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Antioxidant and antibacterial behavior of sediment removed ethanol extract from sea buckthorn seed

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Abstract–The purpose of this study was to develop the separation process removing sediment in sea buckthorn seed ethanol extract, and to investigate the antioxidant and antibacterial behavior for the sediment removed ethanol extract (SBS extract). Sediment such as low solubility materials and oil component was simply removed by filtering after storing the crude extract containing celite for 16 h in a refrigerator at 4 °C. The SBS extract displayed strong antioxidant activity in the stability test as a function of both storage time and temperature when compared to vitamin C and BHA (Butylated hydroxyanisole) as typical antioxidant ingredients. The bactericidal ratio of the SBS extract against *E. coli* increased with an increase in the addition of the test agent, and this was particularly significant at concentrations greater than 1,000 μg/ml addition. The bactericidal ratio of 1,000 μg/ml SBS extract against *B. subtilis*, *S. aureus*, *P. aerusinosa* and *E. coli* was 96.4, 98.6, 91.5, and 52.2% after 2 h, and 98.2, 99.7, 99.3, and 82.6% after 4 h, respectively. These results suggest that the SBS extract may be used as a functional food, pharmaceutical, and cosmetic agent.

Key words: Sea Buckthorn Seed, *Hippophae rhamnoides* L., Sediment Separation, Antioxidant and Antibacterial Behavior, Ethanol Extract, Stability

INTRODUCTION

Sea buckthorn is a deciduous spiny shrub or small tree of the genus *Hippophae* that is between two to four meters high. It is widely distributed throughout the temperate zone of Asia and Europe. It bears fruit that are yellow or orange in color. In addition, it is hard, drought and usually cold tolerant and is useful for land reclamation and farmstead protection. The genus belongs to the family Elaeagnaceae, which consists of six species and 10 subspecies, among which the most economically important one is *Hippophae rhamnoides* Linn., commonly known as sea buckthorn [1-3].

Sea buckthorn fruit contains 60-80% juice that is rich in sugar, organic acids, amino acids, and vitamins. The maximum vitamin C content is 2,500 mg/100 g, which is five to 100 times higher than any other fruit or vegetable known. In addition, the sea buckthorn fruit is rich in proteins such as globulins and albumins, fatty acids, such as linoleic acids and linolenic acid, and antioxidants, such as polyphenols, tocopherol, carotenoids, and flavonoids, etc. There are 24 chemical elements in sea buckthorn juice, including calcium, magnesium, phosphorous, iron, manganese, sodium, potassium, and aluminum, among others. In addition, sea buckthorn berries, leaves, and bark contain β-sitosterol, tocopherol, and many other bioactive compounds [4-13]. The oil content ranges from 1.5% to 3.5% in fruit pulp and about 9.9% to 19.5% in seeds. The oil from the juice and pulp is rich in palmitic (16 : 0) and palmitoleic (16 : 1) acids, which are linoleic (18 : 2) and linolenic (18 : 3) acids. The oil from the seed and juice also contains vitamin E and carotene [14-16]. Many health benefits have been associated with sea buckthorn such as preventive effects against flu, cardiovascular problems, mucosa injuries, and skin problems [17,18]. Recently, interest has increased considerably in finding naturally occurring antioxidant and antimicrobial agents for use in foods, cosmetics and medicinal materials to replace synthetic antioxidants and antimicrobial agents, which are being restricted due to their adverse side effects such as carcinogenicity [19-23]. As mentioned above, many studies have been carried out to assess the biological activities of different parts (stems, leaves, roots, fruits) of sea buckthorn. However, extraction process of the sea buckthorn seed using ethanol has not yet been studied. In addition, sediment separation from crude extract has economic and technical difficulties because of the oil and low solubility components of sea buckthorn seeds, which results in phase separation due to sedimentation.

In this study, the sediment such as oil and low solubility materials was simply removed from the sea buckthorn seed ethanol extract by filtering after storing the crude extract containing celite for 16 h at 4 °C, and the antioxidant and antibacterial behavior of the extract were investigated at each process step, storage time, and temperature, and also compared to vitamin C and BHA as typical antioxidant ingredient.

MATERIALS AND METHODS

1. Preparation of SBS Extract

Sea buckthorn seed was purchased at Ulaanbaatar, Mongolia. 500 g of sea buckthorn seed crushed using a hammer mill (purchased

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by KOREA PULVERIZING MACHINERY CO. LTD, Korea) was extracted in 10 L of ethanol (94% purity, DAEJUNG Chemicals and Metals, Korea) at 50 °C and 500 rpm. After 18 h of extraction, the supernatant was recovered. The residue was the re-extracted using the same procedure as the first extraction. The obtained extraction solutions were combined and concentrated to a dry weight of 2.5-3.0% using a vacuum-evaporator at 60 °C. The concentrate was then mixed with 10% (w/v) of celite (No. 545, DAEJUNG Chemicals and Metals, Korea) and stored in a refrigerator at 4 °C for 16 h and filtered using a vacuum filtering apparatus with filter paper (Whatman No. 2). The extract (SBS extract) was then used in experiments.

2. Antioxidant Activity of SBS Extract

The antioxidant activity was measured using DPPH free radical-scavenging assay. 1 ml of DPPH (1.1-diphenyl-2-picrylhydrazyl, Sigma, USA) and 2.5 ml of the extract was reacted at 37 °C in an incubator for 30 min and the absorbance was measured at 517 nm. DPPH inhibition (%) was calculated as follows:

$$\text{DPPH inhibition (\%)} = [1 - (\text{Exp} - \text{Blank})/\text{Control}] \times 100$$

3. Antibacterial Activity of SBS Extract

Staphylococcus aureus KCCM 11764, *Bacillus subtilis* KACC 10111, *Pseudomonas aerusinosa* KACC 10232, and *Escherichia coli* KCCM 12119 was obtained from the KCCM (Korean Culture Center of Microorganisms, Korea) and KACC (Korean Agricultural Culture Collection, Korea), and used as a reference strain during the antibacterial tests. Approximately 10^3-10^5 CFU/ml of these strains were inoculated on 100 ml Tris-HCl buffer (10 mM, pH 7.0) that was supplemented with the SBS extract and control group. The culture flask was then shaken at 200 rpm on a rotary shaker at each temperature. The antibacterial efficacy was determined by spreading 0.1 ml of the appropriate dilution of the strain suspension on agar plates and the number of colonies was counted. The bactericidal ratio was calculated as follows:

$$\text{Bactericidal ratio (\%)} = (N_0 - N_1)/N_0 \times 100$$

Where, N_0 and N_1 are the number of colonies of the strain suspension at the initial and culture time, respectively.

RESULTS AND DISCUSSION

1. Antioxidant Activity, Extraction Yield, and Stability

DPPH is a free radical that accepts an electron or hydrogen radical to become a diamagnetic molecule and has maximum absorbance between 515 and 520 nm. The scavenging activity of antioxidants by hydrogen donation decreases the absorbance and the color changes from purple to yellow. Thus, DPPH is stable in alcohol solutions due to hydrogen bonding between the alcohol and nitrogen in DPPH although secondary and tertiary oxidation reactions occur in non-polar solvents such as dioxane or CCl_4 [24]. The antioxidant activity at each step of the separation process was evaluated by measuring the DPPH radical scavenging activity. The results of these assays are displayed in Table 1 and Fig. 1. CR is the crude ethanol extract without separation and CE is the extract separated by the use of celite, and CE-CS is the extract separating CR, which contains celite, after storage for 16 h in a refrigerator at 4 °C. The DPPH radical scavenging activity increased with an increase in the test extract concentration and all extracts obtained at the different steps of extraction

Table 1. Comparison of the extraction yield, antioxidant activity (IC_{50}), and stability at the different steps of the separation process used for ethanol extraction of sea buckthorn seed

	CR	CE	CE-CS
Extraction yield (%)	14.0	13.1	12.6
IC_{50} (μg/ml)	5.5	7.5	8.0
Phase separation in stability test during 70 days under room temperature	○	○	×

CR: the crude ethanol extract without separation
CE: the extract separated by the use of celite
CE-CS: the extract filtering CR, which contains celite, after the storage of 16 h in refrigerator of 4 °C
IC_{50}: 50% inhibition concentration

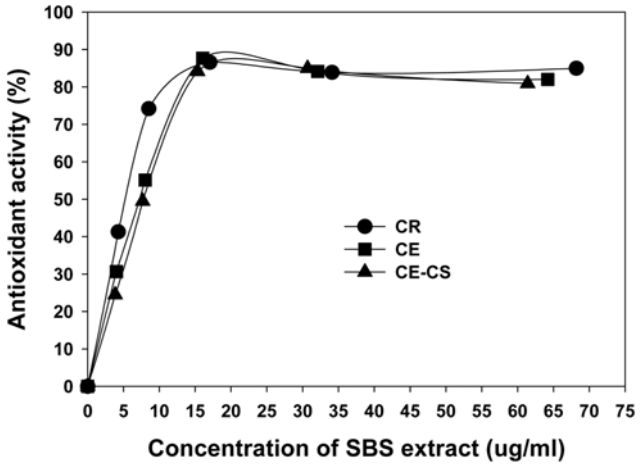

Fig. 1. Antioxidant activity at different steps of ethanol extraction from sea buckthorn seed (CR: the crude ethanol extract without separation, CE: the extract separated by the use of celite, CE-CS: the extract filtering CR, which contains celite, after the storage of 16 h in refrigerator of 4 °C).

exhibited effective antioxidant activity. The IC_{50} (50% inhibition concentration), which was calculated from Fig. 1, was 5.5 μg/ml for CR, 7.5 μg/ml for CE, and 8.0 μg/ml for CE-CS. From Table 1, the extract yield was calculated to be 14.0, 13.1, and 12.6% for CR, CE, and CE-CS, respectively. The separation process resulted in an increase in the IC_{50} i.e., a decreased antioxidant activity, and decreased extract yield. However, phase separation was not observed for CE-CS in the stability test after storage for 70 days at room temperature. This was most likely due to the aggregation of low solubility materials and oil component, which were separated by celite at low temperature (4 °C).

2. Comparison with Other Antioxidant Agent

The antioxidant activity of SBS extract (CE-CS) was compared with vitamin C and BHA, which are typical antioxidant ingredients. As shown in Fig. 2, SBS extract showed strong antioxidant activity, which was similar to vitamin C and higher than BHA. We also compared the antioxidant activity after storage for 70 days at room temperature. As shown in Fig. 3, the antioxidant activity of SBS extract and BHA did not decrease after 70 days. However, the antioxidant activity of vitamin C significantly decreased after 70 days. These results indicate that SBS extract may be useful as an

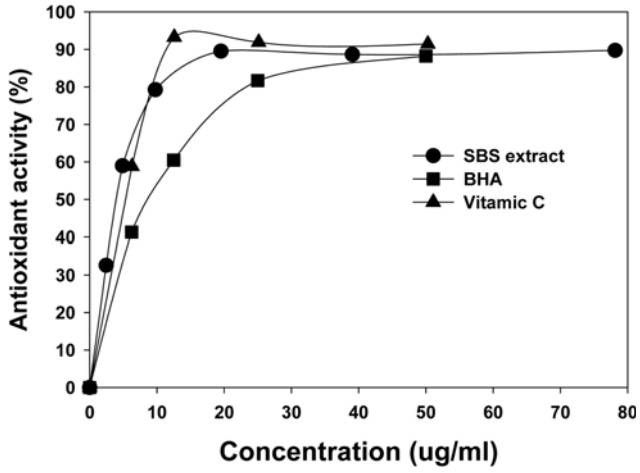

Fig. 2. Comparison of the SBS extract with other antioxidant agents.

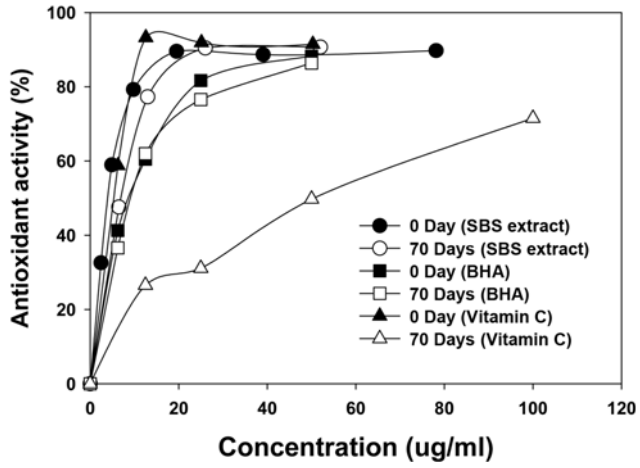

Fig. 3. Effect of storage time on the antioxidant activity.

effective alternative antioxidant agent. In addition, it was stable against high temperature. Fig. 4 shows the antioxidant activity after 1 h of incubation at different temperatures. The test agents without treatment were used as a control. The maximum temperature at which SBS extract, BHA and vitamin C were still stable was 150 °C, 100 °C and 200 °C, respectively. This result indicates that the antioxidant components in the extract, such as phenolics, may be stable at high temperature. Vitamin C is also stable at high temperature since it has a high melting point of 190-192 °C. BHA is unstable at high temperature because it has a relatively low melting point of 48-55 °C. Stable temperature can be used to determine the kind of dry process such as freeze drying, vacuum drying, or spray drying.

3. Antibacterial Efficacy of SBS Extract

Comparisons of the antibacterial activities of the SBS extract, vitamin C and BHA are shown in Fig. 5. The SBS extract and BHA showed strong antibacterial activity against *E. coli*. However, vitamin C did not show any antibacterial activity. BHA is a synthetic antioxidant and antimicrobial agent that causes carcinogenicity due to their mutagenicity and toxicity against enzymes and lipids [25]. It has been suggested that the main factors contributing to the antibacterial activity of the plant extract are phenolics such as flavonoid [26]. Phenolic compounds are extensively distributed throughout

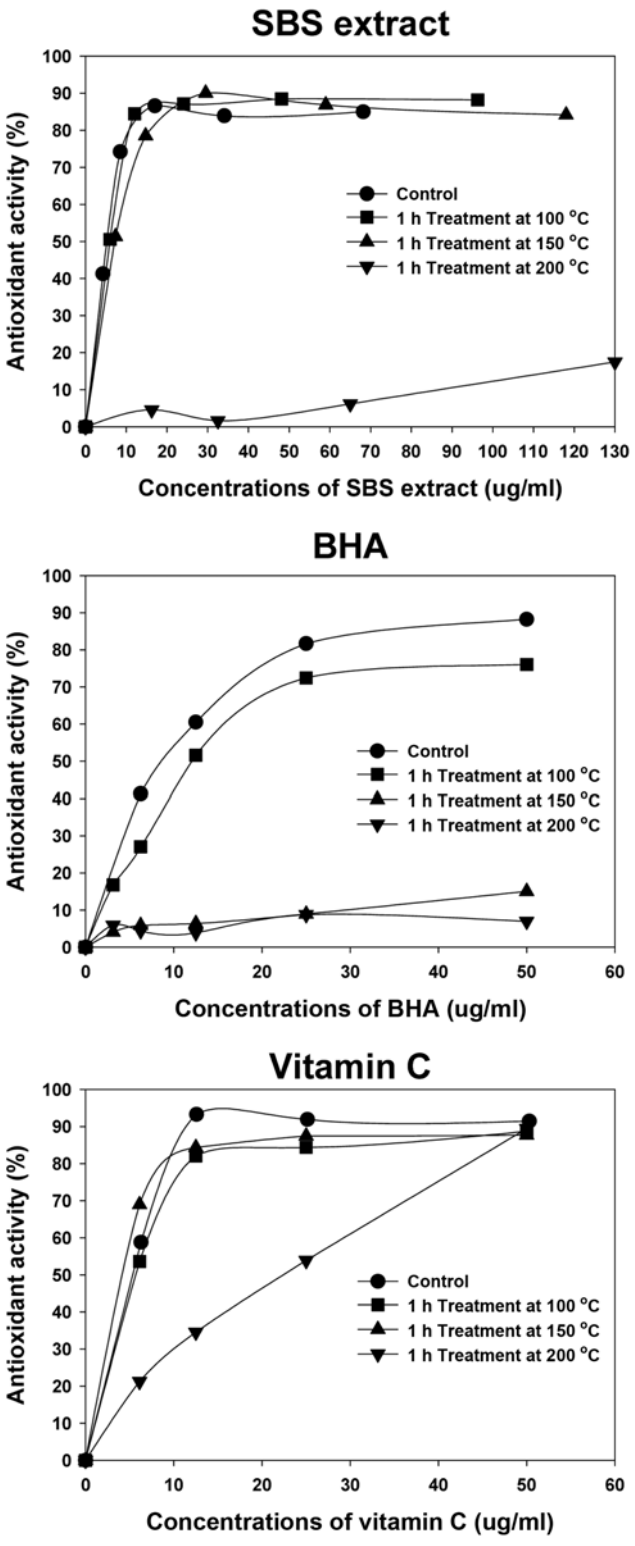

Fig. 4. Effect of temperature on the antioxidant activity.

plants and can bind to proteins, enzymes, or other macro-molecules as well as divalent metal ions, due to the phenolic hydroxyl group [27].

4. Effect of Temperature on Antibacterial Activity

The effect of temperature on the antibacterial activity against *E. coli* was evaluated. As shown in Fig. 6, the bactericidal ratio reached 82.6% at 37 °C after 4 h, and 100% after 8 h; however, the bacteri-

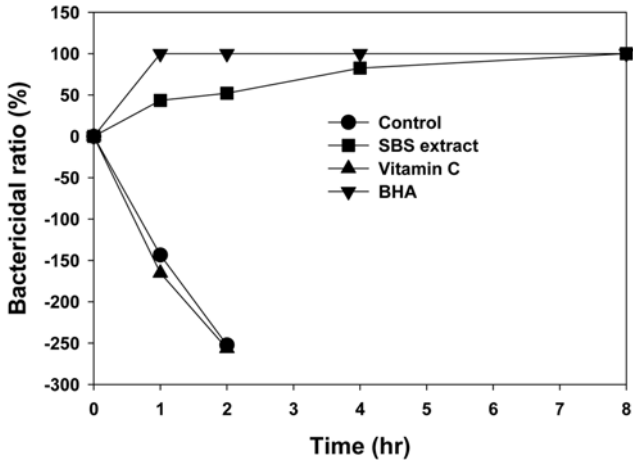

Fig. 5. Comparisons of the antibacterial activities of the SBS extract, vitamin C and BHA (*E. coli*, 200 rpm, 37 °C).

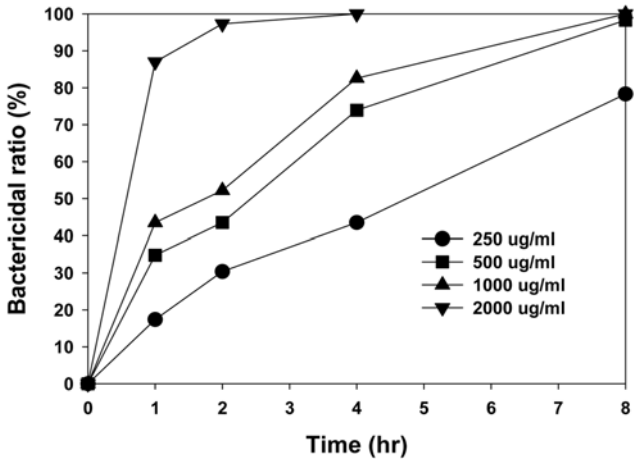

Fig. 7. Effect of SEB extract concentration on the antibacterial activities (*E. coli*, 200 rpm, 37 °C).

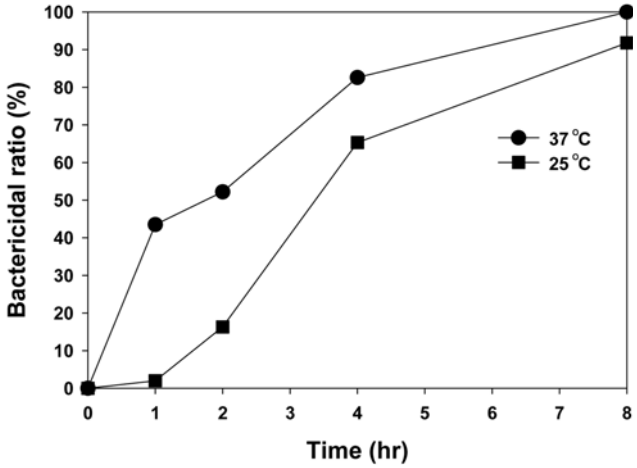

Fig. 6. Effect of culture temperature on the antibacterial activities (*E. coli*, 200 rpm).

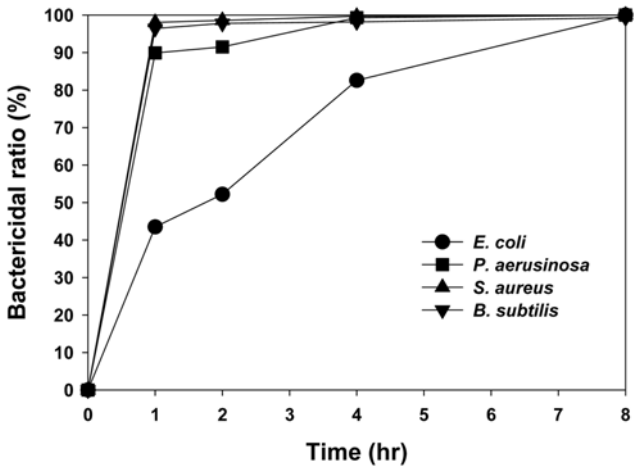

Fig. 8. Antibacterial activity against other bacteria (200 rpm).

cidal ratio only reached 65.3% at 25 °C after 4 h, and 91.8% after 8 h. The bactericidal ratio at 37 °C was obviously higher than at 25 °C. This result is in agreement with a previous study, which reported that the activity of bacteria and the adaptability to the environment changed with temperature [28].

5. Effect of Concentrations on Antibacterial Activity

The bactericidal ratio against *E. coli* at different concentrations was measured and the results are shown in Fig. 7. The bactericidal ratio at 250, 500, 1,000, and 2,000 μg/ml was 30.4, 43.5, 52.2, and 97.3% after 2 h, and 43.5, 73.9, 82.6, and 100% after 4 h, respectively. The bactericidal ratio increased with an increase in the addition of the test agent, and this was particularly significant at concentrations greater than 1,000 μg/ml addition.

6. Antibacterial Activities for Other Bacteria

The antibacterial effects of the extract were tested using *S. aureus* and *B. subtilis* as gram-positive bacteria and *E. coli* and *P. aerusinosa* as gram-negative bacteria. *S. aureus* and *E. coli* were cultured on 10 mM Tris-HCl buffer at pH 7.0 and 37 °C. *B. subtilis* was cultured on the same Tris-HCl buffer at 30 °C. Fig. 8 shows the antibacterial effect of the SBS extract at 1,000 μg/ml. The bactericidal ratio at an SBS concentration of 1,000 μg/ml against *B. subtilis*, *S. aureus*, *P. aerusinosa* and *E. coli* was 96.4, 98.6, 91.5, and 52.2% after 2 h, and 98.2, 99.7, 99.3, and 82.6% after 4 h, respectively. The SBS extract produced a strong bactericidal ratio against *B. subtilis*, *S. aureus*, and *P. aerusinosa*, but weak bactericidal ratio against *E. coli.* These results indicate that the SBS extract inhibited the growth of both gram-positive and gram-negative bacteria without selectivity. In the case of gram-positive bacteria, which have a thicker cell wall structure than the gram-negative bacteria, the synthesis of bacterial cell wall and antibacterial molecule such as phenolics and flavonoids could show antibacterial activity, causing the destruction from asymmetric distribution to the charge on the cell wall and cell membrane. In the case of gram-negative bacteria, direct penetration of the antibacterial molecules into the cells could result in antibacterial activity, which would disrupt the normal physiological activities of cells from complexing with materials such as proteins, enzymes and other macromolecules in cells [29].

CONCLUSIONS

A simple separation process was developed to remove sediment,

such as low solubility materials and oil components, in sea buckthorn seed ethanol extract, and the antioxidant and antibacterial activity for the sediment removed ethanol extract (SBS extract) was investigated. The activity was also compared to vitamin C and BHA as typical antioxidant ingredients. Sediment material was simply removed by filtering after storing the crude extract for 16 h with celite in a refrigerator at 4 °C. The antioxidant activity of the SBS extract remained strong over long storage times and at high temperatures when compared to vitamin C and BHA. The SBS extract was also found to possess antibacterial activity. The bactericidal ratio of the SBS extract against *E. coli* increased with an increase in the concentration of the test agent, and this was particularly significant at concentrations greater than 1,000 μg/ml. The bactericidal ratio at 1,000 μg/ml of the SBS extract against *B. subtilis*, *S. aureus*, *P. aerusinosa* and *E. coli* was 96.4, 98.6, 91.5, and 52.2% after 2 h, and 98.2, 99.7, 99.3, and 82.6% after 4 h, respectively. These results demonstrate that the SBS extract can be used as a cost-effective functional food, pharmaceutical and cosmetic agent. However, further work is needed to identify the bioactive constituents in the extract of the sea buckthorn seed and to optimize the SBS extract separation process.

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