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Research Article

Effect of Light-Activated Hypocrellin B on the Growth and Membrane Permeability of Gram-Negative *Escherichia coli* Cells

Yuan Jiang,[1] Wingnang Leung,[2] Qingjuan Tang,[3] Hongwei Zhang,[2] and Chuanshan Xu[2,4]

[1] *Department of Rehabilitation Medicine, Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, China*
[2] *School of Chinese Medicine, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong*
[3] *College of Food Science and Engineering, Ocean University of China, Qingdao 266003, China*
[4] *Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen 518057, China*

Correspondence should be addressed to Wingnang Leung; awnleung@cuhk.edu.hk and Chuanshan Xu; xcshan@163.com

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Aim. To investigate the effect of light-activated hypocrellin B on the growth and membrane permeability of Gram-negative bacteria. *Methods*. *Escherichia coli* (*E. coli*) as a model bacterium of Gram-negative bacteria was incubated with various concentrations of hypocrellin B for 60 min and was subsequently irradiated by blue light with wavelength of 470 nm at the dose of 12 J/cm^2. Colony forming units were counted and the growth inhibition rate of *E. coli* cells was calculated after light-activated hypocrellin B. Membrane permeability was measured using flow cytometry and confocal laser scanning microscopy (CLSM) with propidium iodide (PI) staining. Bacterial morphology was observed using transmission electron microscopy (TEM). Reactive oxygen species in bacterial cells were measured using flow cytometry with DCFH-DA staining. *Results*. Significant growth inhibition rate of *E. coli* cells was observed after photodynamic action of hypocrellin B. Remarkable damage to the ultrastructure of *E. coli* was also observed by TEM. Flow cytometry and CLSM observation showed that light-activated hypocrellin B markedly increased membrane permeability of *E. coli*. Flow cytometry showed the intracellular ROS increase in *E. coli* treated by photodynamic action of hypocrellin B. *Conclusion*. Light-activated hypocrellin B caused intracellular ROS increase and structural damages and inhibited the growth of Gram-negative *E. coli* cells.

1. Introduction

Bacterial infection is a threat to human beings and the problem can be exacerbated by the emergence of resistant strains through the use of antibiotics. Thus, there is an urgent need to explore alternative strategies for combating pathogenic bacteria.

Photodynamic inactivation (PDI) is a promising method to eradicate pathogenic bacteria because PDI kills pathogenic bacteria via cytotoxic reactive oxygen species (ROS) produced by the photosensitive drug after light irradiation. The nonspecific damages of ROS on bacteria are unlikely to cause the resistance of bacteria to PDI [1–4]. Therefore, PDI draws our interest to explore its role in combating bacterial infections.

In our previous study we investigated photodynamic activity of some naturally occurring photosensitive compounds from traditional Chinese herbs [5–7]. Hypocrellin B is one of the most frequently explored photosensitive drugs from traditional Chinese herb *Hypocrella bambuase* [8, 9]. Our studies observed that light-activated hypocrellin B could produce intracellular ROS level, which can cause significant cell death and damage to Gram-positive bacteria *Staphylococcus aureus* [5, 6, 10]. However, growing evidence shows that Gram-negative bacteria are more resistant to PDI than Gram-positive bacteria [11]. To kill Gram-negative bacteria many photosensitizers were investigated, including methylene blue, toluidine blue, phthalocyanines, chlorins, porphyrins, chlorophyll, bacteriochlorophyll, fullerenes, and their nanoparticles [12]. In the present study, we chose

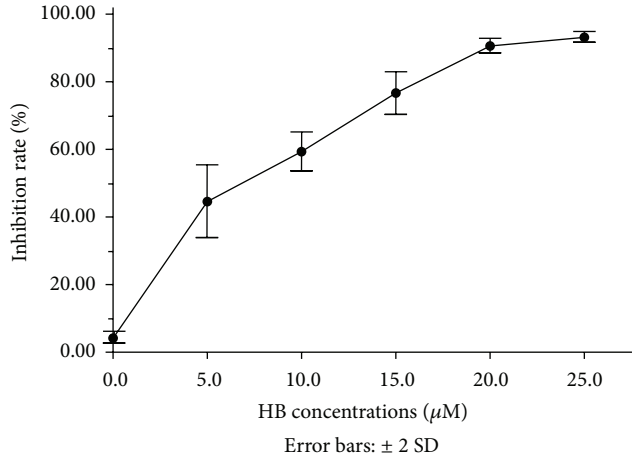

Figure 1: Inhibition of *E. coli* induced by photodynamic treatment of hypocrellin B. *E. coli* cells were incubated with different concentrations of hypocrellin B (0, 5, 10, 15, 20, and 25 μM) and irradiated by blue light with wavelength of 470 nm and energy density of 12 J/cm^2.

E. coli as a Gram-negative model bacterium and focused on observing the effect of light-activated hypocrellin B on the growth and membrane permeability of Gram-negative bacteria.

2. Materials and Methods

2.1. Bacterial Strain. *E. coli* strain DH5α was a generous gift from Dr. Wu Junfeng, P2 Lab of Children's Hospital of Chongqing Medical University, China. After *E. coli* cells were cultured overnight at 37°C in Luria-Bertani (LB) medium at 200 rpm, *E. coli* suspension (10^{-5} cfu/mL, 100 μL) was spread over LB-Agar plates and then incubated for 16 h at 37°C in a humidified atmosphere containing 5% CO_2.

2.2. Colony Forming Unit Assay. *E. coli* cells growing in exponential phase were harvested by centrifugation (at 4000 rpm, 5 min). Bacterial suspension (10^8 cfu/mL) was prepared and incubated with different concentrations of hypocrellin B (0, 5, 10, 15, 20, and 25 μM) in 6-well plate. After incubation for 60 min in the dark at room temperature, bacterial suspension was irradiated by blue light from a novel LED light source with the wavelength of 470 nm and the power density of 60 mW/cm^2. After light irradiation, bacteria cells were serially diluted 10-fold in PBS. Each sample (50 μL) was spread on LB-Agar plates and incubated for 16 h at 37°C in the dark. The colony forming units (CFU) were counted and the inhibition rate of bacterial growth was calculated by the following formula: the growth inhibition rate (%) = (CFU of the control group − CFU of the treatment group)/CFU of the control group × 100%.

All experiments were randomly divided into 4 groups.

Group A: Sham Control. The bacteria in the control were treated by neither hypocrellin B nor light irradiation.

Group B: Hypocrellin B Treatment Alone. The bacteria in the group were treated by hypocrellin B without light irradiation.

Group C: Light Irradiation Alone. The bacteria in the group were irradiated by LED light without hypocrellin B treatment.

Group D: Light-Activated Hypocrellin B. The bacterial cells in this group were incubated by various concentrations of hypocrellin B and irradiated by LED light with the energy density of 12 J/cm^2.

2.3. Membrane Permeability Measurement. Bacterial cells were incubated with hypocrellin B (25 μM) for 60 min in the dark at 37°C and were then exposed to blue light with light dose of 12 J/cm^2. After light irradiation, bacterial cells were immediately harvested and incubated with propidium iodide (PI, 10 μg/mL) for 20 min in the dark. Membrane permeability of the stained cells was observed immediately using a confocal laser scanning microscopy (CLSM) and the images were recorded using a colorful charge-coupled device camera. At the meantime, bacterial cells were also analyzed using flow cytometry (SE, Becton Dickinson) with the excitation setting at 488 nm.

2.4. Bacterial Morphology. After light activation of hypocrellin B, the bacterial cells were immediately fixed, post-fixed, dehydrated, and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA) and were cut into ultrathin sections (100 nm) to be stained in uranyl acetate and lead citrate. Ultrastructural changes were observed using an electron microscopy (H-600; Hitachi, Japan).

2.5. ROS Measurement. After light-activation of hypocrellin B, bacterial cells were immediately harvested and incubated with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, 10 μM, Beyotime, Jiangshu, China) for 20 min at 37°C in the dark and were then analyzed using a flow cytometry (SE, Becton Dickinson) with the excitation setting at 488 nm.

2.6. Statistical Analysis. All data expressed as mean ± SD were statistically analyzed using SPSS 18.0 for Windows.

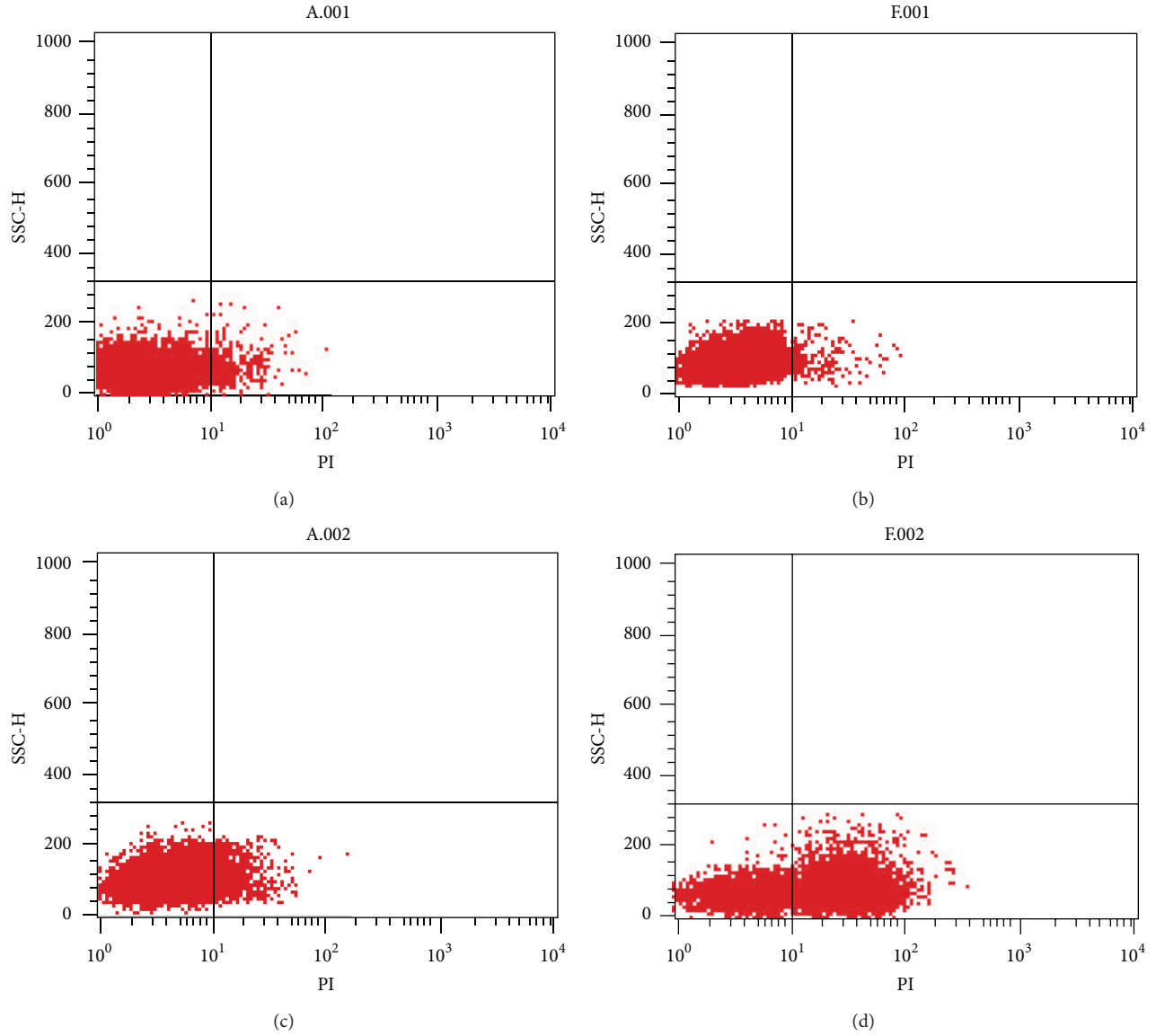

Figure 2: Membrane permeability of *E. coli* cells was measured using flow cytometry with PI staining after photodynamic treatment of hypocrellin B (25 μM), in which the energy density of blue light was 12 J/cm^2. (a) Sham control; (b) hypocrellin B treatment alone; (c) blue light irradiation alone; and (d) photodynamic treatment of hypocrellin B.

The differences between groups were analyzed using one-way ANOVA (analysis of variance). A P value < 0.05 was considered as significant difference.

3. Results

3.1. Colony Forming Unit Assay. Colony forming units of *E. coli* were investigated after treatment of light-activated hypocrellin B and the growth inhibition rate of *E. coli* cells was calculated. Figure 1 showed a significant growth inhibition rate of *E. coli* cells treated by light-activated hypocrellin B in hypocrellin concentration-dependent manner ($P < 0.05$). No significant difference was shown in the cells treated by hypocrellin alone or light irradiation alone ($P > 0.05$).

3.2. Membrane Permeability. The membrane permeability of *E. coli* cells was observed using a CLSM and flow cytometry with PI staining. Flow cytometry showed the positive rate of cells stained by PI was 3.71% in the sham group, 2.24% in the hypocrellin B treatment alone group, and 8.04% in the light irradiation group. The positive rate of cells remarkably increased up to 69.24% in the light-activated hypocrellin treatment group (Figure 2). CLSM also observed that more red fluorescence was found in *E. coli* cells treated by light-activated hypocrellin B than those of the control cells including sham control, hypocrellin treatment alone, and light irradiation alone. No marked difference was found between sham control, hypocrellin treatment alone, and light irradiation alone (Figure 3).

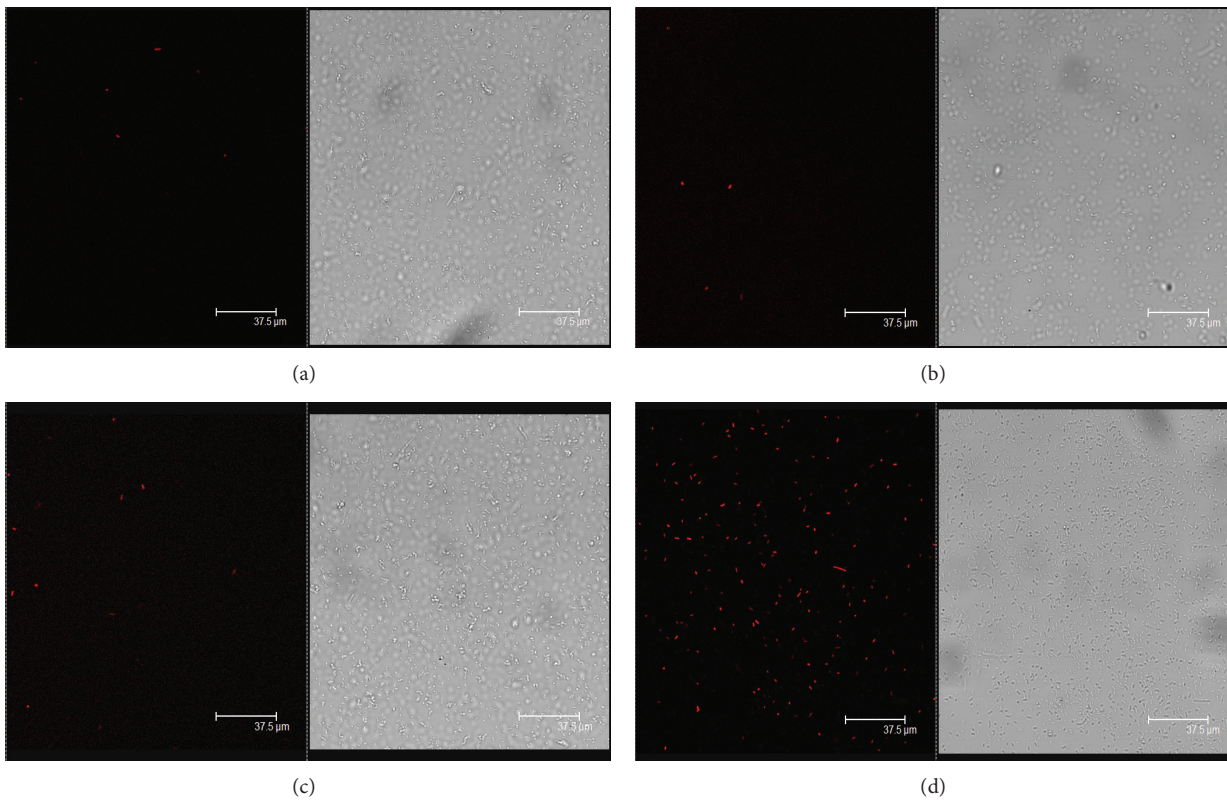

FIGURE 3: Membrane permeability of *E. coli* cells was measured using confocal laser scanning microscopy with PI staining after photodynamic treatment of hypocrellin B (25 μM), in which the energy density of blue light was 12 J/cm^2. (a) Sham control; (b) hypocrellin B treatment alone; (c) blue light irradiation alone; and (d) photodynamic treatment of hypocrellin B.

3.3. Bacterial Morphology. The ultrastructural changes of bacteria were observed using TEM. Intact and smooth cells were observed in sham control, hypocrellin B treatment alone, and light irradiation alone (Figures 4(a), 4(b), and 4(c)), whereas partial cytoplasm leakage was found in cells treated by light-activated hypocrellin B (Figure 4(d)).

3.4. Reactive Oxygen Species. The level of reactive oxygen species (ROS) in *E. coli* cells was analyzed using flow cytometry with DCFH-DA staining. Flow cytometry showed the spectral shift of the fluorescence curves to the right after light activation of hypocrellin B, indicating significant increase of the intracellular ROS level in *E. coli* (Figure 5).

4. Discussion

Traditional Chinese herbs have a long history as folk medicine for treating infectious diseases. Growing evidences have shown that many active compounds isolated from traditional Chinese herbs are capable of producing anti-infection and anti-inflammation effect [13, 14]. Hypocrellin B as an active component of a traditional Chinese herb *Hypocrella bambuase* has been confirmed to have significant activities against pathogenic microbes and malignant tumors [5, 6, 10, 11, 15–17]. Our recent study showed that hypocrellin B as a naturally occurring photosensitizer could cause significant damage to Gram-positive bacteria *S. aureus* cells as well as cancer cells while it was activated by blue light [5, 6, 10]. Our preliminary study also observed that *E. coli* cells were more resistant to photodynamic action of hypocrellin B than *S. aureus*. Thus, in the present study, we chose higher concentrations of hypocrellin B (0, 5, 10, 15, 20, and 25 μM) and higher energy density of blue light (12 J/cm^2) to investigate the effect of light-activated hypocrellin B on *E. coli* cells. Colony forming unit assay showed hypocrellin B had significantly photodynamic inhibition on *E. coli* cells.

Our previous studies reported that blue light could activate hypocrellin B to increase the intracellular ROS level in tumor cells and Gram-positive bacteria strain *S. aureus* [5, 6, 10]. In the present study flow cytometry with DCFH-DA staining also showed the ROS increase in Gram-negative bacteria strain *E. coli* cells. Our TEM observed notable cytoplasm leakage in *E. coli* cells after the treatment of light-activated hypocrellin B. Flow cytometry with PI staining showed that the positive rate of cells stained by PI increased remarkably up to 69.24% in the light-activated hypocrellin treatment group and confocal laser scanning microscopy also found more red fluorescence of PI in cells after light-activated hypocrellin B, demonstrating that light-activated hypocrellin B increased membrane permeability of the treated *E. coli* cells. The results were consistent with our past report on *S. aureus* treated by photodynamic action of hypocrellin B [10]. These findings demonstrated that nonspecific damage of ROS

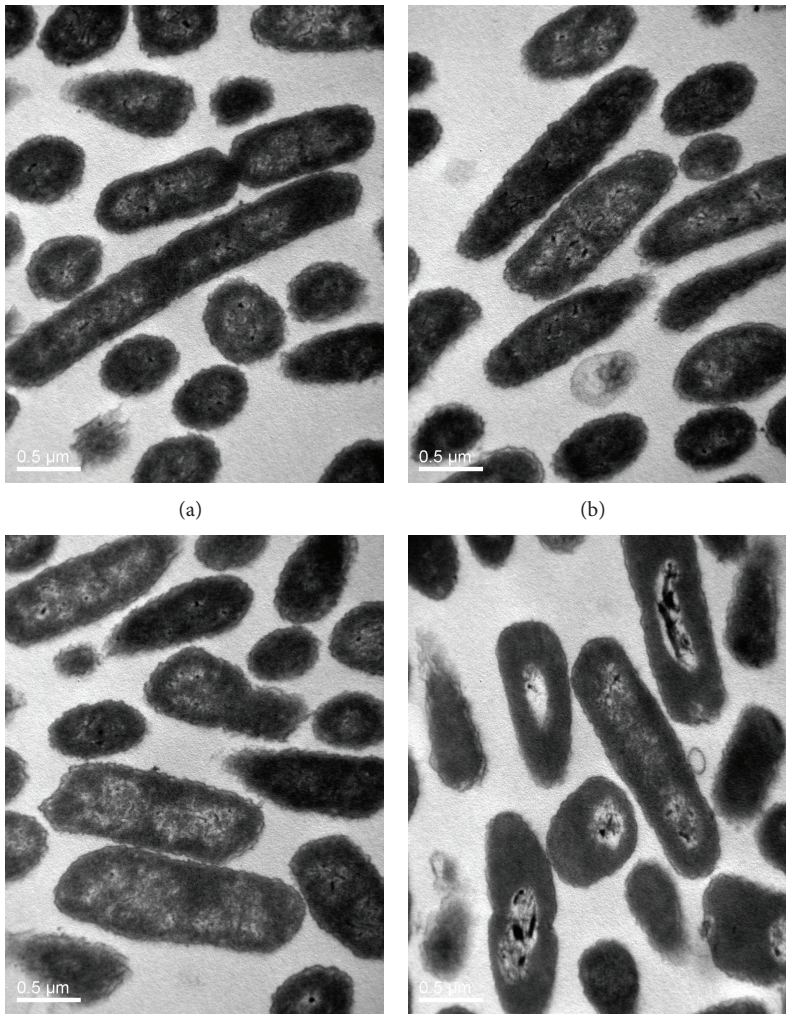

Figure 4: The ultrastructural morphology of *E. coli* cells was observed under a transmission electron microscopy (TEM) after photodynamic action of hypocrellin B (25 μM), in which the energy density of blue light was 12 J/cm^2. (a) Sham control; (b) hypocrellin B treatment alone; (c) blue light irradiation alone; and (d) photodynamic treatment of hypocrellin B (×30000).

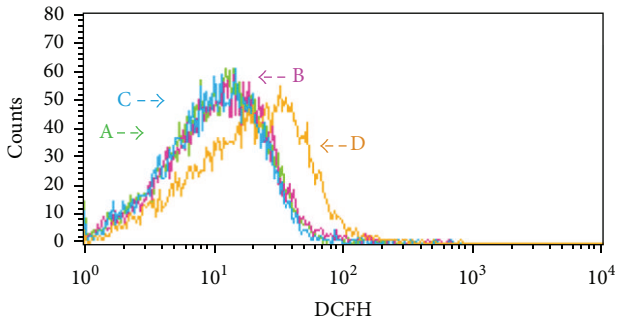

Figure 5: Reactive oxygen species (ROS) in *E. coli* cells were analyzed using flow cytometry with DCFH-DA staining after treatment of hypocrellin B (25 μM) and blue light irradiation (12 J/cm^2). A: sham control; B: hypocrellin B treatment alone; C: blue light irradiation alone; and D: photodynamic treatment of hypocrellin B.

generated by photosensitization of hypocrellin B to bacterial cells was an important cause of damage in the morphological structure of bacteria and in their general inhibition.

In summary, our study found that Gram-negative bacterial strain *E. coli* cells were more resistant to photodynamic treatment of hypocrellin B than Gram-positive bacteria, but light-activated hypocrellin B could also increase the ROS level in *E. coli* cells to cause damage of bacterial structure and inhibit growth of Gram-negative bacteria while higher concentrations of hypocrellin B and higher energy density of light irradiation were used in photodynamic treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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