

# Effect of *Monascus*-Fermented Soybean Extracts on Antioxidant and Skin Aging-Related Enzymes Inhibitory Activities

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**ABSTRACT:** We investigated the *in vitro* inhibitory activity against skin aging-related enzymes and antioxidant activity of *Monascus*-fermented soybean extracts (MFSEs) obtained by using different solvents. The highest Trolox equivalent (TE) antioxidant capacity ( $3.13 \pm 0.06$  mM TE/g) and oxygen radical absorbance capacity ( $2.79 \pm 0.09$  mM TE/g) of MFSEs were evaluated for the methanol and 80% ethanol extracts, respectively. The antioxidant capacities increased with increasing concentration (0.5 ~ 50 mg/mL). In addition, the methanol and 80% ethanol extracts showed an effective inhibition against tyrosinase, hyaluronidase, and elastase compared with those of acetone and hot water extracts ( $P < 0.05$ ). Results indicate that the inhibitory activities against skin aging-related enzymes and antioxidant properties provide evidence for the nutricosmetic potentials of *Monascus*-fermented soybeans.

**Keywords:** *Monascus*-fermented soybean, skin aging-related enzyme, antioxidant activity

## INTRODUCTION

The cosmetic industry is constantly in search of ingredients from natural sources because of their competitive effectiveness and lower toxicity effects (1). Nutricosmetics are nutritional supplements, which purpose is to support the function and the structure of the skin (2). However tyrosinase, elastase, collagenase, and hyaluronidase are key enzymes along with the reactive oxygen species (ROS) involved in skin deterioration (1). Skin aging is known to be caused by both, an intrinsic, natural or cellular mechanism, and the extrinsic mechanism from exposure to external factors, mainly over exposure to solar radiation (3). ROS, arising from oxidative cell metabolism causes damage to cellular components like cell walls, lipid membranes, mitochondria, and DNA, playing a major role in both processes (3,4). Many micronutrients and natural phenolic compounds have an antioxidant potential and tend to reduce free radicals generation when the skin is exposed to UV radiation (5). Therefore, the inhibitors of elastase, hyaluronidase, and tyrosinase can be potential cosmetic ingredients in the treatment of skin aging thereby restoring the skin elasticity, increase moisture content, stimulate collagen synthesis, and skin lightening effects (1,2).

The filamentous fungus genus *Monascus* has been used in solid state fermentation in Asia for centuries to pro-

duce several bioactive metabolites such as color pigments, isoflavones, monacolins, and  $\gamma$ -amino butyric acid (6,7). *Monascus*-fermentation metabolites have been reported to be beneficial for cholesterol management, blood glucose management, blood pressure management, and cancer in animal and cell models (8-10). We also demonstrated that aglycone isoflavones and coenzyme Q10 (CoQ10) levels in soybeans fermented with *Monascus pilosus* increased about 33.4- and 3.0-fold compared to unfermented samples, respectively (11). These compounds are well-known antioxidants used in many healthcare products for anti-metabolic syndrome or anti-aging purposes (12,13). However, information on the nutricosmetic potentials of *Monascus*-fermented soybeans (MFSS) is not yet available. In this study, the inhibitory activity against skin aging-related enzymes and antioxidant activity of *Monascus*-fermented soybean extracts (MFSEs) obtained by using different solvents were examined.

## MATERIALS AND METHODS

### Sample preparation and reagents

MFSS were produced by using a two-stage fermentation process including a seed culture stage (liquid culture) and a metabolite production stage in solid state fermentation. In brief, soybeans were washed, soaked, and auto-

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claved. After cooling, the substrate was inoculated with nutrient broth including *Monascus pilosus* KCCM 60084 [Korean Federation of Culture Collections (KCCM), Seoul, Korea], and incubated at 30°C for 20 days (11). MFSs were collected, lyophilized, and powdered. A sub-sample was then extracted with acetone, methanol, and 80% ethanol with stirring at 150 rpm for 24 h at 30°C, respectively, and lyophilized. The same quantity of sample was boiled at 100°C for 2 h with deionized distilled water to obtain the hot water extract. The freeze-dried extracts (moisture content: <5%) were kept in a -20°C freezer and used for further analyses. All chemical reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

#### **In vitro assay of enzyme inhibitory activities**

The anti-tyrosinase assay was performed according to Wu et al. (14), with slight modifications. Briefly, MFSEs in different solvents were pre-incubated with the tyrosinase solution (100 units/mL) for 10 min. After L-tyrosine (2 mM) was added, the reaction kinetics were evaluated at a wavelength of 480 nm using a UV-Vis spectrophotometer (DU-650, Beckman Coulter Inc., Brea, CA, USA). The reaction mixture without the samples was used as a negative control, whereas kojic acid (1 mmol/mL) was used as a positive control. Percent of inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{A-B}{A} \times 100$$

where A is the absorbance of the negative control and B is the absorbance of the test solution.

The anti-hyaluronidase assay was performed by the method described by Chompoo et al. (15) with slight modifications. A mixture of acetate buffer (pH 3.9), bovine serum albumin (1 mg/mL in acetate buffer), sample solution dissolved in the same buffer, and hyaluronidase (5 mg/mL in acetate buffer) was incubated for 20 min at 37°C. The enzymatic reaction was initiated by the addition of hyaluronic acid (HA) (1.0 mg/mL in acetate buffer). After 40 min incubation at 37°C, the reaction was quenched by the addition of alkaline borate solution. The absorbance was determined at 600 nm, and the enzyme inhibitory activity (%) was quantified. Epigallocatechin gallate (EGCG, 1 mmol/mL) was used as a reference inhibitor.

$$\text{Inhibition (\%)} = \frac{(A-B) - (C-D)}{A-B} \times 100$$

where A is the absorbance with hyaluronidase and without the sample, B is the absorbance without the sample and hyaluronidase, C is the absorbance with the sample

and hyaluronidase, and D is the absorbance with the sample and without hyaluronidase.

To evaluate the inhibitory effect of MFSEs in different solvents on elastase enzyme activity, the method described by Kraunsoe et al. (16) was employed with slight modifications. The sample was pre-incubated with the substrate for 20 min after which porcine pancreatic elastase (1.2 units/mL) was added. The substrate N-succinyl-tri-L-alanine-4-nitroanilide was diluted in Tris buffer at pH 8 in order to obtain a final concentration of 0.6 mM. Reaction kinetics were evaluated at a wavelength of 410 nm using a UV-Vis spectrophotometer (DU-650, Beckman Coulter Inc.). The reaction mixture without sample was evaluated as a negative control, whereas EGCG was used as a positive control at a concentration of 1 mmol/mL. Relative anti-elastase activity (%) was evaluated using the following formula:

$$\% \text{ Inhibition} = \frac{A-B}{A} \times 100$$

where A is the absorbance of the negative control and B is the absorbance of the test solution.

#### **Determination of antioxidant capacity**

The Trolox equivalent antioxidant capacity (TEAC) assay of the extracts was measured using the ABTS<sup>+</sup> [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] radical following the method reported by Re et al. (17), with slight modifications. Quantifications were calculated using a calibration curve with known concentrations of Trolox standards. The results were expressed as mmol Trolox equivalents per g of extract (mmol TE/g).

The hydrophilic oxygen radical absorbance capacity (ORAC) assay was adapted from previously described procedures (18). AAPH [2,2'-azobis(2-amidinopropane) dihydrochloride] was used as the peroxy radical generator, and Trolox was used as a standard. Fluorescein was used as the fluorescent probe. Fluorescein, AAPH, and the samples were prepared in phosphate buffer (75 mM, pH 7.4). A sample solution or Trolox standard was mixed with fluorescein (1 μM) and pre-incubated at 37°C for 30 min before the addition of the AAPH solution (200 mM). The fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 520 nm was measured every 2 min for 120 min using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). The relative ORAC values were calculated using the differences of areas under the decay curves and were expressed as mmol TE/g of extract.

#### **Statistical analysis**

Mean values and standard deviations were calculated from

the data obtained from triplicate experiments. Means were compared using Duncan's multiple range test method in SAS, version 8 (SAS Institute, Cary, NC, USA). Significant difference was considered at the level of  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Antioxidant activity

The TEAC and ORAC assays are the most popularly used electron transfer and hydrogen atom transfer methods, respectively (18). Antioxidant activities of each MFSEs were tested by TEAC and ORAC at concentrations of 0.5~50 mg/mL. The results of the antioxidant capacity are shown in Table 1. At 0.5~50 mg/mL, the antioxidant activities of the acetone, methanol, 80% ethanol, and hot water extracts of samples ranged from 0.11~1.94, 0.16~3.13, 0.18~2.79, and 0.04~1.65 mmol TE/g, respectively. The results suggest that the methanol and 80% ethanol extracts showed higher antioxidant activities with increasing concentration, whereas acetone and hot water extracts showed lower antioxidant activities. The maximum TEAC and ORAC values of these extracts increased by 61.8 to 67.5% compared with those of acetone and hot water extracts, respectively ( $P < 0.05$ ). The highest TEAC ( $3.13 \pm 0.06$  mM TE/g) and ORAC value ( $2.79 \pm 0.05$  mM TE/g) of MFSEs were evaluated for the methanol and 80% ethanol extracts, respectively, at 50 mg/mL. In this study, antioxidant activity values from TEAC and ORAC were proportional between the two tests. Our findings indicate that various extracts might react with free radicals, particularly peroxy radicals, which are the major propagators of the fat autoxidation chain, thereby terminating the chain reaction (19). We recently reported that isoflavone aglycones (genistein, daidzein, and glycitein), CoQ10, and  $\alpha$ -tocopherol levels in the MFSEs increased about 33.4-, 3.0-, and 1.5-fold after 20 days of fermentation, respectively (11,20). Several reports have convincingly shown a close rela-

tionship between antioxidant capacity and these compounds (12,13,20). Kagan et al. (12) demonstrated that both vitamin E and coenzyme Q possess distinct lipoprotective antioxidant properties in biological membranes. Their combined antioxidant activity, however, is markedly synergistic when both are present (12,13). In particular, isoflavones and CoQ10 have been shown to improve dermal health by direct and downstream influences at different steps of the oxidative stress cascade (5,12). Therefore, the higher antioxidant properties of methanol and 80% ethanol extracts might be due to more electron and hydrogen-donating components such as isoflavones aglycones, CoQ10, and  $\alpha$ -tocopherol contained within the extracts.

### In vitro anti-skin aging activity

Aging of skin is characterized by progressive loss of skin tissue as well as changes in macromolecules of the extracellular matrix of the dermis (3). The tyrosinase, elastase, and hyaluronidase inhibitory activities of MFSEs at 50 mg/mL are shown in Fig. 1. It was found that methanol and 80% ethanol extracts showed enhanced inhibitory activities (80.2~90.1%) against these skin aging-related enzymes compared with the acetone and hot water extracts (28.2~45.6%). In particular, inhibition of tyrosinase activity (90.1%) was abrogated by methanol, which was compared with the results of treatment with 1 mmol of kojic acid (94.5%) as a positive control (Fig. 1). It is known that methanol is an efficient extraction media for a broad spectrum of compounds (15). The inhibition of tyrosinase might depend on the number of hydroxyl groups in the phenolic compounds from MFSEs that are available to form a hydrogen bond with an enzyme site, leading to lower enzymatic activity (21). Tyrosinase inhibition is the most common approach to find out a skin lightening agent as this enzyme catalyses the rate-limiting step of pigmentation (14).

HA is a naturally occurring glucose-based polymer that plays an important role as skin rejuvenant because it holds moisture, increases viscosity, and reduces perme-

**Table 1.** Antioxidant properties of *Monascus*-fermented soybean extracts obtained using different solvents at various concentrations (unit: mmol Trolox equivalent/g)

Solvent	Sample concentration (mg/mL)									
	0.5		5.0		10.0		25.0		50.0	
	TEAC	ORAC	TEAC	ORAC	TEAC	ORAC	TEAC	ORAC	TEAC	ORAC
Acetone	0.12±0.01 <sup>b</sup>	0.11±0.02 <sup>b</sup>	0.58±0.03 <sup>b</sup>	0.42±0.05 <sup>b</sup>	1.18±0.09 <sup>b</sup>	1.12±0.05 <sup>b</sup>	1.52±0.06 <sup>ab</sup>	1.71±0.04 <sup>b</sup>	1.94±0.07 <sup>b</sup>	1.88±0.08 <sup>ab</sup>
Methanol	0.18±0.01 <sup>ab</sup>	0.16±0.02 <sup>a</sup>	0.95±0.06 <sup>a</sup>	0.79±0.07 <sup>a</sup>	1.72±0.06 <sup>a</sup>	1.65±0.08 <sup>a</sup>	2.12±0.07 <sup>a</sup>	2.05±0.07 <sup>a</sup>	3.13±0.06 <sup>a</sup>	2.76±0.09 <sup>a</sup>
80% Ethanol	0.22±0.07 <sup>a</sup>	0.18±0.03 <sup>a</sup>	0.82±0.04 <sup>ab</sup>	0.72±0.05 <sup>a</sup>	1.69±0.05 <sup>a</sup>	1.61±0.06 <sup>a</sup>	2.06±0.08 <sup>a</sup>	1.98±0.04 <sup>a</sup>	2.68±0.05 <sup>ab</sup>	2.79±0.05 <sup>a</sup>
Hot water	0.09±0.02 <sup>c</sup>	0.04±0.01 <sup>c</sup>	0.42±0.02 <sup>c</sup>	0.31±0.02 <sup>c</sup>	0.73±0.03 <sup>c</sup>	0.61±0.04 <sup>c</sup>	1.04±0.09 <sup>b</sup>	1.18±0.06 <sup>b</sup>	1.65±0.08 <sup>b</sup>	1.43±0.04 <sup>b</sup>

Each value is mean±SD (n=3).

Different letters (a-c) in the same column indicate a significant difference at  $P < 0.05$ .

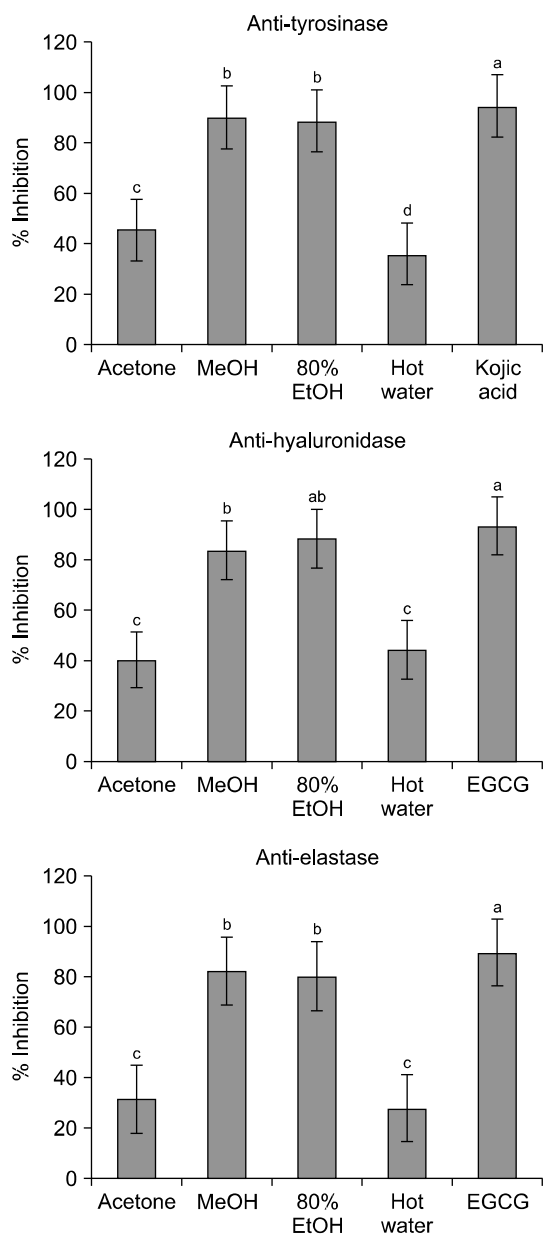


Fig. 1. Inhibitory effects of *Monascus*-fermented soybean extracts (50 mg/mL) in different solvents and positive controls (1 mmol/mL) against the skin aging-related enzymes. Each value is expressed as the mean  $\pm$  SD ( $n=3$ ). Different letters (a-d) on the bars show a significant difference at  $P<0.05$ .

ability of extracellular fluid (22). Hyaluronidase mediates the degradation of HA by increasing the permeability of the connective tissue and lowering the viscosity of bodily fluids (3,22). Therefore, hyaluronidase inhibition is key in maintaining the HA level and the moisture retention of the skin (3). In the hyaluronidase assay, all of MFSEs in different solvents showed more than 40% inhibition when evaluated at 50 mg/mL. The proportion of tested extracts with inhibitory activity was highest for the extracts of 80% ethanol. Maximum inhibitory activity ( $88.5 \pm 2.28\%$ ) of the extract increased by 78.3% to 87.4% when compared with those of acetone and hot water extracts, respectively ( $P<0.05$ ). The data indicate

that the ethanol and water mixture will cover a wide range of polarities for polyphenol extraction such as isoflavones. As a positive control, EGCG showed excellent hyaluronidase inhibition of 93.8% at the concentration of 1 mmol/mL.

The inhibition of elastase activity could also be used as a method to protect against skin aging (3,16). Elastin is an extracellular matrix protein that has an influence on skin elasticity, and elastase is the proteinase enzyme capable of degrading elastin, which can lead to aging process (3,4). As shown in Fig. 1, anti-elastase activity in methanol extracts increased by 2.9-fold compared to the acetone and hot water extracts. The inhibitory rate for the methanol extract was  $82.48 \pm 1.89\%$ , which can be considered as a good inhibitor. However its values were lower than EGCG ( $89.65 \pm 1.68\%$ ) used as positive control. EGCG has an effective inhibition on collagenase enzyme, expression of mRNA stromelysin induced by IL-1 $\beta$  activities and protection from skin damage caused by UV rays (21). It is known that the key enzyme capable of attacking all the major connective tissue matrix proteins is elastase (3,21).

Taken together, the results suggest that the enzymatic inhibitory activities of methanol and 80% ethanol extracts against tyrosinase, elastase, and hyaluronidase, as well as their antioxidant capacities, have provided evidence for the nutricosmetic utility of MFSEs. Nutricosmetics are the newest trend in skin care and involve the consumption of dietary and nutritional supplements to produce a visual appearance benefit and improve the health of the skin (2,4). Although synergistic processes and interactions among various components in MFSEs cannot be ruled out, the results could be explained by the presence of different types of *Monascus*-fermentation metabolites with inhibitory activity toward skin aging-related enzymes and antioxidant properties. However, considering the possibility of cosmetic applications and finished cosmetics products, the combination of non-toxic or moderately toxic solvents, such as water or ethanol-water mixtures should be evaluated.

In conclusion, the methanol and 80% ethanol extracts of MFSEs showed enhanced inhibitory activities against skin aging-related enzymes and antioxidant capacity. The results may be a key for the successful development of nutricosmetic ingredients that will be able to protect and defend the skin against free radicals, inhibit tyrosinase, elastase, and hyaluronidase associated with hyperpigmentation, wrinkle, and skin aging.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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