Anti-oxidant, skin whitening, and antibacterial effects of *Canavalia gladiata* extracts

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Canavalia gladiata, a Chinese medicine known as sword bean, has been traditionally used for anti-inflammatory and anti-oxidant properties. This study evaluated the pharmacologic effects of *C. gladiata* on anti-oxidant, skin whitening, and antibacterial activities. The 80% ethanol extracts were prepared from ripe or unripe *C. gladiata*. We also made activity comparison between ripe and unripe *C. gladiata*. DPPH and nitro blue tetrazolium/xanthine oxidase (XO) assays were used to evaluate the anti-oxidant activity. *C. gladiata* showed antioxidant effect in a dose dependent manner. Next, mushroom tyrosinase activity was measured to evaluate the skin whitening effect. *C. gladiata* showed inhibitory effect on tyrosinase activity in a dose dependent manner. *C. gladiata* extracts also inhibited XO activity. In addition, the antibacterial effects were evaluated by using the minimum inhibitory concentration test and disk diffusion assay. *C. gladiata* showed antibacterial activity to *Vibrio vulnificus*. In conclusion, *C. gladiata* showed the anti-oxidant, tyrosinase inhibition, and antibacterial activities. *C. gladiata* extracts may be useful for the development of skin whitening and anti-bacterial agents.

Keywords: *Canavalia gladiata*; Anti-oxidant; Skin whitening; Anti-bacterial

INTRODUCTION

Canavalia gladiata (Leguminosae family) is widely cultivated in tropical regions such as Southeast Asia and other regions. It is called sword bean since its fruits have a shape similar to a straw cutter. It contains components such as urease, canavanine, hemagglutinine, and *C. gibberellin* I and II [1,2]. In folk medicine, its seeds, pods, stems, and roots are known to have efficacy in treating dysentery, nausea, hemorrhoids, sinusitis, back pain, obesity, diarrhea and hiccups [1]. In addition, several studies have reported the effects of *C. gladiata* on allergy [3], inflammation [4,5], cancer [6], gastritis [7], antioxidant [8], and skin whitening [9]. However, there has

not been sufficient research to verify the differences between ripe and unripe green sword beans.

The antioxidant activity has recently become a target for product development in the pharmaceutical and cosmetics industry [10]. Sword bean extracts have been reported to have a skin whitening effect [9]. These patents only verified the skin whitening effects of sword bean extract, but few studies discussed the effects in relation to ripeness or unripeness of sword beans.

Recently, *Vibrio vulnificus* food poisoning has occurred frequently due to the rising water temperature of the sea level in Korea [11]. In order to prevent food poisoning caused by fish and shellfish including summer sashimi, chlorine- and

oxygen-based disinfectants are used in fish farms. However, the problem of residual toxicity due to these chemical disinfectants has been raised. In this situation, the natural antimicrobial agent using sword bean extract is expected not only to prevent food poisoning caused by *V. vulnificus*, but also to increase the freshness of fish and shellfish while reducing the toxicity. Studies on the antibacterial effect of sword bean extracts against *Streptococcus mutans*, which is known as a causative organism of dental caries, are insufficient. Additional verification of the antibacterial effect of sword bean extracts against *S. mutans* will determine the clinical applicability of sword bean extracts.

In this study, the anti-oxidant effect of sword beans was tested by DPPH and nitro blue tetrazolium (NBT)/xanthine oxidase (XO) assay, focusing on the differences in the anti-oxidant activity between ripe and unripe sword beans. In addition, the skin whitening effect experiments were conducted by the in vitro mushroom tyrosinase activity method. We also investigated the differences in the XO inhibitory activity between ripe and unripe sword beans by the in vitro XO method. Finally, the antibacterial effects of ripe and unripe sword beans were studied on *Vibrio* species and the antibacterial activity against *S. mutans*.

MATERIALS AND METHODS

Experimental materials

1) Materials

Ripe and unripe *C. gladiata* (each 2 kg) supplied from Hwanggeum Nongwon Co. (Jangheung, Korea) were extracted twice with 80% ethanol in a heating mantle (MS-E109; Hanil Lab Tech Co., Ltd., Yangju, Korea) and the extracts were filtered. The extracts were concentrated with a rotary vacuum evaporator (N-1000; EYELA, Tokyo, Japan) and the concentrates were lyophilized using a freeze dryer (TFD8503; Ilshin Lab Co., Ltd., Busan, Korea). The yields of ripe and unripe *C. gladiata* extracts were 23.13% and 5.45%, respectively.

2) Used strains and culture media

The strains used in this experiment were *V. vulnificus* MO6-24/O [12,13], *Vibrio cholerae* N16961 [14], and *S. mutans* ATCC 25175 [15]. These strains were pre-cultured in LB Broth

(1% tryptone, 0.5 % yeast extract, 1% sodium chloride, pH 7.0±0.2) (LB Broth; Becton Dickinson, East Rutherford, NJ, USA) or brain heart infusion (BHI) supplemented with 5% sucrose (BHI Broth; Becton Dickinson).

Experimental methods

1) Measurement of the DPPH radical scavenging activity

The antioxidant capacity was analyzed by measuring their free radical scavenging activity by using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH; 0.2 mM) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Butylated hydroxyanisole (ascorbic acid; 10 µg/mL) (Sigma-Aldrich Chemical Co.) was used as a positive control. The sword bean extracts and other drugs dissolved in methanol were mixed with 0.2 mM DPPH solution (1:1 ratio), and incubated in the dark at room temperature for 30 minutes. Absorbances were measured at 470 nm using an ELISA microplate reader (ELx 808; BioTek Instruments, Inc., Winooski, VT, USA) [16].

2) NBT/XO (superoxide scavenging activity) measurement

Hypoxanthine, ethylenediaminetetraacetic acid (EDTA), NBT and XO were purchased from Sigma-Aldrich Chemical Co. Potassium phosphate buffer (pH 7.4, 1 M) was purchased from Bio-Solution (Suwon, Korea). Superoxide produced while XO causes hypoxanthine to convert to uric acid reacts with NBT forming NBT-Diformazan. At this time, superoxide scavenging capacity can be confirmed through the degree of the increase in absorbance [17].

Potassium phosphate buffer with 0.6 mM hypoxanthine, 1 mM EDTA, and 0.2 mM NBT was prepared. Ripe or unripe *C. gladiata* extract (10 µL), 140 µL of buffer, 50 µL of 0.1 U/mL XO were added to each well of 96-well plates (SPL Life Science Co., Pocheon, Korea). The samples were incubated in the dark at 37°C for 20 minutes, and the absorbance was measured at 490 nm using an ELISA microplate reader. Allopurinol (Sigma-Aldrich Chemical Co.) was used as the positive control.

3) Measurement of the skin whitening effect of *C. gladiata* through the in vitro mushroom tyrosinase activity

In vitro mushroom tyrosinase activity was measured in order to confirm the skin whitening effects of ripe and unripe *C. gladiata* extracts. The extracts were treated with tyrosinase (25 U/mL; Sigma-Aldrich Chemical Co.) in an incubator at 37°C for

10 minutes. In addition, the samples were treated with L-DOPA (50 mM; Sigma-Aldrich Chemical Co.) in the dark at 37°C for 10 minutes, and the absorbance was measured at 490 nm using an ELISA microplate reader. Ascorbic acid (20 µg/mL) was used as a positive control.

4) XO activity of *C. gladiata*

Xanthine, XO, and allopurinol were purchased from Sigma-Aldrich Chemical Co. To measure XO activity, the amount of xanthine converted to uric acid was compared using measurements of absorbance. After 77 µL of 100 mM potassium phosphate buffer (pH 7.4) and 50 µL of ripe and unripe *C. gladiata* extract were added to ultraviolet-transparent 96 well plates (Corning Incorporated, NY, USA), 7 µL of XO (200 mU/mL) was added and the solutions were incubated in the dark at 37°C for 10 minutes in an incubator (Benchmark, Angleton, TX, USA). Then, 66 µL of 0.4 mM xanthine was added and the solutions were incubated in the dark at 37°C for 10 minutes. The absorbance was measured at 290 nm using an ELISA microplate reader. Allopurinol (10 µM) was used as a positive control.

5) Measurement of antibacterial effects of *C. gladiata*

To confirm the antibacterial effects of ripe and unripe *C. gladiata*, the microplate dilution method [18] was performed. *V. vulnificus* and *V. cholerae* were cultured in LB Broth and *S.*

mutans was grown in BHI broth containing 5% sucrose.

We also measured the antibacterial activity by the paper disk diffusion method. Sterilized paper disks (6 mm diameter) were placed on the plate, and each of the samples were dripped [19].

Statistical analysis

All experiments were repeated 3 times or more and statistical analysis was performed with the significant results of three experiments among them. The experiment results were expressed as the mean±standard error, and statistical differences were determined using the Student's t-test. p-value <0.05 was considered statistically significant.

RESULTS

Antioxidant effects through the DPPH radical scavenging activity

The antioxidant capacity was measured by the reaction of ripe and unripe *C. gladiata* extracts with DPPH. As a result, dose-dependent antioxidant activity was observed at the concentrations ranging from 2 to 1, 0.5 and 0.25 mg/mL, and unripe *C. gladiata* showed better antioxidant capacity than ripe *C. gladiata* (Fig. 1). *C. gladiata* extract at the concentration of 2 mg/mL showed an antioxidant effect comparable to that of ascorbic acid of the control group (Fig.

Fig. 1. Anti-oxidant activity of ripe and unripe *Canavalia gladiata* extracts. *C. gladiata* extracts dissolved in methanol were mixed with DPPH (0.2 mM in methanol) in 96 well plate for 30 minutes and then the absorbance was measured at 490 nm using an ELISA microplate reader. Ascorbic acid (10 µg/mL) was used as a positive anti-oxidant drug. *p<0.05, **p<0.01 compared with the untreated group. A: ripe *C. gladiata*, B: unripe *C. gladiata*.

Fig. 2. Anti-oxidant activities of ripe and unripe *Canavalia gladiata* extracts in nitro blue tetrazolium (NBT)/xanthine oxidase assay. The scavenging potential for superoxide radicals was analyzed via a hypoxanthine/xanthine oxidase generating system coupled with NBT. Allopurinol (10 mM) was used as a positive anti-oxidant drug. *p<0.05, **p<0.01, and ***p<0.001 compared with the untreated group. A: ripe *C. gladiata*, B: unripe *C. gladiata*.

1).

Antioxidant effects by reactive oxygen species inhibitory activity

The NBT/XO assay was performed to verify the reactive oxygen species (ROS) inhibitory activity of ripe and unripe *C. gladiata* extracts. ROS inhibitory activity was increased in a dose dependent manner at concentrations from 4 mg/mL to 2, 1, 0.5, and 0.25 mg/mL in both ripe and unripe *C. gladiata* extracts. Unripe *C. gladiata* extracts showed better ROS inhibitory activity than ripe *C. gladiata* extracts (Fig. 2). However, the ROS inhibitory activity of unripe *C. gladiata* extracts was not better than that of Allopurinol (Fig. 2).

Verification of the skin whitening effect through the tyrosinase inhibitory activity

The tyrosinase inhibitory activity of ripe and unripe sword bean extracts was 33% and 45%, respectively at 4 mg/mL (Fig. 3). Ascorbic acid used as the positive control showed tyrosinase inhibitory activity of 68% on average. In vitro mushroom tyrosinase activity experiments at the final concentrations of 4, 2, 1, 0.5, and 0.25 mg/mL of ripe and unripe sword bean extracts, both ripe and unripe sword bean extracts showed tyrosinase inhibitory activity in a dose dependent manner. Based on these experiment results, the

Fig. 3. Effect of ripe and unripe *Canavalia gladiata* extracts on tyrosinase activity. Ripe and unripe *C. gladiata* extracts dissolved in Dulbecco's phosphate-buffered saline were mixed with mushroom tyrosinase in 96 well plate and incubated for 10 minutes at 37°C and then mixtures were mixed with L-DOPA in the same way. The absorbance was measured at 490 nm using an ELISA microplate reader. Ascorbic acid (20 µg/mL) was used as positive control. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the untreated group. A: ripe *C. gladiata*, B: unripe *C. gladiata*.

amounts (IC_{50}) of ripe and unripe *C. gladiata* extracts required to inhibit the tyrosinase activity by 50% were determined. The IC_{50} of unripe *C. gladiata* extract was 6.1 mg/mL, which was higher than the IC_{50} of ripe *C. gladiata* extract, 4.3 mg/mL.

XO inhibitory activity

Ripe and unripe *C. gladiata* extracts showed the XO inhibitory activities of 35% and 24%, respectively at 4 mg/mL (Fig. 4). Allopurinol used as the positive control showed high inhibitory activity of 80% on average against XO. In vitro XO activity experiments, ripe and unripe *C. gladiata* extracts inhibited XO in a concentration dependent manner at final concentrations of 4, 2, and 1 mg/mL.

Based on these experiment results, the amounts of ripe and unripe *C. gladiata* extracts required to inhibit the XO activity by 50% (IC_{50}) were determined. The IC_{50} of unripe sword bean extracts was 5.7 mg/mL, and it was lower than the IC_{50} of ripe sword bean extracts, 8.3 mg/mL.

The antibacterial effects of ripe and unripe *C. gladiata* extracts on *V. vulnificus*, *V. cholerae* and *S. mutans*

To investigate the antibacterial activity of *C. gladiata* extracts against *V. vulnificus*, *V. cholerae* and *S. mutans*, we measured the absorbance after treating each strain with sword bean extracts. As a result, unripe sword bean extract

Fig. 4. Effect of ripe and unripe *Canavalia gladiata* extracts on xanthine oxidase activity. Ripe and unripe *C. gladiata* extracts dissolved in Dulbecco's phosphate-buffered saline were mixed with xanthine oxidase in 96 well plate and incubated for 10 minutes at 37°C and then mixtures were mixed with xanthine. The absorbance was measured at 290 nm using an ELISA microplate reader. Allopurinol (10 µM) was used as a positive control. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the untreated group. A: ripe *C. gladiata*, B: unripe *C. gladiata*.

showed an antibacterial effect on *V. vulnificus* at 4 mg/mL compared to other concentrations (Fig. 5). In contrast, sword bean extracts did not show antibacterial activity against *V. cholerae* and *S. mutans* (Figs. 6 and 7).

In the disk diffusion assay for *V. vulnificus*, the diameters

Fig. 5. Antibacterial effect of ripe and unripe *Canavalia gladiata* extracts on *Vibrio vulnificus*. *V. vulnificus* (90 μ L) cultured in LB Broth was mixed with ripe and unripe *C. gladiata* extracts (10 μ L) in 96 well microplate. The 96 well microplate was cultured in incubator at 37°C overnight. Each growth of bacteria (bact.) was observed by ELISA microplate reader on 630 nm optical density. Tetracycline (TC, 1 μ g/mL) was used as a positive control. * p <0.05 and *** p <0.001 compared with the untreated group. A: ripe *C. gladiata*, B: unripe *C. gladiata*.

Fig. 6. Antibacterial effect of ripe and unripe *Canavalia gladiata* extracts on *Vibrio cholerae*. *V. cholerae* (90 μ L) cultured in LB Broth was mixed with ripe and unripe *Canavalia gladiata* extracts (10 μ L) in 96 well microplate. The 96 well microplate was cultured in incubator at 37°C overnight. Each growth of bacteria (bact.) was observed by ELISA microplate reader on 630 nm optical density. Tetracycline (TC, 1 μ g/mL) was used as a positive control. * p <0.05, ** p <0.01, and *** p <0.001 compared with the untreated group. A: ripe *C. gladiata*, B: unripe *C. gladiata*.

of growth inhibitory zones were 8 ± 0.17 mm and 9 ± 0.22 mm, respectively for ripe and unripe sword bean extracts at the concentration of 4 mg/disk (Table 1). The diameters of growth inhibitory zones for ripe and unripe sword bean extracts were 7 ± 0.31 mm and 8 ± 0.28 mm, respectively at the concentration of 4 mg/disk in the culture media for *V. cholera*, compared to the negative control. Growth inhibitory zones were not observed in the culture media for *S. mutans*

Fig. 7. Antibacterial effect of ripe and unripe *Canavalia gladiata* extracts on *Streptococcus mutans*. *S. mutans* (90 μ L) cultured in LB Broth was mixed with ripe and unripe *C. gladiata* extracts (10 μ L) in 96 well microplate. The 96 well microplate was cultured in an incubator at 37°C overnight. Each growth of bacteria (bact.) was observed by ELISA microplate reader on 630 nm optical density. Tetracycline (TC, 1 μ g/mL) was used as a positive control. * p <0.05 and ** p <0.01 compared with the untreated group. BHI: brain heart infusion, A: ripe *C. gladiata*, B: unripe *C. gladiata*.

Table 1. Antibacterial activity of *Canavalia gladiata* extracts against bacterial strains

Sample	Concentration per disk	Zone of inhibition (mm)		
		Tested microorganisms		
		<i>Vibrio vulnificus</i>	<i>Vibrio cholerae</i>	<i>Streptococcus mutans</i>
Tetracycline (positive control)	2 μ g/disk	17 $\pm$ 0.08	16 $\pm$ 0.05	23.3 $\pm$ 0.10
Ripe <i>C. gladiata</i>	4 mg/disk	9 $\pm$ 0.22	8 $\pm$ 0.28	–
	2 mg/disk	8 $\pm$ 0.29	–	–
	1 mg/disk	–	–	–
	0.5 mg/disk	–	–	–
Unripe <i>C. gladiata</i>	4 mg/disk	8 $\pm$ 0.17	7 $\pm$ 0.31	–
	2 mg/disk	7 $\pm$ 0.20	–	–
	1 mg/disk	–	–	–
	0.5 mg/disk	–	–	–

Values are mean $\pm$ standard deviation of three parallel measurements. –: no inhibition zone observed.

Fig. 8. Inhibition zone of *Canavalia gladiata* extract against 3 bacterial strains (*Vibrio vulnificus*, *Vibrio cholerae*, *Streptococcus mutans*). *V. vulnificus* (plates 1 and 2), *V. cholerae* (plates 3 and 4), and *S. mutans* (plates 5 and 6) were loaded on the plates. Disks with ripe or unripe *C. gladiata* extracts were dropped on the plates. Each 20 μ L was dropped on the plates: tetracycline (1 μ g/mL), DPBS, *C. gladiata* extracts (4, 2, 1 and 0.5 mg/mL). Tetracycline was used as a positive control drug and DPBS was for a solvent control. LB: LB Broth, BHI: brain heart infusion.

(Fig. 8). The diameter of each growth inhibitory zone is shown in Table 1. Unlike the MIC measurement, *C. gladiata* extracts showed antibacterial activity against *V. vulnificus* and *V. cholerae* at the concentration of 4 mg/disk. On the other hand, no growth inhibitory zone was observed in the case of *S. mutans*.

DISCUSSION

Recently, *C. gladiata* is a natural product having attracting attention as an anti-aging and skin-whitening material. In this study, we confirmed anti-aging, skin whitening, and antibacterial activities of *C. gladiata* and investigated the differences in efficacy between ripe and unripe sword beans. With respect to antioxidant effects, it was confirmed that both ripe and unripe *C. gladiata* extracts have antioxidant effects in a concentration dependent manner. Unripe *C. gladiata* extract showed better antioxidant effects than ripe *C. gladiata* extract. In terms of skin whitening effects, it was found that both ripe and unripe *C. gladiata* extracts had positive effects, and ripe *C. gladiata* extract showed better skin whitening effects than unripe *C. gladiata* extract. For XO inhibitory effects, both ripe and unripe *C. gladiata* extracts exhibited XO inhibitory effects in a concentration dependent manner, and unripe *C. gladiata* extract showed better inhibitory effects than ripe *C. gladiata* extract. In terms of antibacterial effects, only unripe *C. gladiata* extract at the concentration

of 4 mg/mL had the antibacterial effect of 50% inhibition in the culture media for *V. vulnificus*. The results of the disk diffusion assay showed significant antibacterial effects on *V. vulnificus* and *V. cholerae*, but no significant antibacterial effect on *S. mutans* was observed.

Therefore, *C. gladiata* is thought to have sufficient potential for development as a raw material for dietary supplements and cosmetics through its antioxidant, skin whitening, and antibacterial effects. In this respect, it is necessary to verify various pharmacologic activities of *C. gladiata* through additional cell and animal experiments in the future.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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