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NITRIC OXIDE RADICAL SCAVENGING ACTIVITIES IN FISH CAKE WITH BLACK SOYBEAN

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ABSTRACT

The soybean is a species of legume native to East Asia, widely grown for its edible bean, which is a significant and important source of plant protein in humen. This study was to evaluate the nitric oxide (NO) radical scavenging activities for aqueous and ethanoic extracts from fish cakes with two black soybean species, *Glycine max* and *Rhynchosia volubilis*. NO scavenging activity of the fish-pasta was evaluated 38.5% on 10 mM solution with 15% *G. max* addition and control was 8.2% at same concentration. There was significant difference among control groups without *G. max* and *G. max* added groups (t = 49.14, p <0.001). 1.0 mM extract of the fish cake was evaluated 6.0% on 5% *R. volubilis* and those of the 10% and 15% black soybeans were 10.0% and 16.2% at same concentration, respectively. There was significant difference among black soybean added groups (t = 79.12, p <0.001). The 2.5 mM, 5.0 mM, and 10.0 mM treatment groups also showed significant differences. Overall, ethanol extracts were slightly higher in NO inhibitory activity than those of aqueous extracts. However, there was no significant differences (p > 0.05). *R. volubilis* extracts were slightly higher in NO inhibitory activity than those of *G. max* extracts.

KEYWORDS: *Glycine max*, nitric oxide radical scavenging, *Rhynchosia volubilis*.

INTRODUCTION

The soybean [*Glycine max* (L.) Merr.] is the most important commercial species among the grain legume in the family Fabaceae (or Leguminosae). The soybean is a native of eastern Asia and a major source of protein for the people of China, Korea, Japan and Indonesia for thousands of years.[1] Legumes are a significant source of protein, dietary fiber, carbohydrates and dietary minerals.

Soybeans are generally differentiated based on the color of their seed coats. In general, soybean cultivars have yellow, black, green, brown or mottled seed coats.[2] Most soybean cultivars have a yellow-colored seed coat. Seed coats of soybeans are colored with red, black, or brown soybeans.[3] Black and brown soybeans are the anthocyanins of the bean coats and exhibit a dark color. Dark colored soybeans are also becoming popular worldwide due to their potentially high functional characteristics.[4]

Anthocyanins are included in red, purple, and blue colored flavonoid pigments in the seed coats of colored soybeans.[5] Physiological and genetic studies indicated that the color variation is due to the difference in the accumulation of pigment-stimulating metabolites.[6]

In Korea, black soybeans are classified as small, medium, and large seeds based on their seed weights.[7] *Rhynchosia volubilis* is a climbing, perennial plant with twining stems and seeds are usually two, black, lustrous, elliptic or subreniform. Seeds of *R. volubilis* belong to a small group.

Small seeds are favored for soybean sprouts, while medium and large seeds are preferred to augment vegetables, cooked rice, and home-made fermented pastes.[8-9]

Fish cakes (also called fishcakes or fish pastes) products are products made by adding salt to fish meat, kneading the fish meat, and heating it to solidify. In the Korean Food Standards Codex, fish cakes are defined as a processed marine product containing salt-soluble protein from fish meat.[10-11] Especially, Korean fish paste is not chemically broken down by a fermentation process until it reaches the consistency of a soft creamy purée or paste.[12]

Nitric oxide (NO) is a gaseous free radical, which has important roles in physiological and pathological conditions. Marcocci et al.[13] reported that scavengers of

nitric oxide compete with oxygen, leading to a reduction in the production of nitric oxide. Around 80,000 references invoking NO listed in PubMed.[14]

This study evaluated the NO free radical scavenging activity in the aqueous and ethanoic extracts of the fish cake with black soybeans.

MATERIALS AND METHODS

Preparation of sample

There are many specialty stores in Asian cities offering only fish paste and fish balls. These stores are often known for their own signature variety of fish pastes. The materials of fish cakes purchased on the market. Fillets of Pacific cod (*Gadus macrocephalus*), Atlantic mackerel (*Scomber scombrus*), and shrimp (*Fenneropenaeus chinensis*) were used as raw material for preparation of fish pastes. White fish is smashed until completely disintegrated, and then egg white, flour and vegetables were added.

To make fish mince, solid materials except egg and flour were put in a washing machine and smashed into small pieces. After 0.01% salt added to the sample and grinded the ingredients for about 20 minutes. Fish paste preparation was prepared according to the mixing ratio in Table 1. The two kinds of beans are made with a separate fish cake ingredient. In other words, soybeans were added to the basic fish cake ingredients: 1) basic fish cake ingredients + black *Glycine max* Merr., 2) basic fish cake ingredients + black *Rhynchosia volubilis* Lour.

Table 1: The formula for manufacturing fish cake with black soybean in this study.

Materials	Weight (%)			
	Control	5%	10%	15%
Fish	70	65	60	55
Shrimp	5	5	5	5
Egg	2	2	2	2
Flour	18	18	18	18
Vegetable	5	5	5	5
G. max Merr. (Black soybean)	-	(5)	(10)	(15)
Rhynchosia volubilis Lour. (Black)	-	(5)	(10)	(15)

Sample extract

Distilled water and 80% ethanol were used as the extraction solvent. The fish cake (100 g) was ground with distilled water or 80% ethanol and a grinding mixer to extract effective components for anti-oxidants. An aliquot was further mixed with 100 mM Tris-HCl buffer (pH 7.4). The sample was further stirred with a magnetic bar at 60℃ for 120 minutes. The sample was treated with ultrasound at room temperature for 60 minutes. The ultrasound extraction was carried out using an ultrasonic bath (5510, Branson, USA). The mixture was shaken vigorously for one hour at room temperature. Extracted sample was filtered. The sample was evaporated to remove solvent under reduced pressure and controlled temperature by using rotary vacuum evaporator (N-1001S-W, Eyela, Tokyo, Japan). To get dry powder, samples placed in a low temperature vacuum chamber.

Assay of nitric oxide (-NO) scavenging activity

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat.[15] The procedure of NO scavenging assay is based on the principle that scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Under aerobic conditions, NO reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of which can be determined using Griess reagent. The nitric oxide scavenging activity was measured by the method described by Marcocci et al.[13]

For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of each extracts dissolved in water and incubated for an hour at 37°C. After the incubation period, 1 ml of the reactant was mixed with 3 ml of 2% acetic acid and 400 µl of the Griess reagent and it was reacted for 15 minutes at room temperature.

The amount of nitrite remaining was measured by checking the absorbance at 520 nm using Microplate Reader (VersaMax, California, USA). Quercetin was used as a positive control.[16]

Inhibitory analysis

All experiments were performed thrice and the results averaged data were expressed as mean ± SD. The percent inhibition was calculated as the decolourization percentage of the test sample using the following formula:

$$\text{Inhibition (\%)} = (IA\text{-}As)/IA \times 100$$

Where IA is the absorbance of the 100% initial and As is the absorbance of the sample. IA and As were the values which were subtracted the average absorbance of the blank wells.

The concentration of the extract that inhibits 50% of the enzyme activity (IC_{50}) was calculated. Extracts with high inhibitory activity were analyzed using a series of suitable extract concentrations. IC_{50} values were determined by plotting percent inhibition (Y axis) versus log10 extract concentration (X axis) and calculated by logarithmic regression analysis from the mean inhibitory values. Regression analysis by a dose response curve was plotted to determine the IC_{50} values.

Data was conducted using Microsoft Excel and SPSS 21.0 for Windows (Chicago, IL, USA). A one-way and a two-way analysis of variance (ANOVA) followed by the Tukey post hoc test were used to analyze statistical significance ($p < 0.05$).

RESULTS

The nitric oxide (NO) radical activity in fish past with black soybean was obtained by setting up the equations. The antioxidant activity for NO radical of aqueous extraction groups was shown in Tables 2. It was observed that inhibition percentage values go on increasing with enhancements in concentration of research extracts in the assay mixture. NO scavenging activity of the fish-pasta was evaluated 38.5% on 10 mM solution with 15% *G. max* addition and control (pure fish, starch, shrimp, squid, egg, and vegetable) was 8.2% at same concentration. There was significant difference among control groups without *G. max* and *G. max* added groups (t = 49.14, p <0.001). The 1.0 mM and 5.0 mM treatment groups also showed significant differences. There was no significant difference in the 2.5 mM treatment group.

Table 3 was shown the results of antioxidant activity for ethanol extracts of fish cake with black soybeans. 1.0 mM extract of the fish cake was evaluated 6.0% on 5% *R. volubilis* and those of the 10% and 15% black soybeans were 10.0% and 16.2% at same concentration, respectively. There was significant difference among three black soybean added groups (t = 79.12, p <0.001). The 2.5 mM, 5.0 mM, and 10.0 mM treatment groups also showed significant differences.

Figure 1 was shown the rate of NO inhibitory of Quercetin (positive control) and its relative NO scavenging ability (%) according to the concentration of aqueous extract of fish cake with two black soybeans. The values for the *G. max and R. volubilis* were 33.0% and 39.6% on 10.0 mM, respectively. Figure 2 was shown the rate of NO inhibitory of Quercetin and relative inhibitory rate for the ethanol extract. The values for the *G. max and R. volubilis* were 38.4% and 46.9% on 10.0 mM, respectively.

IC_{50} value was inversely related to the antioxidant activity of fish cake with two black soybeans. The values of IC_{50} for the aqueous extracts of *G. max and R. volubilis* were 151.8 and 141.8 μg/ml, respectively (Table 4). The values of IC_{50} for the ethanol extracts of *G. max and R. volubilis* were 123.1 and 152.6 μg/ml, respectively.

Overall, ethanol extracts were slightly higher in NO inhibitory activity than those of aqueous extracts. However, there was no significant differences ($p > 0.05$). *R. volubilis* extracts were slightly higher in NO inhibitory activity than those of *G. max* extracts. They was also no significant differences ($p > 0.05$).

Table 2: The degree of inhibition (%) of NO by fish cake with black soybean of aqueous extracts at different concentrations.

Species	Concentration (mM)	Black soybean (%)			*t*-test
		5	10	15	
G. max	1.0	3.08±1.12	7.25±0.37	10.67±0.33	41.73***
	2.5	8.70±0.96	12.33±1.16	16.25±1.21	1.43
	5.0	12.51±2.42	17.95±1.36	27.17±2.04	33.32***
	10.0	17.11±1.15	21.95±1.45	38.53±2.54	49.14***
Rhynchosia volubilis	1.0	4.55±1.67	8.57±1.23	14.10±1.97	23.54**
	2.5	11.67±2.33	14.67±2.27	23.86±1.49	0.02
	5.0	19.23±2.01	22.89±2.36	28.72±3.62	3.52*
	10.0	24.10±2.55	29.67±2.54	39.64±3.16	24.18**
F-test		0.09	2.12	2.14	

Data represented the mean ± SD from three replicates. *: p<0.05, **: p<0.01, ***: p<0.001.

Table 3: The degree of inhibition (%) of NO by fish cake with black soybean of ethanol extracts at different concentrations.

Species	Concentration (mM)	Black soybean (%)			*t*-test
		5	10	15	
G. max	1.0	4.55±1.26	8.57±2.91	12.99±1.59	62.11***
	2.5	11.27±1.54	14.67±1.53	22.09±2.38	13.75**
	5.0	19.23±3.02	21.95±1.48	31.63±2.47	50.65***
	10.0	24.10±2.61	29.67±3.22	40.18±2.33	5.95*
Rhynchosia volubilis	1.0	5.97±2.15	9.86±3.16	16.25±2.40	79.12***
	2.5	14.87±3.07	16.88±2.85	26.37±3.01	13.91**
	5.0	24.10±2.24	26.44±2.78	33.66±1.95	66.79***
	10.0	31.52±3.04	39.62±3.17	43.70±2.96	93.96***
F-test		0.05	2.04	2.11	

Data represented the mean ± SD from three replicates. *: $p<0.05$,**: $p<0.01$, ***: $p<0.001$.

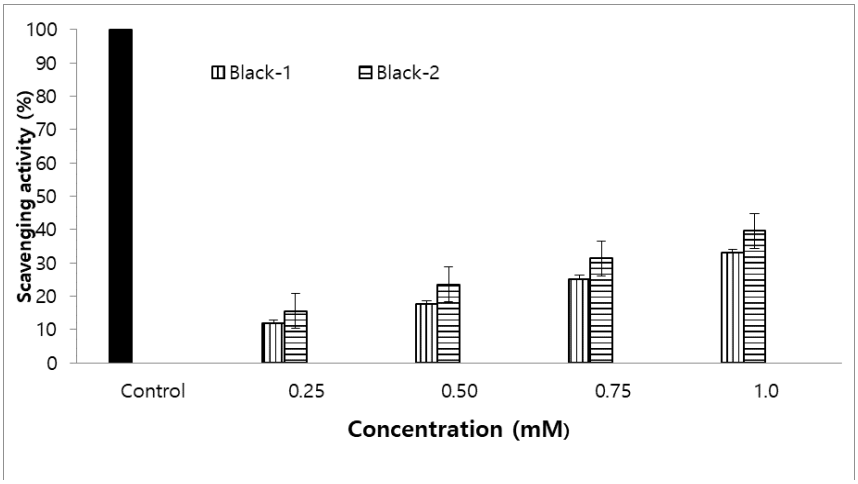

Figure 1. Control group and its relative NO scavenging ability (%) according to the concentration of aqueous extract of fish cake with black soybean. Black-1: *Glycine max*, Black-2: *Rhynchosia volubilis.*

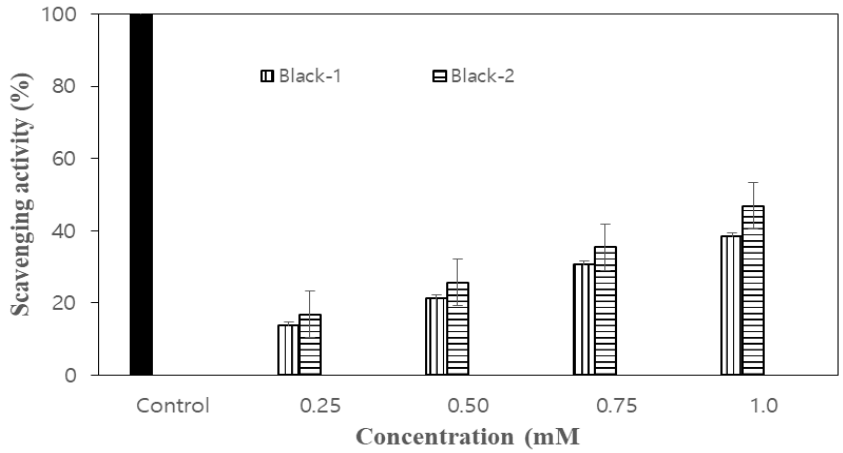

Figure 2: Control group and its relative NO scavenging ability (%) according to the concentration of ethanol extract of fish cake with black soybean. Black-1: *G. max*, Black-2: *Rhynchosia volubilis.*

Table 4. The 50% inhibition (IC_{50}) of NO on solvents and fish cake with back soybeans.

Solvent	Fish cake with *Glycine max*	Fish cake with *Rhynchosia volubilis*
Water	151.8±13.1	141.8±14.1
Ethanol	123.1±12.6	152.6±15.3

DISCUSSION

Soybeans are the seeds from flowering plants in the Fabaceae family and are classified as legumes. Soybeans contain amino acids, which are the protein building blocks that the body uses to heal and to make new tissues, such as bone, muscle, hair, skin, and blood. Protein is an essential nutrient that contribute to structural and mechanical function, regulate processes in the cells and body, and provide energy if necessary.[17] Proteins are composed of amino acids and are available in food sources like meats, dairy foods, legumes, vegetables, and grains.[18]

Black soybeans are classified as legumes and are popular as a functional food rather than yellow soybean. It provides a nutritional profile of the black bean and an in-depth look at its possible health benefits. The iron, phosphorus, calcium, magnesium, manganese, copper, and zinc in black beans all contribute to building and maintaining bone structure trusted source and strength.

Generally, the anti-oxidant activity is elevated during digestion and absorption of the common beans in the intestine.[19] Black soybean grains contain large amounts of monounsaturated fatty acids, which gives them greater oxidative stability.

Juan and Chou[20] reported that fermentation enhanced the total phenolic and flavonoid content as well as antioxidant activity of the black soybean extract. They also reported lipid solvent such as acetone extract and the methanol extract of fermented black soybeans showed

higher DPPH free radical-scavenging effect than aqueous solvents. The phenolic contents should be also considered as an important characteristic feature of soybean seeds, and as a potential selection criterion for antioxidant activity in soybean.[21]

Although antioxidant effects of the extract from the mixture of germs and cotyledons were similar in both soybeans, black soybeans may be more effective in inhibiting LDL oxidation than yellow soybeans because of total polyphenols contents in its seed coat.[22] Many soybean extract at varied concentrations showed remarkable inhibitory effect of nitric oxide radical scavenging activity.[23-25], A significant and linear relationship was found between the antioxidant activity and fish cake with black soybean content, indicating that black soybeans could be major contributors to antioxidant activity.

CONCLUSIONS

The results of the present study would certainly help to ascertain the potency of the crude extracts from black *Glycine max* and *Rhynchosia volubilis* as potential source of natural antioxidants.

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