have uncovered a biochemical mechanism that may potentially be involved in the immune modulatory pathways exploited by a variety of herbs [5,7,8]. Clinically, hundreds of trials have examined the effects of CAM on asthma, allergic rhinitis, and atopic dermatitis [26-28]. In our cytotoxicity assay, MLE did not affect the growth or survival of normal murine splenocytes. However, MLE doses up to 500 μg/mL reduced the percentage of tumor cell growth in B16BL6 (67% alive) and colon26-M5.1 (62% alive) cells. These results demonstrate that MLE has no effect on normal cell, but does demonstrate its cytotoxic effects against tumor cells.

Pine pollen extract showed strong antioxidant and anti-inflammatory activity [29], and the results of this study also demonstrated that MLE has strong antioxidant activity by measuring reducing power, DPPH radical scavenging, and hydroxyl radical scavenging. MLE may prove to be useful in preventing lung tumor metastasis, because it appears to exert no additional side effects. Therefore, MLE may be useful in the development of new plant materials as CAMs against diseases whose principal pathology is oxidative stress.

References

1. Fiorentino A, D'Avrosca B, Pacifico S, Mastellone C, Piscopo V, Caputo R, Monaco P. Isolation and structure elucidation of antioxidant polyphenols from quince (Cydonia vulgaris) peels. J Agric Food Chem 2008;56:2660-7.
2. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006;160:1-40.
3. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; 39:44-84.
4. Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev 2002;13:155-68.
5. Fisher M, Yang LX. Anticancer effects and mechanisms of polysaccharide-K (PSK): Implications of cancer immuno-therapy. Anticancer Res 2002; 22:1737-54.
6. Saiki I, Saito S, Fujita C, Ishida H, Iida J, Murata J, Hasegawa A, Azuma I. Induction of tumoricidal macrophages and production of cytokines by synthetic muramyl dipeptide analogues. Vaccine 1998;6:238-44.
7. Yoon TJ, Yoo YC, Lee SW, Shin KS, Choi WH, Hwang SH, Ha ES, Jo SK, Kim SH, Park WM. Anti-metastatic activity of Acanthopanax senticosus extract and its possible immunological mechanism of action. J Ethnopharmacol 2004;93:247-53.
8. Effert H, Kaha S, Paulus K, Adams M, Rauh R, Boechzelt H, Hao X, Kaina B, Bauer R. Phytochemistry and pharmacogenomics of natural products derived from traditional Chinese medicine and Chinese materia medica with activity against tumor cells. Mol Cancer Ther 2008;7:152-61.
9. Schantz SP, Brown BW, Lira E, Taylor SL, Beddingfield N. Evidence for the role of natural immunity in the control of metastatic spread of head and neck cancer. Cancer Immunol Immunother 1987;25:141-8.
10. Kim SH, Choi HS, Lee MS, Chung MS. Flavor pattern and sensory characteristics of Adenophora remotiflora. Annals of Plant Resources Research, Dukssung Women's University 2007; 260:112-20.
11. Kim SH, Choi HS, Lee MS, Chung MS. Volatile compounds and antioxidant activities of Adenophora remotiflora. Korean Journal of Food Science and Technology 2007;39:109-13.
12. Kim SS, Kim SJ, Kim GH. Quality characteristics of Adenophora remotiflora for increasing the value as a food resource. Institute of Natural Science Research, Dukssung Women's University 2001;7:117-25.
13. AOAC. Official methods of analysis of AOAC. Washington DC: Association of official analytical chemicals; 2005.
14. Oyaizu M. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. Japanese Journal of Nutrition 1986;44:307-15.
15. Velioglu YS, Mazza G, Gao L, Oomah BD. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J Agric Food Chem 1998;46:4113-7.
16. Huang D, Ou B, Prior RL. The chemistry beind antioxidant capacity assays. J Agric Food Chem 2005;53:1841-56.
17. Chung SK, Osawa T, Kawakishi S. Hydroxyl radical scavenging effects of species and scavengers from brown mustard (Brassica nigra). Biosci Biotechnol Biochem 1997;61:118-23.
18. Yoon TJ, Yoo YC, Choi OB, Do MS, Kang TB, Lee SW, Azuma I, Kim JB. Inhibitory effect Of Korean mistletoe (Viscum album coloratum) extract on tumour angiogenesis and metastasis of haematogenous and non-haematogenous tumour cells in mice. Cancer Lett 1995;97:83-91.
19. Lee SJ, Saiki I, Hayakawa Y, Nunome S, Yamada H, Kim SH. Antimetastatic and immunomodulating properties of a new herbal prescription, Bojung-bangam-tang. Int Immunopharmacol 2003;3:147-57.
20. Johnson TM, Yu Z, Ferrangs VJ, Lowenstein RA, Finkel T. Reactive oxygen species are downstream mediators of p53 dependent apoptosis. Proc Natl Acad Sci U S A 1996;93:11848-52.
21. Meir S, Kanner J, Akiri B, Hadas SP. Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. J Agric Food Chem 1995;43:1813-7.
22. Cho MJ. A study on the antioxidant nutrients analysis and antioxidative activities of Adenophora remotiflora and Aster glehni. [master’s thesis]. Seoul: Duk sung Women’s University; 2001.
23. Rice-Evan C, Miller N, Paganga. Antioxidant properties of phenolic compounds. Trends Plant Sci 1997;2:152-9.
24. Rininger JA, Kickner S, Chigurupati P, McLean A, Franck Z. Immunopharmacological activity of Echinacea preparations following simulated digestion on murine macrophages and human peripheral blood mononuclear cells. J Leukoc Biol 2000;68:503-10.
25. Schepetkin IA, Faulkner CL, Nelson-Overton LK, Wiley JA, Quinn MT. Macrophage immunomodulatory activity of polysaccharides isolated from Juniperus scopolorum. Int Immunopharmacol 2005;5:1783-99.
26. Gillis CN. Panax ginseng pharmacology: A nitric oxide link? Biochem Pharmacol 1997;54:1-20.
27. O’Hara M, Kiefer D, Farrell K, Kemper K. A review of 12 commonly used medicinal herbs. Arch Fam Med 1998;7:523-36.
28. Timothy M, Simi K, Leonard B. Complementary and alternative medicine: herbs, phytochemicals and vitamins and their immunological effects. J Allergy Clin Immunol 2009;123:283-94.
29. Lee KH, Kim AJ, Choi EM. Antioxidant and anti-inflammatory activity of pine pollen extract in vitro. Phytother Res 2009;23:41-8.