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Enhanced Antimicrobial Activities and Physicochemical Characteristics of Isoliquiritigenin Encapsulated in Hydroxypropyl- β -Cyclodextrin

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초 록

아이소리퀴리티게닌은 감초의 구성 성분으로서 항산화, 항균 활성과 같은 다양한 약리학적 활성을 가지고 있는 소수성 물질이다. 본 연구에서는 소수성 약물의 용해도를 증진시키기 위해서 아이소리퀴리티게닌/2-하이드록시프로필-베타사이클로덱스트린 복합체를 freeze-drying 방식으로 제조하였고, 상평형 연구, DSC, SEM, 1H NMR을 통하여 그 특성을 확인하였다. 항균 활성은 *Staphylococcus aureus* (*S. aureus*)와 *Escherichia coli* (*E. coli*)에 대해 broth dilution assay 측정법으로 최소성장억제농도(minimum inhibitory concentration, MIC)를 확인하였다. 상평형 연구를 통해 아이소리퀴리티게닌/2-하이드록시프로필-베타사이클로덱스트린 복합체는 1 : 1 몰비율로 형성되는 것을 확인하였다. *S. aureus*와 *E. coli*에 대한 MIC는 아이소리퀴리티게닌/2-하이드록시프로필-베타사이클로덱스트린 복합체의 경우 각각 1.25 및 2.5 mg/mL이었으며, free한 아이소리퀴리티게닌은 각각 5, 5 mg/mL로 아이소리퀴리티게닌/2-하이드록시프로필-베타사이클로덱스트린 복합체가 더 높은 항균 활성을 나타내었다. 이는 아이소리퀴리티게닌/사이클로덱스트린 복합체가 식품 및 화장품 산업에서 합성 방부제를 대체하고 천연 방부제로서 응용 가능성이 있음을 시사한다.

Abstract

Isoliquiritigenin (ILG) is a hydrophobic component in licorice and has a variety of pharmaceutical and biological activities. In this study, we prepared an isoliquiritigenin-hydroxypropyl- β -cyclodextrin (ILG/HP- β -CD) complex by freeze-drying method to enhance its water solubility. The complex was characterized by phase solubility studies, DSC, SEM, and 1H NMR. Antimicrobial activities against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) were evaluated by broth dilution method. The results showed that the stoichiometry of ILG/HP- β -CD complex was 1 : 1. The antimicrobial activity of ILG/HP- β -CD complex was higher than that of using free ILG against *S. aureus* and *E. coli*. Therefore, we suggest that ILG/HP- β -CD complex may be used as a natural antiseptic and could potentially replace synthetic preservatives in cosmetic and food industries.

Keywords: isoliquiritigenin, hydroxypropyl- β -cyclodextrin, complexation, characterization, antimicrobial activity

1. Introduction

Isoliquiritigenin (2',4',4-trihydroxychalcone, ILG) is a chalcone-type flavonoid (Figure 1) and one of the bioactive molecules in licorice[1]. It has a variety of pharmaceutical and biological activities such as anti-

oxidative[2], anti-inflammatory[3], anticancer[1], and whitening effects[4]. ILG also exhibits antimicrobial activities, which are superior to those of other licorice ingredients (glycyrrhizin, liquiritin, liquiritigenin), against *Bacillus subtilis*, *Propionibacterium acnes*, *Escherichia coli*, and *Pseudomonas aeruginosa*[5]. However, because ILG is a hydrophobic molecule with a water solubility of only 6.66 μ g/mL, the pharmaceutical application of this substance as a drug has been hindered.

Cyclodextrins (CDs) are cyclic oligosaccharides composed of D-glucopyranose with a hydrophobic cavity and a hydrophilic surface. Depending on the number of glucopyranose units, CDs are classified as α -CD (6 units), β -CD (7 units), and γ -CD (8 units). CDs can

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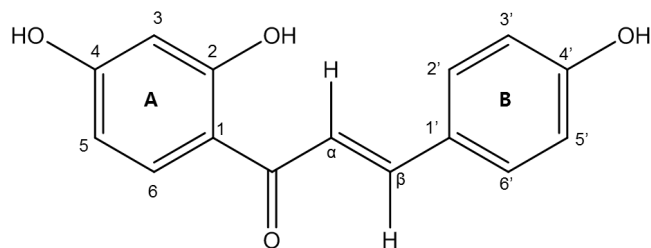


Figure 1. The structure of ILG.

be used to encapsulate hydrophobic molecules in their cavity, thus enhancing the water solubility, and with it biological activity, and stability, of drugs[6]. In addition, complexation with cyclodextrins is used to improve drug stability against UV radiation, light, and heat[7]. Hydroxypropyl- β -cyclodextrin (HP- β -CD) is a hydroxylalkylated form of cyclodextrin. It is characterized by high water solubility, low toxicity, and great stability[8].

Encapsulation of hydrophobic molecules in cyclodextrins has been reported in many articles. When complexed with cyclodextrins, a variety of activities of hydrophobic drugs were increased as the water solubility is enhanced[6,7]. Notably, when essential oils, which are poorly water soluble, were encapsulated in cyclodextrin, their antimicrobial activities were enhanced compared to free essential oils. In addition, genistein, one of isoflavones, cyclodextrins complexes showed an improvement of the antimicrobial activity on *Bacillus subtilis* in previous study[9]. These results suggested that enhancement of water solubility could improve their antimicrobial activity in aqueous solutions[10,11].

Therefore, in this study the ILG/HP- β -CD complex was prepared by freeze-drying method for improving pharmaceutical activity. This complex was characterized by differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and nuclear magnetic resonance (^1H NMR). Water solubility of ILG in the presence of various concentrations of HP- β -CD was evaluated by phase solubility studies and antimicrobial activities against *S. aureus* and *E. coli* were evaluated by broth dilution method.

2. Materials and Methods

2.1. Materials

Isoiquiritigenin, hydroxypropyl- β -cyclodextrin, methyl paraben were purchased from Sigma (St. Louis, MO, U.S.A.). Tween 80® (polysorbate 80) surfactant was kindly provided by Saimdang, Co. Ltd. (Cheongju, Korea). *S. aureus* (ATCC 6538) and *E. coli* (ATCC 23726) was provided by the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea). Mueller-Hinton broth and agar were purchased from Becton, Dickinson and Company (BD, Franklin Lakes, NJ, USA). Water was deionized and Milli-Q filtered. Other solvents used such as ethanol, DMSO- d_6 were analytical grade.

2.2. Preparation of ILG/HP- β -CD complex

ILG/HP- β -CD complex was prepared by freeze-drying method. 5 mM ILG was added to a 5 mM HP- β -CD solution (100 mL) in a 1

: 1 molar ratio. The resulting mixture was stirred for 48 h at 25 °C to reach equilibrium. The mixture was filtered through a 0.45 μm filter (25 mm, Minisart, Sartorius) to remove free ILG. After filtration, the solution was frozen at -60 °C for 24 h and lyophilized at -60 °C for 48 h.

2.3. Differential scanning calorimetry (DSC) analysis

The crystallinity of ILG and ILG/HP- β -CD complex was analyzed by differential scanning calorimetry (DSC) analysis using a Shimadzu DSC-60 series instrument (Dong-il Shimadzu Corp., Seoul, Korea). The analysis was performed from 25 to 250 °C at a heating rate of 10 °C/min.

2.4. Scanning electron microscopy (SEM) analysis

The surface morphology of ILG, HP- β -CD, physical mixture and inclusion complex was examined using SEM (TESCAN VEGA3, Cranberry TWP, PA, USA) with 20 Kv accelerating voltage. Dried samples were attached to carbon tapes. The tapes were then attached to a metal stub and coated with gold by using a Cressington 208HR sputter coater (Cressington Scientific Instruments Ltd., UK) at 20 mA for 300 s.

2.5. Nuclear magnetic resonance (^1H NMR) analysis

^1H NMR experiments were performed at 298 K using Broadband MERCURY, 400 MHz NMR system (Varian, USA). ^1H NMR spectra of ILG, HP- β -CD, and inclusion complex were obtained by dissolving them in 0.65 mL of DMSO- d_6 .

2.6. Phase solubility study of ILG/HP- β -CD complex

Phase solubility studies were carried out according to the previously reported method by Higuchi and Connors[12]. First, excess ILG was added to various concentrations of HP- β -CD solution (0-10 mM). Then, the mixtures were stirred for 48 h at 25 °C and 37 °C, respectively. After equilibrium was reached, the solutions were centrifuged (13,000 rpm, 10 min), and supernatants were analyzed by UV-Vis spectrometry (Cary 50, Australia) at ILG's maximum absorbance wavelength of 371 nm.

The stability constants (K_s) were calculated from phase solubility diagrams, following Eq. (1), where S_0 is the concentration of ILG in the absence of HP- β -CD. The slope in the equation is the slope of the phase solubility diagram.

$$K_s = \frac{\text{Slope}}{S_0(1 - \text{Slope})} \quad \text{Eq. (1)}$$

Thermodynamic factors were obtained using temperature and stability constants. When ILG was encapsulated in HP- β -CD, Gibb's free energy (ΔG_i) was calculated by Eq. (2), where R is the universal gas constant (8.314 Jmol $^{-1}$ K $^{-1}$) and T is temperature in Kelvin.

$$\Delta G_i = -RT_i \ln K_i \quad \text{Eq. (2)}$$

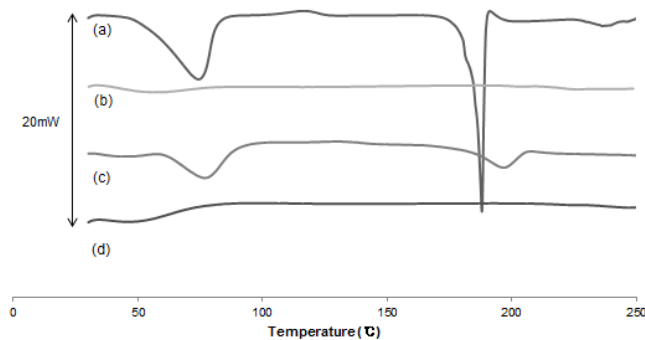


Figure 2. DSC thermograms (a) ILG, (b) HP- β -CD, (c) ILG/HP- β -CD physical mixture, and (d) ILG/HP- β -CD complex.

The enthalpy change (ΔH) was determined using the Van't Hoff equation (Eq. (3)), and the entropy change (ΔS) was calculated by Eq. (4).

$$\ln \frac{K_2}{K_1} = \frac{\Delta H}{R} \times \frac{T_2 - T_1}{T_2 T_1} \quad \text{Ep. (3)}$$

$$\Delta S_i = \frac{\Delta H_i - \Delta G_i}{T_i} \quad \text{Ep. (4)}$$

2.7. Evaluation of antimicrobial activities

The antimicrobial activities of ILG/HP- β -CD complex were studied against *S. aureus* (ATCC6538) and *E. coli* (ATCC23726), two strains that are parts of the skin flora. *S. aureus* and *E. coli* were incubated aerobically at 37 °C for 24 h in Mueller-Hinton-broth (MH-broth). Minimum inhibitory concentrations (MICs) for free ILG and ILG/HP- β -CD complex were determined via broth dilution inhibition assay with some modifications as described by [10,13]. *S. aureus* and *E. coli* were diluted in double-strength MH-broth to obtain an initial inoculum of 5.0 log₁₀ CFU/mL. MIC analysis was conducted in 96-well microtiter plates (300 μ L capacity/well). Methyl paraben as a positive control, ILG and ILG/HP- β -CD complex were emulsified in water using 1 % Tween 80 and 20 % EtOH solution. The samples added to the test wells ranged from 0.125-10 mg/mL. Bacterial suspensions were added to each well and then they were incubated at 37 °C for 18 h. The suspensions were spread onto Mueller-Hinton-agar (MH-agar) and grown in agar plates, which were incubated at 37 °C for 18 h. Lastly, the number of bacterial colonies formed on each plate was determined. The lowest concentration that completely inhibited the microorganism's growth was considered as the MIC values.

2.8. Statistical analysis

All the experiments were performed in triplicate, and statistical data were analyzed by the Student's t-test at the level of $P = 0.05$.

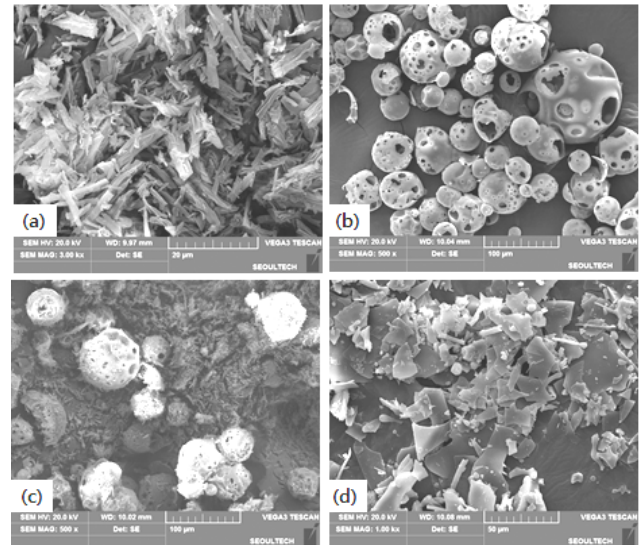


Figure 3. Scanning electron microphotographs (a) ILG, (b) HP- β -CD, (c) ILG/HP- β -CD physical mixture, and (d) ILG/HP- β -CD complex.

3. Results and Discussion

3.1. Physicochemical Characteristics of ILG/HP- β -CD Complex

3.1.1. DSC analysis

DSC analysis was used as a first line of evidence to test whether the drug was encapsulated in cyclodextrin. If a guest molecule is encapsulated in cyclodextrin, its characteristics (e.g., boiling point, melting point, crystallinity, etc.) are changed. The DSC thermograms of ILG, HP- β -CD, ILG/HP- β -CD physical mixture, and ILG/HP- β -CD complex are shown (Figure 2). Figure 2 (a) shows the DSC thermogram of ILG alone. ILG displayed a sharp endothermic peak at 188.14 °C, which is characteristic for crystalline sample. The DSC thermogram of HP- β -CD did not show any peaks which indicates that HP- β -CD is an amorphous substance (Figure 2 (b)). The DSC thermogram of the ILG/HP- β -CD physical mixture displayed an endothermic peak around 188 °C, which is likely caused by the crystalline transition of ILG (Figure 2 (c)). In contrast, ILG/HP- β -CD complex did not show any endothermic peaks suggesting that it is an amorphous substance like HP- β -CD (Figure 2 (d)). In the case of the physical mixture, both ILG and HP- β -CD's characteristics appeared, which suggests that there is no interaction between ILG and HP- β -CD. The endothermic peak of the complex disappeared in the thermogram suggesting that ILG was encapsulated into the cavity of HP- β -CD and formed an amorphous or disordered structure [14].

3.1.2. SEM analysis

SEM images of ILG, HP- β -CD, ILG/HP- β -CD physical mixture and ILG/HP- β -CD complex are shown (Figure 3). ILG appeared as narrow needle-like crystals, whereas HP- β -CD appeared as irregular spherical particles with multiple cavities. ILG/HP- β -CD physical mixture displayed characteristic of both ILG and HP- β -CD. ILG crystals and irregular spheres of HP- β -CD were founded in physical mixture.

Table 1. The Stability Constant (K_s), and the Thermodynamic Parameters of ILG/HP- β -CD Inclusion Complexes Obtained from the Phase Solubility Diagram

T (°C)	S_0 (mM)	Slope	K_s (M^{-1})	ΔG ($kJ\ mol^{-1}$)	ΔH ($kJ\ mol^{-1}$)	ΔS ($J\ mol^{-1}K^{-1}$)
25	0.029	0.1051	4058	-20.60	23.32	147.31
37	0.026	0.1318	5840	-22.36	23.32	147.28

Table 2. The Chemical Shift Values of Free ILG and Inclusion Complex in HP- β -CD ($\Delta\delta^*$)

H assignment	δ ILG free (ppm)	δ ILG complexed (ppm)	$\Delta\delta^*$
H-3	6.283 (1 H, s)	6.271 (1 H, brs)	-0.012
H-5	6.398 (1 H, d)	6.383 (1 H, brd)	-0.015
H-6	7.774 (1 H, d)	7.760 (1 H, brd)	-0.014
H- α	7.748 (1 H, d)	7.744 (1 H, brd)	-0.004
H- β	8.162 (1 H, d)	8.158 (1 H, brd)	-0.004
H-2',6'	6.857 (2 H, d)	6.848 (2 H, brd)	-0.009
H-3',5'	6.420 (2 H, d)	6.415 (2 H, brd)	-0.005

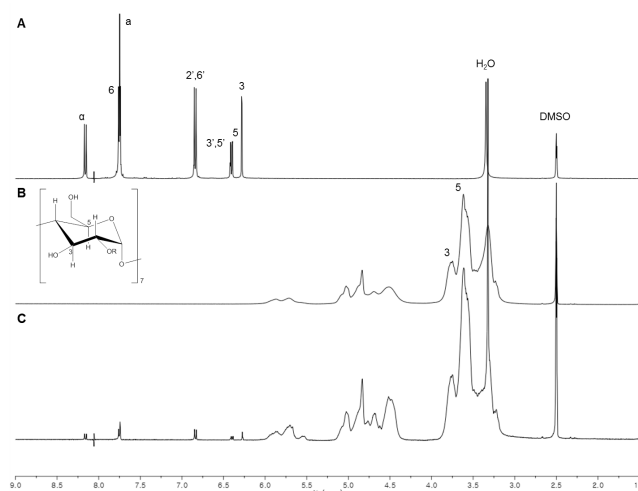
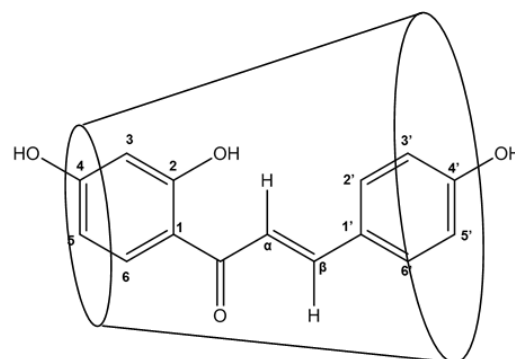
H assignment	δ HP- β -CD free (ppm)	δ HP- β -CD complexed (ppm)	$\Delta\delta^*$
H-3	3.757 (dd)	3.759 (brdd)	0.002
H-5	3.614 (m)	3.620 (brm)	0.006

$$\Delta\delta^* = \Delta\delta(\text{complexed}) - \Delta\delta(\text{free})$$

However, the ILG/HP- β -CD inclusion complex appeared as irregular plate-like structure in which the original morphology of two components disappeared. This change in size and morphology of ILG and HP- β -CD suggests that the formation of the inclusion complex between ILG and HP- β -CD[14,15].

3.1.3. 1H NMR analysis

1H NMR spectra can provide direct evidence that inclusion complex is formed. We analyzed the 1H NMR spectra of ILG, HP- β -CD, and ILG/HP- β -CD (Figure 4). All samples were dissolved in DMSO- d_6 . The major hydrogen protons of ILG and HP- β -CD were assigned and their protons displayed signals at 6.0-8.2 ppm and 1.0-6.0 ppm, respectively. If ILG had been encapsulated into the HP- β -CD cavity, their chemical shifts will be changed. Especially, HP- β -CD protons inside the cavity (H-3 and H-5) will be more sensitive to the changed environment[16]. Table 2 illustrate the hydrogen chemical shift values of ILG and HP- β -CD in the free and in the complexed form. $\Delta\delta^*$ is the variation of the chemical shifts of ILG and HP- β -CD before and after forming inclusion complexes. When ILG complexed with HP- β -CD, we observed changed $\Delta\delta^*$ for aromatic proton of ILG H-3 (-0.012), H-5 (-0.015), H-6 (-0.014), H-2',6' (-0.009), H-3',5' (-0.005), and H- α (-0.004), H- β (-0.004) of ethylene groups. These results indicated that all portions of ILG entered into the cavity of HP- β -CD. The H-5 (0.006) of HP- β -CD had a little larger $\Delta\delta^*$ than H-3 (0.002), which illustrated that the ILG entered into the cavity of HP- β -CD from the small port[17]. Therefore, we can predict the structure of ILG/HP- β -CD complex (Figure 5).

**Figure 4. 1H NMR spectrum (a) ILG, (b) HP- β -CD, and (c) ILG/HP- β -CD complex in DMSO- d_6 .****Figure 5. The structure of ILG/HP- β -CD complex.**

3.1.4. Phase solubility study

The phase solubility diagram of ILG at various concentrations of HP- β -CD (0-10 mM) at 25 °C and 37 °C is shown (Figure 6). With increasing HP- β -CD concentration (0-10 mM), the solubility of ILG was increased linearly within the studied concentration range. Following Higuchi and Connor's theory, it can be classified as an A_L type phase diagram. The stoichiometry of the ILG and HP- β -CD complex is 1 : 1 within the studied concentration range (0-10 mM). The stability constants (K_s) and thermodynamic parameters ΔG , ΔH , ΔS were calculated and are shown (Table 1). Commonly, K_s value means the strength of complexation of drug within the CD cavity[14]. The stability constants (K_s) of ILG/HP- β -CD were 4058 M^{-1} and 5840 M^{-1} at 25 °C and 37 °C, respectively. This indicates that there were more interactions between ILG and HP- β -CD at 37 °C than at 25 °C, representing the complexation was enhanced at relatively high

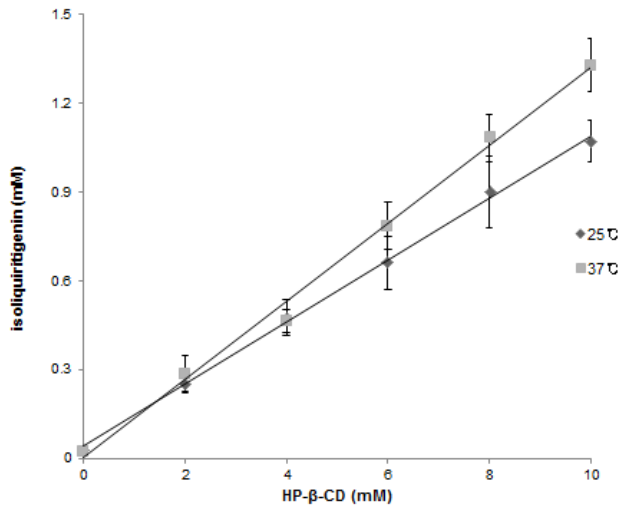


Figure 6. Phase solubility diagrams of ILG with HP- β -CD, in aqueous solution at 25 °C and 37 °C.

temperature. The thermodynamic properties were calculated and are shown (Table 1). The Gibb's free energy (ΔG) was used to determine if the reaction was spontaneous. ΔG values of ILG/HP- β -CD were $-20.60 \text{ kJmol}^{-1}$ and $-22.36 \text{ kJmol}^{-1}$ at 25 °C and 37 °C, respectively. The negative ΔG values indicate that complexation of ILG/HP- β -CD is a spontaneous reaction at both temperatures. The enthalpy (ΔH) is a parameter between endothermic and exothermic reactions. ΔH values at both temperatures were positive ($+23.32 \text{ kJmol}^{-1}$) which means that the reaction is endothermic reaction. Thus, the system was received energy upon complexation forming non-covalent bonds. The change in entropy (ΔS) is a measure of disorder in the system. We determined positive ΔS , which means that disorder of the system increased upon complexation[18]. In summary, inclusion complexation of ILG and HP- β -CD occurred spontaneously at relatively high temperature.

3.2. Evaluation of antimicrobial activities

The Minimum Inhibitory Concentration (MIC) assay is a well-known for evaluating antimicrobial activity. The MIC is determined by identifying the lowest concentration of a particular drug needed to kill bacteria. The MICs of ILG and ILG/HP- β -CD inclusion complex against *S. aureus* and *E. coli* are shown (Table 3). The MICs of free ILG against *S. aureus* and *E. coli* were both 5 mg/mL in both cases. The MICs of HP- β -CD inclusion complex against *S. aureus* and *E. coli* were 1.25 mg/mL and 2.5 mg/mL, respectively. That is, the MICs of inclusion complex were lower than those of free ILG. For methyl paraben, MICs showed the same values with inclusion complex in both bacterias. This suggests that HP- β -CD enhanced ILG access to membrane and inside of the cytoplasm of bacteria by increasing ILG aqueous solubility[10,11]. Therefore, the superior solubilizing effect of HP- β -CD can be a major influence on enhanced antimicrobial activities.

Table 3. The Minimum Inhibitory Concentration (MIC) of Methyl Paraben, ILG and ILG/HP- β -CD Complex against *S. aureus* and *E. coli*

Minimum inhibitory concentration (MIC, mg/mL)		
	<i>S. aureus</i>	<i>E. coli</i>
Methyl paraben	5	5
ILG	5	5
ILG-HP- β -CD complex	1.25	2.5

4. Conclusion

In this study, inclusion complex of ILG and HP- β -CD was prepared by freeze drying method with a 1 : 1 molar ratio. The physicochemical characteristics of this complex were evaluated and its antimicrobial activities against *S. aureus* and *E. coli* were determined. The formation of inclusion complex was confirmed by phase solubility studies, DSC, SEM, and ^1H NMR. The water solubility of hydrophobic ILG was enhanced by complexation with HP- β -CD. The antimicrobial activities of ILG/HP- β -CD inclusion complex were improved against *S. aureus* and *E. coli*. Therefore, ILG/HP- β -CD complex could be used as a natural antiseptic and potentially replace synthetic preservatives in cosmetic and food industry.

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