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# Coicis Semen Reduces *Staphylococcus aureus* Persister Cell Formation by Increasing Membrane Permeability

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**ABSTRACT**

Unlike resistant cells, persister cells resist antibiotics due to a decreased cellular metabolic rate and can transition back to normal susceptible cells when the antibiotic is removed. These persister cells contribute to the chronic symptoms of infectious diseases and promote the emergence of resistant strains with continuous antibiotic exposure. Therefore, eliminating persister cells represents a promising approach to significantly enhance antibiotic efficacy. Here, we found that Coicis Semen extract reduced *Staphylococcus aureus* persister cells at a concentration of 0.5 g/L. Linoleic acid and oleic acid, the major components of Coicis Semen extract, exhibited a comparable reduction in persister cells when combined with three antibiotics: ciprofloxacin, oxacillin, and tobramycin. Conversely, these effects were nullified in the presence of the surfactant Tween 80 (1%), suggesting that the hydrophobic characteristics of linoleic acid and oleic acids play a pivotal role in reducing the number of *S. aureus* persister cells. Considering the concentration-dependent effects of linoleic acid and oleic acid, the persister-reducing activity of Coicis Semen extract was primarily attributed to these fatty acids. Moreover, Coicis Semen extract, linoleic acid, and oleic acid increased the cell membrane permeability of *S. aureus*. Interestingly, this effect was counteracted by 1% Tween 80, indicating a close association between the reduction of persister cells and the increase in cell membrane permeability. The identified compounds could thus be used to eliminate persister cells, thereby enhancing therapeutic efficacy and shortening treatment duration. When used in conjunction with antibiotics, they may also mitigate chronic symptoms and significantly reduce the emergence of antibiotic-resistant bacteria.

***Keywords:*** Coicis Semen, persister cell, *Staphylococcus aureus*, membrane permeability, linoleic acid, oleic acid

## 1. INTRODUCTION

Bacterial infections pose a significant threat to human health, and the rise of drug-resistant bacteria has made the treatment of these infections increasingly difficult (Doron and Gorbach, 2008; Martínez and Baquero, 2002). One of the major challenges in the treatment of bacterial infections is the occurrence of persister cells, a small subpopulation of bacteria highly tolerant to antibiotics and other antimicrobial agents (Balaban *et al.*, 2019). Similar to dormant cells, persister cells exhibit reduced metabolism, rendering antibiotics that target their metabolism ineffective (Lewis, 2007). Upon completion of antibiotic treatment, these cells can revert back to their normal antibiotic-sensitive states (Carvalho *et al.*, 2017). The persister cell state enables bacteria to

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withstand antibiotic treatment without the emergence of resistant strains, making it a major contributor to chronic and recurrent infections and creating opportunities for resistant strains to emerge (Hansen *et al.*, 2008; Wu *et al.*, 2017). Persister cells can lead to prolonged hospital stays, increased healthcare costs, and heightened morbidity and mortality (LaFleur *et al.*, 2010; Mulcahy *et al.*, 2010). Therefore, the development of novel strategies targeting persister cells is crucial for the treatment of infectious diseases (Conlon, 2014). Several methods to reduce the number of persister cells have been reported, such as activating the ClpP protease with acyldepsipeptides (Lee *et al.*, 2010; Li *et al.*, 2010), boosting the proton-motive force with metabolites to enhance the uptake of aminoglycoside antibiotics (Allison *et al.*, 2011), conjugating antibiotics with antimicrobial peptides (Brezden *et al.*, 2016), and discovering new chemicals targeting cell membrane (Ooi *et al.*, 2009).

*Staphylococcus aureus*, a pathogenic Gram-positive bacterium, is a significant cause of infectious diseases, including skin and soft tissue infections, as well as more serious systemic infections such as pneumonia, sepsis, and endocarditis (Oliveira *et al.*, 2018; Otto, 2014). In a survey of bloodstream infections conducted in 45 countries from 1997 to 2016, *S. aureus* was the most frequently encountered strain, accounting for 20.7% of all cases (Diekema *et al.*, 2019). *S. aureus* is also a common cause of persistent infections and poses a particular challenge, with numerous reports of antibiotic-resistant strains (Chang *et al.*, 2015). Analysis of chronic *S. aureus* infection revealed that many cells were persister cells (Mulcahy *et al.*, 2010).

Plants contain a wide variety of constituent compounds, many of which exhibit antibacterial (Ham *et al.*, 2020), antifungal (Adfa *et al.*, 2020; Jang *et al.*, 2012; Lee *et al.*, 2021; Yoon and Kim, 2023), anti-inflammatory (Myeong *et al.*, 2023; Yang *et al.*, 2019), and antioxidant effects (Lee *et al.*, 2021; Nam *et al.*, 2018; Yang *et al.*, 2022). Coicis Semen, the seeds of the *Coix lacryma-jobi* plant, is a traditional medicinal herb commonly used in East Asian countries such as China, Japan, and Korea. Coicis Semen has been reported to possess anti-inflammatory cytokines (Yun *et al.*, 2009), as well as antioxidant (Park and Kang, 2000) and anticancer (Das *et al.*, 2017) properties. Additionally, several studies have suggested that Coicis Semen possesses bactericidal activity against a wide range of bacterial species, including *S. aureus* (Das *et al.*, 2017). The main components of Coicis Semen oil are linoleic acid, oleic acid, and palmitic acid, constituting 38%–51%, 30%–38%, and 14%–18% of its total composition, respectively (Xi *et al.*, 2016).

In this study, we evaluated the change in the number of persister *S. aureus* when an ethanol extract of Coicis Semen was co-administered with three antibiotics: oxacillin, ciprofloxacin, and tobramycin. Additionally, we explored the inhibitory effect of Coicis Semen extract components on persister cells and elucidated the biological mechanism of persister cell reduction.

## 2. MATERIALS and METHODS

### 2.1. Bacterial strains and culture conditions

*S. aureus* ATCC 6538 was acquired from the Korean Collection for Type Cultures (Jeongeup, Korea) and stored at −80℃ with 25% glycerol (catalog number: 4066-4400, Daejung Chemical & Metals, Siheung, Korea). The cells were cultured using Tryptic Soy Broth (TSB, catalog number: 211825, Becton Dickinson Korea, Seoul, Korea), and Tryptic Soy Agar (TSA) was prepared by mixing TSB with 1.5% Bacto Agar (catalog number: 214010, Becton Dickinson Korea). For the cell cultures, the cells were cultured at 37℃ with continuous shaking at 250 rpm. A saline solution was prepared by dissolving sodium chloride (catalog number: S0476, Samchun Chemicals, Seoul, Korea) at a concen-

tration of 0.85% (w/v). All solutions were autoclaved at 121℃ for 20 minutes.

## 2.2. Chemicals

Ciprofloxacin hydrochloride monohydrate (catalog number: C2227, Tokyo Chemical Industry, Tokyo, Japan), oxacillin sodium salt (catalog number: sc-224180B, Santa Cruz Biotechnology, Dallas, TX, USA), and tobramycin (catalog number: T2503, Tokyo Chemical Industry) were dissolved in distilled water prior to use. Linoleic acid (catalog number: L07949, Alfa Aesar, Thermo Fisher Scientific Korea, Seoul, Korea), oleic acid (catalog number: O0180, Tokyo Chemical Industry), and palmitic acid (catalog number: 129702500, Acros Organics, Thermo Fisher Scientific Korea) were dissolved in ethyl alcohol (catalog number: 000E1367, Samchun Chemicals) before use. Tween 80 (catalog number: 000T09) was obtained from Samchun Chemicals. SYTOX™ Green Nucleic Acid Stain (catalog number: S7020) was purchased from Invitrogen (Thermo Fisher Scientific Korea).

## 2.3. Preparation of Coicis Semen ethanol extract

Coicis Semen was acquired from the Jiundang Oriental Pharmacy (Seoul, Korea), and its ethanol extract was prepared as described in a previous study (Ham and Kim, 2018) with slight modifications. Thirty grams of Coicis Semen were crushed to a diameter of ≤ 3 mm and soaked in 300 mL of 95% ethyl alcohol (catalog number: E0219, Samchun Chemicals) at 50℃ for 3 hours, with periodic shaking every 30 minutes. The resulting extract solution was filtered using Whatman™ qualitative filter paper Grade 1 (catalog number: 1002-110, Cytiva™, Sigma-Aldrich, St. Louis, MO, USA). The filtrate was then concentrated using a rotary concentrator (RV-10, IKA Korea, Seoul, Korea) and freeze-dried with a lyophilizer (FDU-2110, EYELA, SunilEyela, Seongnam, Korea) over the course of 3 days. The extract was stored at −80℃ and reconstituted in ethanol before use.

## 2.4. Effect of Coicis Semen ethanol extract and its chemical compounds on *Staphylococcus aureus* persister cell population

*S. aureus* stored at −80℃ was streaked on TSA plates and incubated at 37℃ for 24 hours. A single colony was precultured in 5 mL TSB and incubated at 37℃ with continuous shaking at 250 rpm for 24 hours. The precultured cells, with an $Abs_{600}$ of 0.05, were inoculated in 5 mL of culture medium and incubated at 37℃ with shaking at 250 rpm for 3 hours. Afterward, the cells were diluted in saline and spread on TSA plates. The plates were then incubated at 37℃ for 24 hours to determine the initial colony count. Each antibiotic was added to the main culture medium at 10 times the minimal inhibition concentration (MIC): 2.5 mg/L for oxacillin, 20 mg/L for ciprofloxacin, and 80 mg/L for tobramycin. Ethanol was added as a control, and the extract or its chemical components were added to assess their impact on the persister cell population. After incubating at 37℃ for 24 hours, the number of colony-forming units (CFUs) was examined on TSA plates as described in a previous study (Wang *et al.*, 2018). The percentage of persister cells was calculated by dividing the number of CFUs after antibiotic treatment by the number of CFUs before antibiotic treatment. The experiment was conducted in triplicate. To evaluate the offsetting effect of the surfactant Tween 80, the change in the percentage of persister cells was measured with an additional treatment of 1% Tween 80 under the same conditions.

## 2.5. Chemical composition analysis of Coicis Semen ethanol extract

The components of the Coicis Semen ethanol extract

were analyzed using gas chromatography as described in a previous study with slight modifications (Jandacek *et al.*, 2004). The Coicis Semen ethanol extract (6.74 mg) was saponified with 4 mL of 0.5 N methanolic sodium hydroxide at 80℃ for 5 minutes, followed by cooling at 25℃. The samples were then methylated with 3 mL of 14% boron trifluoride (catalog number: 021-06171, FUJIFILM Wako Pure Chemical, Osaka, Japan) at 80℃ for 5 minutes, followed by cooling to 25℃. Next, the methylated samples were mixed with 2 mL of saturated sodium chloride solution and 2 mL of hexane. After vortexing for 1 minute, the hexane layer was separated by centrifugation at 455 × g and filtered with syringeless filters (catalog number: MV32ANPPT002TC01, GVS Korea, Namyangju, Korea). The fatty acid content of the Coicis Semen ethanol extracts was then analyzed via gas chromatography (model number: 7890B, Agilent Technologies, Wilmington, DE, USA) using a flame ionization detector with an HP-INNOWax column (catalog number: 19091N-133, Agilent Technologies). Helium was used as the carrier gas with a flow rate of 1 mL/min. The inlet temperature was set at 250℃, and the detector temperature was also maintained at 250℃. The oven temperature was initially set at 140℃ for 1 minute, then increased to 240℃ at a rate of 3℃/min and held for 5 minutes. The overall run time was 26 minutes, with an injection volume of 1 μL analyzed in split mode (1:20).

## 2.6. Analysis of the membrane permeability

Cell membrane permeability was assessed using SYTOX™ Green Nucleic Acid Stain according to the manufacturer's instructions to examine the impact of Coicis Semen ethanol extract and its components (Kim *et al.*, 2015). After an additional 3-hour incubation following the main culture, the *S. aureus* cells were washed twice with saline using centrifugation at 7,280 × g for 5 minutes. SYTOX™ Green Nucleic Acid Stain at a concentration of 0.5 μM was then incorporated into the cell suspensions, followed by the addition of the extract or its chemical components. Fluorescence was measured at 528 nm in a black 96-well plate (catalog number: 30296, SPL Life Sciences, Pocheon, Korea) using a Synergy LX multimode reader (BioTek, Agilent Technologies Korea, Seoul, Korea) with an excitation wavelength of 485 nm. The alteration in cell membrane permeability when treated with an additional 1% Tween 80 was also evaluated under the same conditions.

# 3. RESULTS and DISCUSSION

## 3.1. Reducing the persister cells of *Staphylococcus aureus* by Coicis Semen ethanol extract

Several antibiotic-resistant strains of *S. aureus* have become increasingly notorious, with persister cells being among the main contributors to the emergence of resistant cells (Chang *et al.*, 2020). Through a screening analysis using a library of methanol extracts from 389 forest products, Coicis Semen extract was found to substantially decrease the number of *S. aureus* persister cells in this study. During the screening, the number of persister cells for oxacillin was reduced by 95.5% using a 0.5 g/L methanol extract of Coicis Semen.

The identified Coicis Semen was further extracted with ethanol, a safe solvent, in a yield of 2.82%. The ethanolic extract of Coicis Semen was used to evaluate its persister cell reduction effect using three antibiotics: ciprofloxacin, oxacillin, and tobramycin [Fig. 1(a)]. These antibiotics were chosen due to their distinct bactericidal mechanisms. Ciprofloxacin inhibits DNA gyrase and topoisomerase IV, ultimately inhibiting cell division (Hooper *et al.*, 1987). Oxacillin is a beta-lactam antibiotic that inhibits bacterial cell wall synthesis (Fernandes *et al.*, 2013). Tobramycin binds to the bacterial ribosome

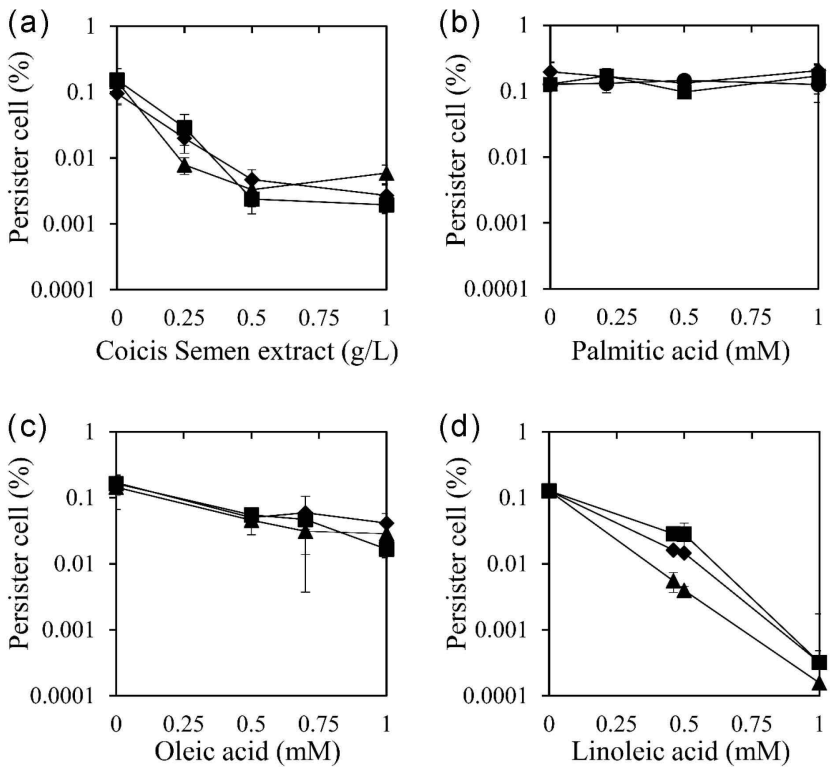


**Fig. 1.** Reduction in *Staphylococcus aureus* persister cells in response to the Coicis Semen extract and its constituent fatty acids. Coicis Semen extract (a), palmitic acid (b), oleic acid (c), and linoleic acid (d) were co-administered along with three antibiotics [20 mg/L of ciprofloxacin (■), 2.5 mg/L of oxacillin (◆), and 80 mg/L of tobramycin (▲)] to observe changes in the number of persister cells. The experiment was conducted in triplicate.

and inhibits protein synthesis (Brötz-Oesterhelt and Brunner, 2008). The ethanol extract of Coicis Semen at 0.5 g/L reduced the number of persister cells by 98.4%, 95.1%, and 97.7% when co-administered with ciprofloxacin, oxacillin, and tobramycin, respectively. Moreover, the persister cell removal activity of the Coicis Semen ethanol extract became the maximum activity at 0.5 g/L when co-administered with ciprofloxacin, oxacillin, and tobramycin. The MIC of the ethanolic extract of Coicis Semen against *S. aureus* was above 1 g/L, and no antibiotic activity was observed below 1 g/L used in this study. These observations suggested that the reducing persister cells of the ethanol extract of Coicis Semen with antibiotics are not due to its antibacterial activity.

## 3.2. Chemicals in the Coicis Semen ethanol extract

The fatty acid composition of Coicis Semen ethanol extract was analyzed using gas chromatography (Fig. 2). The fatty acid content consisted of 39.8% oleic acid, 26.0% linoleic acid, 11.0% palmitic acid, and 0.7% stearic acid. In a previous study, the free fatty acid content of an n-hexane extract of Coicis Semen was reported to consist of 45.8% oleic acid, 26.9% linoleic acid, 16.7% palmitic acid, and 5.5% stearic acid (Xi *et*

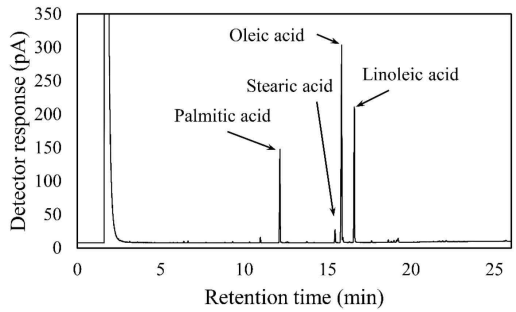


**Fig. 2.** Compositional analysis of ethanol extract of Coicis Semen using gas chromatography. The ethanol extract of Coicis Semen had 39.8% oleic acid, 26.0% linoleic acid, 11.0% palmitic acid, and 0.7% stearic acid. The content in the extract was calculated using the results of gas chromatography analysis with standard chemicals for analysis. The retention times were 12.139 min for palmitic acid, 15.443 min for stearic acid, 15.840 min for oleic acid, and 16.601 min for linoleic acid.

*al.*, 2016). Interestingly, despite the difference in the extraction solvent and analysis method in the aforementioned study, our free fatty acid analysis yielded a similar result.

## 3.3. Effects of fatty acids in the ethanol extract of Coicis Semen on persister cells

To identify the active chemicals in the ethanol extract of Coicis Semen responsible for reducing persister cells, the effect was evaluated using the fatty acid components palmitic acid, oleic acid, and linoleic acid [Fig. 1(b–d)]. Oleic acid and linoleic acid demonstrated a reduction in persister cells for all three antibiotics tested, while palmitic acid did not exhibit this effect. When coupled with oxacillin, ciprofloxacin, and tobramycin, oleic acid at 0.5 mM reduced persister cells by 70.5%, 66.6%, and 68.2%, respectively [Fig. 1(c)]. Linoleic acid at 0.5 mM reduced persister cells by 88.5%, 78.0%, and 96.9% when co-administered with oxacillin, ciprofloxacin, and tobramycin, respectively [Fig. 1(d)]. Both oleic acid and linoleic acid displayed a concentration-dependent persister cell removal activity, with linoleic acid exhibiting a stronger effect than oleic acid. However, palmitic acid did not show a persister cell inhibitory effect [Fig. 1(b)]. Considering the content of palmitic acid, oleic acid, and linoleic acid in the Coicis Semen ethanol extract (0.21 mM palmitic acid, 0.7 mM oleic acid, 0.46 mM linoleic acid), the effectiveness of persister cell reduction at these concentrations was evaluated and depicted in Fig. 1. The assessment of the effect at these concentrations suggests that oleic acid and linoleic acid are the main active components in the Coicis Semen ethanol extract that reduce persister cells.

The reduction of persister cells by oleic acid and linoleic acid is likely related to their antimicrobial activity against *S. aureus*. In previous studies, the MIC of oleic acid and linoleic acid against *S. aureus* was reported as 0.9 mM (Atashbeyk *et al.*, 2014; Fung *et al.*, 2017) and 0.2 mM (Yuyama *et al.*, 2020), respectively. This indicates that the antibacterial potency of linoleic acid is stronger than that of oleic acid, potentially explaining why linoleic acid reduced persister cells more effectively than oleic acid at the same concentration.

## 3.4. Changes in membrane permeability by Coicis Semen extract and its constituent fatty acids

Previous studies have demonstrated the effectiveness of membrane-damaging agents in reducing persister cells (Dombach *et al.*, 2021; Grassi *et al.*, 2017), and recent studies have documented the ability of fatty acids to penetrate the cell membrane (DeMars *et al.*, 2020). Here, we measured the effect of selected substances on cell membrane permeability (Fig. 3). Exogenous, heterogeneous fatty acids may alter the membrane properties (DeMars *et al.*, 2020). In turn, an increase in cell membrane permeability may offer a means to eliminate

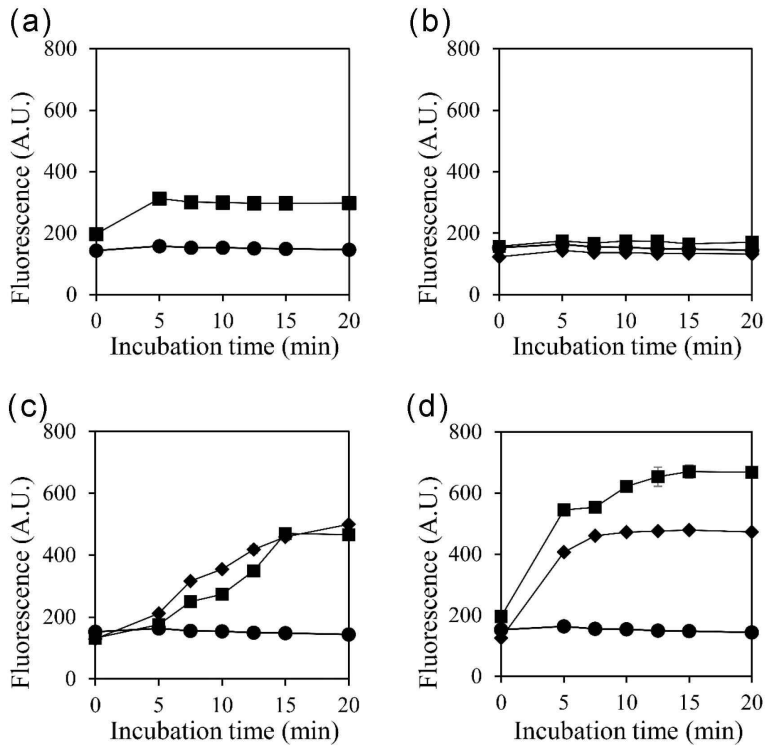


**Fig. 3.** Cell membrane permeability changes in response to Coicis Semen extract and its constituent fatty acids. *Staphylococcus aureus* cells were treated with Coicis Semen extract (a), palmitic acid (b), oleic acid (c), and linoleic acid (d), and the cell membrane permeability was measured via fluorescence. The following treatments were examined: Coicis Semen extract (a) at 0.5 g/L (■) compared to the untreated control (●), palmitic acid (b) at 0.21 mM (◆) and 0.5 mM (■) compared to the untreated control (●), oleic acid (c) at 0.5 mM (■) and 0.7 mM (◆) compared to the untreated control (●), and linoleic acid at 0.46 mM (◆) and 0.5 mM (■) compared to the untreated control (●). The experiment was conducted in triplicate.

persister cells (Defraine *et al.*, 2018). In line with the results in Fig. 1, Coicis Semen extract, oleic acid, and linoleic acid increased cell membrane permeability, whereas palmitic acid did not (Fig. 3). An increase in cell membrane permeability could facilitate the entry of antimicrobial substances into the cell (Brun *et al.*, 2018), leading to the disruption of cell integrity. Additionally, given the nature of persister cells where metabolism is halted, repairing the disrupted cell membrane is presumed to be challenging. Therefore, we propose that the inhibitory effect of Coicis Semen extract on persister cells is associated with changes in membrane permeability.

The hydrophobic nature of fatty acids is suggested to be a crucial factor in enhancing the permeability of cell membranes. To validate this hypothesis, the reduction of persister cells and the increase in cell membrane permeability were evaluated with a surfactant, 1% Tween 80 (Fig. 4). The results demonstrated that the increased permeability of the cell membrane was abolished by 1% Tween 80. This outcome suggests that the hydrophobic properties of oleic acid and linoleic acid are essential for entering the cell membrane and increasing membrane permeability, thereby reducing the occurrence of persister cells. The simultaneous abolishment of both the reduc-

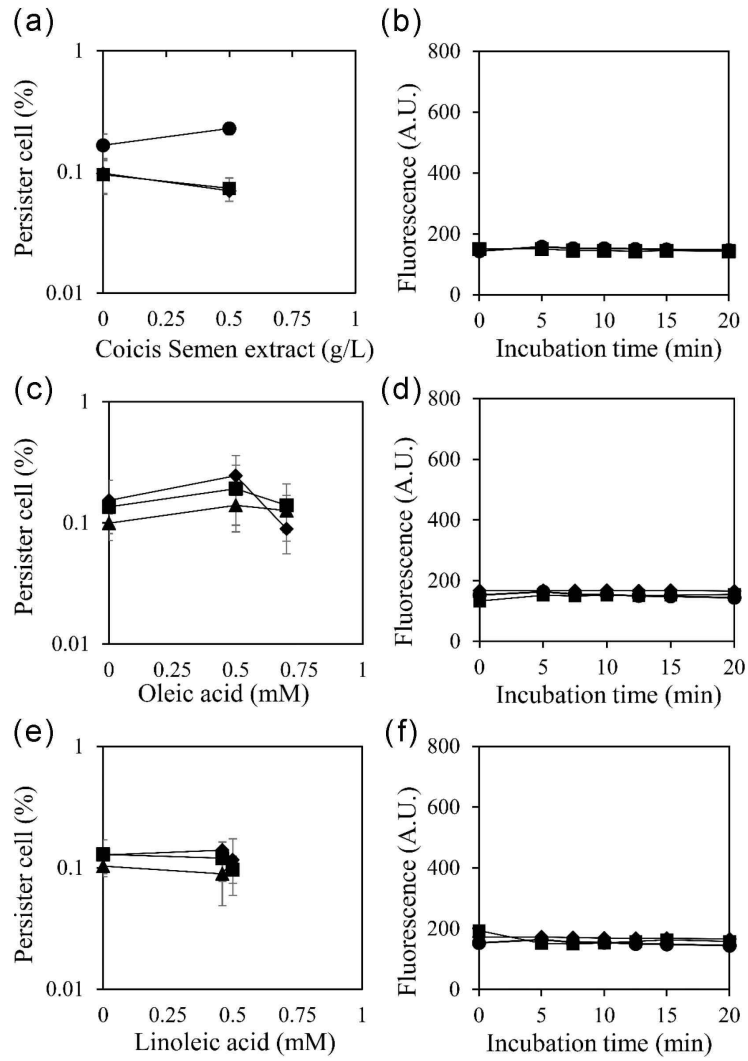


**Fig. 4.** Effects of Tween 80 on the reduction of persister cell and increases in cell membrane permeability in response to Coicis Semen extract, oleic acid, and linoleic acid. Changes in persister cells (a, c, and e) and permeability of *Staphylococcus aureus* cell membranes (b, d, and f) in response to Coicis Semen extract (a and b), oleic acid (c and d), and linoleic acid (e and f) co-administered with 1% Tween 80. Changes in persister cell numbers (a, c, and e) were then examined in cells treated with 20 mg/L of ciprofloxacin (■), 2.5 mg/L of oxacillin (◆), and 80 mg/L of tobramycin (▲). Cell membrane permeability was evaluated under the following treatments: Coicis Semen extract (b) at 0.5 g/L (■) compared to the untreated control (●), oleic acid (d) at 0.5 mM (■) and 0.7 mM (◆) compared to the untreated control (●), and linoleic acid at 0.46 mM (◆) and 0.5 mM (■) compared to the untreated control (●). The experiment was conducted in triplicate.

tion of persister cells and the increase in membrane permeability by Tween 80 suggests a close interrelation between them.

## 4. CONCLUSIONS

In this study, we explored the impact of Coicis Semen, a medicinal herb in the Asian region, on persister cell reducing activity in *S. aureus*. Coicis Semen demonstrated the ability to decrease the number of persister cells by disrupting the bacterial membrane. These findings highlight the potential of Coicis Semen as an adjunct in the antimicrobial treatment of *S. aureus* infections, offering a means to reduce persister cells and significantly curbing recurrence and the emergence of resistant strains. The discovery of novel and effective strategies to diminish persister cells is crucial for the treatment of bacterial infections, and the utilization of natural products such as Coicis Semen may enhance the efficacy of antibiotics.

## CONFLICT of INTEREST

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

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