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A Study on the Cellular Protective Function of Black Soybean Extract

Ji-Yuan Liang, Liang- Yü Chen, Jr-Shiuan Wang and Chien-Wei Cheng*
Department of Biotechnology, Ming Chuan University, Gui-Shan 333, Taoyuan, Taiwan, R.O.C

Abstract

Black soybean has been used as a material for functional foods and traditional medicines in Asia for hundreds of years. Germination of black soybean generates bioactive compounds, which may contribute to improve functionalities. Changes in hydrophilic phenolic compounds of black soybean were characterized in terms of total phenolic contents, scavenging activities of superoxide radicals, DNA damage and bacterial viability analysis. During germination of black soybean, the contents of antioxidants increased in seed kernels, but decreased in seed coats. The increase in phenolic contents of seed kernels may be attributed to the dissolution of seed coat pigments during germination. Not only can water extract of black soybean be used as an antioxidant for the prevention of DNA strand breaks and inactivation of *E. coli* by free radicals, it can also be applied as a functional material for dietary supplement.

Keyword: black soybean; antioxidant; anthocyanin; germination; free radical
Corresponding author: Chien-Wei Cheng [ochien@gmail.com]
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Introduction

Black soybean (*Glycine max* L. Merrill) is one of the major ingredients for making soy sauce, which is widely applied as a flavoring agent to foods in East Asia [1]. However, black soybean and its products are also used as functional and medical foods in East Asian countries including Taiwan.

The antioxidant capacity is an important research topic in different fields of human and animal biology, such as physiology and nutrition science [2]. Polyphenols are

normally polyhydroxy phenolic complexes possess an aromatic ring bearing one or more hydroxyl groups. Structures of polyphenols may range from that of a simple phenolic molecule to that of a complex high-molecular mass polymer [3]. Furthermore, phenolic compounds are regarded as the most important antioxidants in plants and plant-based foods [4].

Many lethal effects on live cells, such as DNA degradation and membrane peroxidation, have been attributed to oxygen-derived free radical species, such as superoxide anion radicals ($O_2^{\bullet-}$) and singlet oxygen, also called as reactive oxygen species (ROS) [5, 6]. $O_2^{\bullet-}$ is the intermediate product generated during oxidation and reduction, and can be formed from hydroxyl radical and hydroperoxide compounds, causing cell damage, inflammation, atherosclerosis and aging [7, 8].

Germination of legume seeds may raise the levels of free amino acids, available carbohydrates, dietary fiber and many other components such as oligosaccharides and phenolic compounds. The bioavailable compounds generated may improve the functionality of legume seeds [9-12]. For example, germination has been shown to increase the phenolic content of lupin seeds and their antioxidant activity [13].

Riboflavin, also known as vitamin B_2, is very sensitive to light. After photolysis, riboflavin is changed into triplet excited-state riboflavin. $O_2^{\bullet-}$ or singlet oxygen is generated by the reaction of the triplet excited-state riboflavin [6, 14]. Our previous research found that cleavage of supercoiled plasmid DNA structure and inactivation of *E. coli* could be induced by the ROS from riboflavin photolysis [15, 16].

The aim of this study was to investigate the variation in total phenolic contents of seed coats (SC) and seed kernels of black soybean (SK) after germination. The deformation level of the supercoiled plasmid DNA and *E. coli* viability after riboflavin photolysis was determined as an antioxidant index. The total phenolic contents and free radical-scavenging abilities in the water extract of SC were also determined and compared with the integrity of plasmid DNA and microbial viability after riboflavin photolysis for validation *in vivo*.

Material and Methods

Chemicals

Folin-Ciocalteu reagent, 2'-deoxy-D-ribose, L-methionine, mono-potassium phosphate, potassium dihydrogen phosphate, riboflavin and sodium carbonate were purchased from Sigma-Aldrich (St. Louis, USA). The reagent used for staining DNA was HealthView Nucleic Acid Stain purchased from Genomics (New Taipei City,

Taiwan). Nitro blue tetrazolium (NBT) was purchased from Bio Basic Inc. (Markham Ontario, Canada). A phosphate buffer was prepared from mono-potassium phosphate and potassium di-hydrogen phosphate, and the pH was adjusted to 7.8. The ultra-pure water by Milli-Q system was used as a solvent in this study.

Germination of black soybean

The black soybean was provided by Tatung Soy Sauce Corp. (Yunlin, Taiwan). As shown in Fig. 1, black soybeans were germinated at a bean-water ratio of 1:1.5 (g/g) at room temperature for 24, 48 and 72 hour in the dark. After germination, the SC and SK were separated and lyophilized. The freeze-dried samples were ground through a 60-mesh sieve.

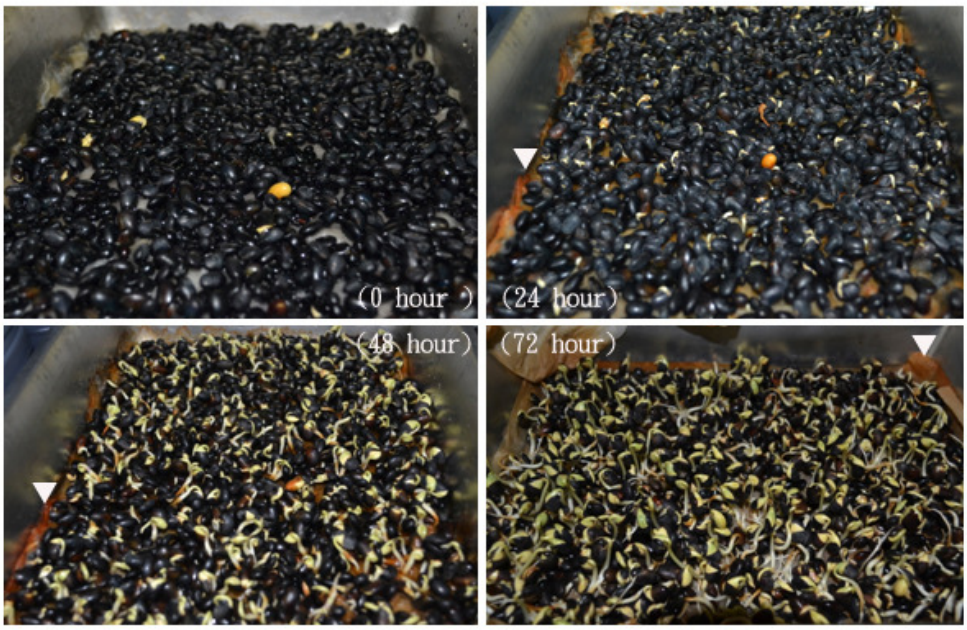

Fig. 1 Photographs of black soybean during germination for 0, 24, 48 and 72 hour. (**∇:** brown substances in soaking water)

Water extract of black soybean (WEBS)

WEBS was prepared by soaking black soybean at a bean-water ratio of 1:1.5 (g/g) for 2, 6, 12, and 24 hour. The soaking water was then collected and stored at -20°C for subsequent analysis of total phenolic contents in WEBS.

Water extract of seed coats of black soybean (WESC)

Preparation of WESC was as follows. In brief, one gram of ground SC and 5 mL water underwent sonication into a centrifuge tube for 20 min. The samples were centrifuged at 4°C, and 3,000 rpm for 10 min, and the supernatant was collected. The process was repeated until the precipitate and the combined extracts were quantitated to 10 mL. The final concentrations of WESC obtained were 0, 10, 100, 1,000, and 10,000 μg/g.

Determination of total phenolic contents

The total phenolic content of each extract was determined by a modified Folin-Ciocalteu method [17]. The ground samples (SC or SK) were extracted as described below. All solutions were prepared in 70% ethanol. The final concentrations of extracts were 0, 100 and 1,000 μg/g.

The extract solution (250 μL) was mixed with 1 N Folin-Ciocalteu reagent (250 μL), and incubated for 5 min. The solution was mixed with 0.5 mL of 20% sodium carbonate and 4 mL water, and incubated at room temperature for 25 min. The mixture was centrifuged at 4°C and 5,000 rpm for 10 min to remove the precipitate. The combined supernatants were collected and quantitated to 10 mL. The supernatants were detected by the UV/Vis spectrophotometer (PerkinElmer Lambda35) at 730 nm. The total phenolic contents of the samples were determined as a gallic acid equivalent (GAE) in mg per gram of dry sample extract.

$O_2^{\bullet-}$-scavenging activity assayed by NBT reduction

The $O_2^{\bullet-}$-scavenging activity of the extract was determined by the modified method [18]. The ground samples of SC were extracted as described in Section 2.4. All solutions were prepared in phosphate buffer (50 mM pH 7.8). The final concentrations of extracts of SC were 0, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10 mg/g.

The photo-induced reactions were performed in a plastic box (104 cm × 74 cm × 55 cm) with a 30 W fluorescent lamp (FCL30D/28, China Electric MGF. Co., Taiwan), and the reaction system was covered with a black cloth. The relative position of the reactant and lamp was fixed. The distance of lamp and sample was 20 cm. The intensity of illumination was adjusted to 4,000 Lux by a digital light meter (YF-170, Tenmars Electronics Co., Taiwan).

The total volume of reactant was 3 mL and the concentrations of riboflavin, methionine and NBT were 2.4×10^{-6} M, 0.01 M and 1.6×10^{-4} M, respectively. The reactant was illuminated for 20 min. The riboflavin photolysis generated $O_2^{\bullet-}$ which reduced NBT to form blue formazan that can be detected at 560 nm. Extract of SC (50 μL) was added into the reaction mixture, in which $O_2^{\bullet-}$ was scavenged, thereby inhibiting reduction of NBT. For the control treatment, the reactant was kept in the dark. The $O_2^{\bullet-}$-scavenging activity was calculated using the following equation.

$$O_2^{\bullet-}\text{-scavenging activity (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100\%$$

where *A* denotes the absorbance of blue formazan measured at 560 nm.

Effects of water extract of seed coats of black soybean (WESC) on DNA integrity assay

The illumination units were setup according to the method developed by Liang *et al*. [15]. Six DC 12V 5050 of light-emitting diode (LED) chips (Vita LED Technologies Co., Tainan, Taiwan) were pasted onto the plastic cylinder of the illumination chamber (opaque plastic, height of 8 cm and diameter of 7 cm). The reaction solution was put in a glass tube of 6 mm in diameter and 100 mm in height, which was set at the top end of the cylinder. The wavelength of maximum emission of blue LED is 462 nm and spectral width at half height ($W_{1/2}$) is 23 nm. The illumination intensity was set at 1.5 mW/cm^2 and validated by a solar power meter (TM-207, Tenmars Electronics Co., Taiwan). The illumination chamber was placed in a cold room (9 ± 1°C).

The DNA integrity assay was determined according to the method developed by Liang *et al*. [16]. Plasmid DNA, pGEM-7Zf(-) as the target molecule, was transformed into *E. coli*, DH5α strain (NCBI Taxonomy ID: 668369), and grown overnight in LB broth at 37°C. The culture was then harvested and the DNA was purified using a Plasmid Miniprep kit (BioKit, Miaoli, Taiwan). After purification, 600 μL plasmid DNA was dissolved in water and 800 μL of riboflavin (120 μM in 100 mM phosphate buffer at pH 7.8) were added into the glass tube. Investigations on effects of WESC on DNA integrity were conducted as follows. (A) The plasmid DNA solution was treated with 23.3 μL of WESC (0, 10, 100, 1,000, and 10,000 μg/g) at 1.5mW/cm^2 by blue-light illumination for 20 min. (B) The plasmid DNA solution was treated with 23.3 μL of WESC (10,000 μg/g) at 1.5mW/cm^2 by blue-light illumination for 2, 4, 6, 8, 10 and 30 min, respectively. For the dark control treatment, the tubes were covered with thick aluminum foil. After illumination, the 10 μL reaction solution was taken out and quenched by adding 2 μL of loading dye (0.25% bromophenol blue and 40% sucrose) for the next electrophoresis by 1.2% agarose gel. Polymorphism of plasmid DNA was visualized by internal staining in gel with the nucleic acid stain (HealthView).

Effects of WESC on viability of E. coli by riboflavin photolysis

Investigations on effects of WESC on viability of *E. coli* via a riboflavin photolysis were determined using the method developed by Liang *et al*. [15]. *E. coli* was grown overnight in LB broth at 37°C. After overnight growth, 500 μL of *E. coli* was loaded in a 1.5-mL centrifuge tube and diluted three times with two-fold sterilized water. Cultures were grown to an optical density at 600 nm (OD_{600}) of 0.5 (ca. 1.2 × 10^{10} CFU/mL). After centrifugation at 10,000 rpm for 5 min, the supernatant was removed. 1,000 μL of riboflavin (30 μM in 100 mM phosphate buffer at pH 7.8) was added into the centrifuge tube containing *E. coli* and mixed well. The *E. coli* solution was treated with or without 20 μL of WESC (10,000 μg/g) at 1.5mW/cm^2 by blue-light illumination for 60 min, respectively. After illumination, the 200 μL reaction solutions were diluted with sterilized water and transferred to LA plates for overnight

cultivation at 37°C. The survival of *E. coli* following treatment was examined by counting the number of viable colony-forming units (CFUs) after overnight incubation. The inactivation rate of *E. coli* was calculated using the reduction percentage, $(1 - I/D) \times 100\%$, where I and D are the numbers of CFUs after illumination (I) and keep in the dark (D), respectively. Thus, the reduction percentage was defined as a negative value of the inactivation rate.

Statistics analysis

Data are represented by mean ± standard deviation of three separate experiments. A homoscedastic two-sample *t*-test was employed to assess whether the two sets of measurements differed, and the values of $P < 0.05$ were considered significant.

Results and Discussion

Effects of germination on total phenolic contents of SC and SK

The total phenolic contents of SC and SK during germination were investigated. As shown in Fig. 2, the total phenolic contents of SC were all higher than those of SK. Moreover, while the total phenolic contents of SC decreased steadily with increase in duration of germination, those of SK increased moderately. Both DPPH radical-scavenging activity and reducing power of SC decreased with increase in germination duration (data not shown here).

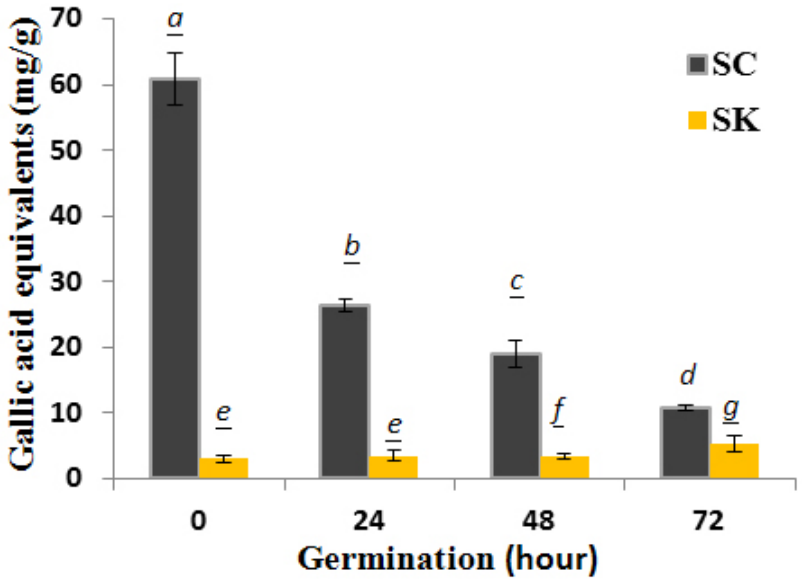

Fig. 2 Changes in total phenolic contents of SC and SK during germination for 0, 24, 48 and 72 hour. Data were represented by mean ± standard deviation, where n = 3. Statistical differences ($p < 0.05$) between groups are indicated by different letters above the bar.

Effects of germination on $O_2^{\bullet-}$-scavenging activity of SC

The dose-response curves for the $O_2^{\bullet-}$-scavenging activity of SC are shown in Fig. 3A. As can be seen, $O_2^{\bullet-}$-scavenging activities of SC reduced with increase in germination duration. The IC_{50} of SC is defined as the equivalent concentration of SC that can remove 50% of $O_2^{\bullet-}$. As shown in Fig. 3B, the IC_{50} of SC increased with increase in germ.

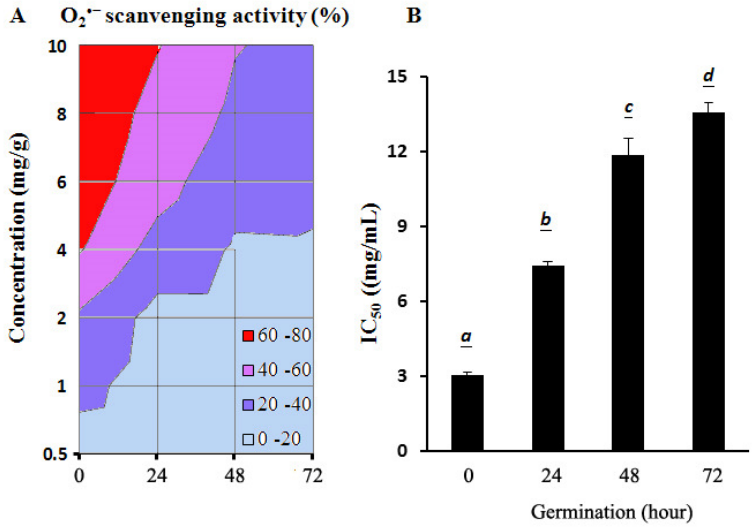

Fig. 3 A. $O_2^{\bullet-}$-scavenging activity of SC during germination for 0, 24, 48 and 72 hour. B. IC_{50} of SC. Data were represented by mean ± standard deviation, where n = 3. Statistical differences ($p < 0.05$) between groups are indicated by different letters above the bar.

Total phenolic contents of WEBS

The total phenolic contents of WEBS under immersion treatment of different durations are shown in Fig. 4. As can be seen, the total phenolic contents of WEBS increased with increase in immersion time. Moreover, the changes in total phenolic contents by 12- and 24-hour immersion treatments were both insignificant.

As seen in Figs. 2 and 3, both total phenolic contents and $O_2^{\bullet-}$ scavenging activities of SC reduced with increase in germination time. Brown substances were produced during germination from pigments of black soybean dissolved into the soaking water as shown in Fig. 1. On the other hand, Fig. 4 shows increase in total phenolic contents of WEBS with immersion time. Anti-oxidative ability of black soybean is associated to its high content of flavonoids, such as anthocyanins and isoflavones [1, 5]. Most of the flavonoids are the lipophilic compounds in soybean kernels [1, 9, 19]. The total phenolic contents and anti-oxidant capacity of SC were far superior to those of SK [20]. The composition of anthocyanins found in the SC of black soybean have been identified as 3-glucosides of delphinidin, cyanidin, and petunidin [21].

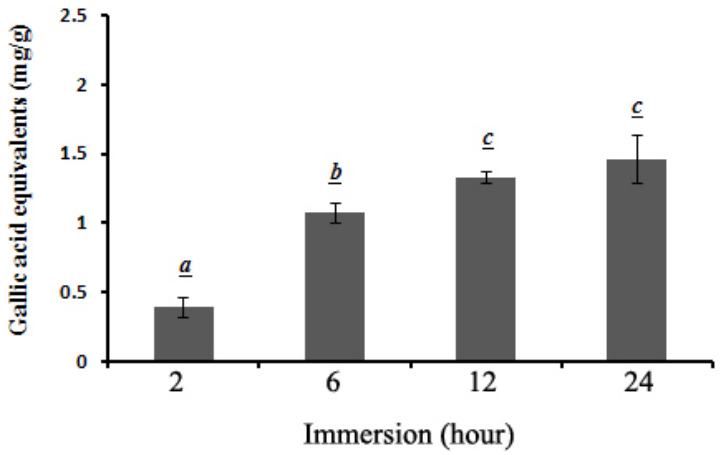

Fig. 4 Total phenolic contents of WEBS after immersion for 2, 6, 12 and 24 hour. Data were represented by mean ± standard deviation, where n = 3. Statistical differences ($p < 0.05$) between groups are indicated by different letters above the bar.

Increased phenolic content of lupin seed after germination as well as their antioxidant activity was reported by Dueňas *et al*. (2009)[13]. Fig. 2 shows increase in total phenolic contents of SK during germination. The seed coat pigments dissolved into water might be adsorbed by seed kernels during germination, contributing to increase in phenolic content of SK.

Effects of WESC on DNA integrity assay

To examine the effects of WESC on protection of DNA strand breaks, supercoiled plasmid DNA was allowed to react with ROS generated via a riboflavin photolysis. The effects of ROS from riboflavin photolysis were evaluated by its impact on plasmid DNA integrity of *E. coli*. As shown in Fig. 5A, supercoiled plasmid DNA could be observed in lane P, and lane D was the plasmid DNA within riboflavin kept in the dark for 30 min. The other lanes were illuminated within riboflavin by blue light at 1.5 mW/cm^2 for different durations. The plasmid DNA within riboflavin illuminated for 2 min remained in supercoiled form. After riboflavin photo-reaction for more than 4 min, the plasmid DNA bands became linear or open circular instead of supercoiled. These results showed that riboflavin photolysis generating ROS enhanced the levels of DNA cleavages. Excessive DNA strand breakage might be associated with depletion of cellular ATP and NAD^+ levels, thus interfering with ATP synthesis and perhaps even leading to cell death [22] .

Lanes 10-10,000 μg/g were the plasmid DNAs treated with riboflavin at blue-light illumination and in presence of 10, 100, 1,000, and 10,000 μg/g WESC, respectively. It seems that treatment with WESC at the level of 10,000 μg/g could scavenge $O_2^{\bullet -}$ via riboflavin photolysis, thus providing protection to plasmid DNA. As shown in Fig. 5B, lanes 2C-30C were the plasmid DNAs treated with riboflavin and 10,000 μg/g WESC at blue-light illumination for different durations. The bands of plasmid DNAs were all

retained for up to 30 min.

WESC could potentially be used as an antioxidant for the prevention of DNA damages by free radicals. Tea polyphenols are natural antioxidants. Previous research reported that green tea exhibited similar protective behaviors under the same DNA integrity assay with the plasmid DNA treated with riboflavin and green tea by blue-LED illumination at 1.5 mW/cm^2 [23]. With the same pattern as green tea, WESC may be retrieved to be a functional material for dietary supplement.

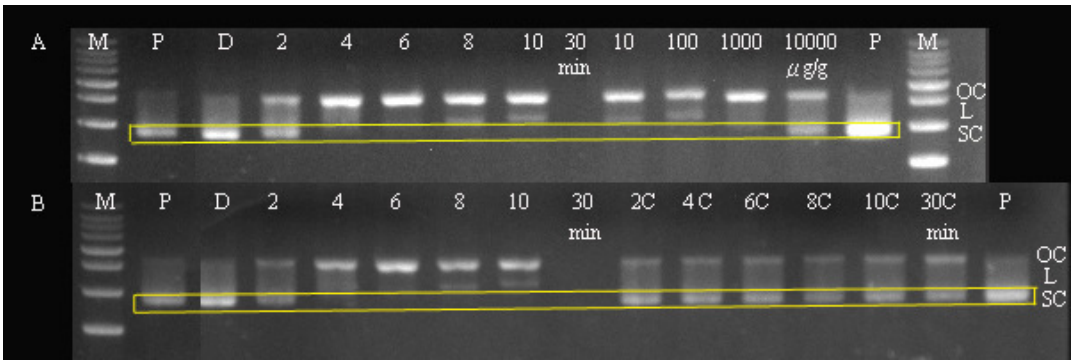

Fig. 5 DNA integrity assay *in vitro*. **A.** Lane M, 1 kb DNA marker. Lane P, plasmid DNA, pGEM-7zf(-). Lane D, plasmid DNA and riboflavin in the dark for 30 min. Lanes 2-30 min, plasmid DNA treated with riboflavin at 1.5mW/cm^2 by blue-light illumination for 2, 4, 6, 8, 10 and 30 min, respectively. Lanes 10-10,000 μg/g, plasmid DNA treated with riboflavin and WESC (10, 100, 1,000 and 10,000 μg/g) at 1.5 mW/cm^2 by blue-light illumination for 20 min. **B.** Lanes 2-30 min, plasmid DNA treated with riboflavin at 1.5mW/cm^2 by blue-light illumination for 2, 4, 6, 8, 10 and 30 min, respectively. Lanes 2C-30C, plasmid DNA treated with riboflavin and 10,000 μg/g WESC at 1.5 mW/cm^2 by blue-light illumination for 2, 4, 6, 8, 10 and 30 min, respectively. OC, L, and SC stand for open-circular, linear, and supercoiled plasmid DNA, respectively.

Effects of WESC on viability of E. coli by riboflavin photolysis

As shown in Fig. 6, the photochemical effect of blue-light illumination (1.5 mW/cm^2, 60 min) without riboflavin added was about 32% inactivation rate for *E. coli*. Blue light excited endogenous intracellular porphyrins to behave as photosensitizers, creating reactive singlet oxygen and other ROS, which resulted in cell death via disruption of organelles and chromosomal genetic materials [24].

The blue light-induced ROS from riboflavin 5'-phosphate has been employed to inactivate *E. coli* [15]. Fig. 6 shows 80% inactivation ratio of *E. coli* in riboflavin after blue-light illumination for 60 min at 1.5 mW/cm^2. The effects of WESC on the viability of *E. coli* by blue-light illumination via riboflavin photolysis were investigated. As shown in Fig. 6, when WESC was added in the presence of riboflavin by blue-light illumination at 1.5 mW/cm^2 for 60 min, a 34% inactivation rate could be achieved. The results of the WESC assay showed that up to 46% of *E. coli* was retained when treated with riboflavin and WESC by blue-light illumination for 60 min. $O_2^{\bullet-}$ generated from riboflavin photolysis can be employed to examine the effect of

luminance on NBT reduction [25]. As shown in Fig. 6, up to 46% of *E. coli* was retained after riboflavin photolysis for 60 min, showing that WESC can inhibit the $O_2^{\bullet-}$ generated from riboflavin photolysis.

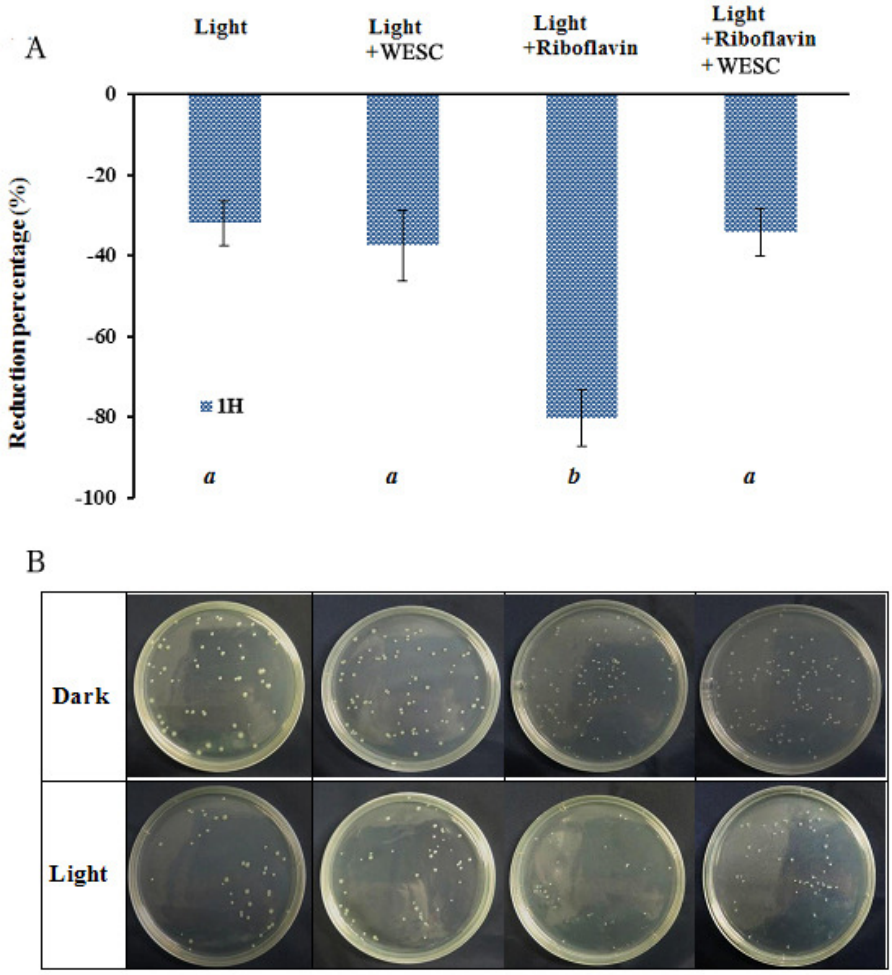

Fig. 6 The effects of riboflavin photolysis on reduction percentage of *E. coli* **A.** Effects of WESC on viability of *E. coli* treated with riboflavin photolysis by blue-light illumination at 1.5 mW/cm^2 for 60 min. **B**. The survival of *E. coli* was examined after riboflavin photolysis reaction by blue-light illumination at 1.5 mW/cm^2 for 60 min. Data were represented by mean ± standard deviation, where n = 3. Statistical differences ($p < 0.05$) between groups are indicated by different letters below the bar.

Black soybean is usually immersed in water for purpose of cleaning before the processing of soy sauce. Soaking water is normally drained after the immersion process as wastewater by the industry. However, total phenolic content of about 40 mg GAE/g was found in the powder obtained by spray-drying the soaking water of black soybean for soy sauce manufacture. According to the results of $O_2^{\bullet-}$ scavenging, DNA damage protection and bacterial viability analysis, the water extract of black soybean contains very good antioxidants for developing into a functional food.

Conclusions

The total phenolic contents of seed kernels of black soybean increased during germination. The pigments in seed coats may be dissolved into water and adsorbed by seed kernels during germination of black soybean. The water extract of black soybean has very good cell protection activities according to the results of $O_2^{\bullet-}$ radical-scavenging, DNA damage and bacterial viability analysis. Soaking water of black soybean can be taken as a bio-resource and an antioxidant to prevent DNA damages by free radicals. The black soybean water extract can be a functional material for dietary supplements.

Acknowledgements

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黑豆萃出物之細胞保護功能研究

梁致遠、陳良宇、王志玄、鄭建瑋*

銘傳大學 健康科技學院 生物科技學系 (中華民國 台灣 桃園)

中文摘要

數百年來，亞洲將黑豆當成功能性食品及傳統的醫葯。發芽的黑豆產生活性物質，能增進功能性。以黑豆水溶性多酚物質的改變，分析其多酚含量、清除超氧自由基能力、DNA 損害及微生物活性。黑豆發芽期間，豆仁的抗氧化活性增加，但種皮的抗氧化活性降低。發芽期間，豆仁的抗氧化活性增加可能是種皮的色素溶解導致。黑豆水溶性萃取物不僅可作為抗氧化物質且可避免因自由基引起 DNA 的斷裂及大腸桿菌的失活。黑豆水萃物可作為日常所須功能性材料。

關鍵字：黑豆、抗氧化活性、花青素、發芽、自由基
通訊作者: 鄭建瑋[ochien@gmail.com]

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