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# Antibacterial Activity of the Phaeophyta *Ecklonia stolonifera* on Methicillin-resistant *Staphylococcus aureus*

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In an effort to discover an alternative therapeutic agent against methicillin-resistant *Staphylococcus aureus* (MRSA), several medicinal plants and seaweeds were evaluated for its antibacterial activity against MRSA. A methanolic extract of the Phaeophyta *Ecklonia stolonifera* exhibited significant antibacterial activity against MRSA. To perform more detailed investigation on antibacterial activity, the methanol extract of *E. stolonifera* was further fractionated with organic solvents such as hexane, dimethylchloride, ethyl acetate, and n-butanol. Among them, the hexane fraction showed the strongest antibacterial activity against MRSA strains with MIC from 500 to 600 μg/mL. The fraction also exhibited a bactericidal activity against MRSA, indicating that *E. stolonifera* contains a bactericidal substance against MRSA.

Key words: Antibiotic resistance, Anti-MRSA activity, Methicillin resistant *Staphylococcus aureus*, MRSA

## Introduction

*Staphylococcus aureus* is one of the most important pathogens that can produce a wide variety of diseases, from relatively benign skin infections such as folliculitis and furunculosis, to deep-seated and life-threatening conditions including erysipelsa, deep abscesses, osteomyelitis, pneumonia, sepsis, and endocrditis (Lowy, 1998). *S. aureus* was proved to be susceptible to the earliest antimicrobial substance such as penicillin. As antibiotic application has been increased, however, staphylococcal resistance rapidly developed. By the end of the 1950s, at least 85% of *S. aureus* strains were resistant to penicillin (Finland, 1979). The introduction in 1959 of the new semi-synthetic penicillins, methicillin and oxacillin, which are not inactivated by penicillinase, was expected to give physicians a break from the problem of resistance. However, methicillin-resistant *S. aureus* (MRSA) were first detected in 1961 and recently MRSA is the most problematic Gram-positive bacterium in public health because it has become resistant to almost all available antibiotics except vancomycin and teicoplanin (Witte, 1999). Vancomycin has been the drug of choice for the treatment of infections caused by MRSA. However, the rapid development of resistance to vancomycin has been reported in several countries. Therefore, there is a pressing need to develop alternative therapeutic agents against MRSA (Hiramatsu, 1997; Hanaki, 1998; Witte, 1999).

The Phaeophyta *Ecklonia stolonifera* Okamura is a member of the family of *Laminariaceae*, belonging to the order *Laminarials* as a perennial brown alga. *E. stolonifera* is distributed in Korea and Japan, and is commonly used as foodstuff along with *Laminaria japonica* and *Undaria pinnatifida*. It has been reported that *E. stolonifera* contains several bio-active compounds useful for human health. The previous phytochemical investigations performed on this species resulted in the isolation of phloroglucinol (Lee et al., 1996), phlorotannins (Taniguchi et al., 1991) and ecklonialactones (Kurata et al., 1993; Kang et al., 2003) worked as antioxidant compounds. This algae was also revealed to have antimutagenic activity (Han et al., 2000), bromoperoxidase activity (Hara and Sakurai, 1998), feeding-deterrent effect (Taniguchi et al., 1991), hepatoprotective activity (Kim et al., 2005), inhibition effect on MMP-1 expression (Joe et al., 2006), and tyrosinase activity (Kang et al., 2004). In this paper, we examine the antibacterial activity of *E. stolonifera* against MRSA and several pathogenic microorganisms.

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# Methods and Materials

## Bacterial strains and medium

The bacterial strains used in this study were *S. aureus* (KCTC 1927), and two MRSA (KCCM 40510 and KCCM 40511), *Bacillus subtilis* (KCTC 1028), *Escherichia coli* (KCTC 1682), *Salmonella typhimurium* (KCTC 1925), *Vibrio parahaemolyticus* (KCTC 2729), and *Klebsiella pneumoniae* (KCTC 2242). These strains were from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea) and the Korea Culture Center of Microorganisms (KCCM; Seoul, Korea). All strains were grown aerobically at 37°C in Mueller Hinton broth (MHB; Difco, USA) for minimal inhibitory concentration (MIC) assay and in Mueller Hinton agar (MHA; Difco, USA) for disc diffusion assay.

## Preparation of sample

*E. stolonifera* was collected from a Jeju aquaculture farm (Jeju, Korea) in March 2007 and other natural resources such as herbs and seaweeds used in this study were purchased from a commercial market located at Busan, Korea. Each sample was dried in an oven at 40°C for 2 days and finely powdered. Each powdered sample (100 g) was extracted with 1 L of 70% methanol twice at 80°C for 3 hr and filtered. The combined filtrate was concentrated by rotary evaporation at 40°C. A liquid/liquid solvent fractionation procedure was performed to fraction an antibacterial substance according to relative polarity. The methanol extract of *E. stolonifera* was evaporated with a rotary evaporator and then dissolved in 2 L of distilled water. The hydrated solution was fractionated

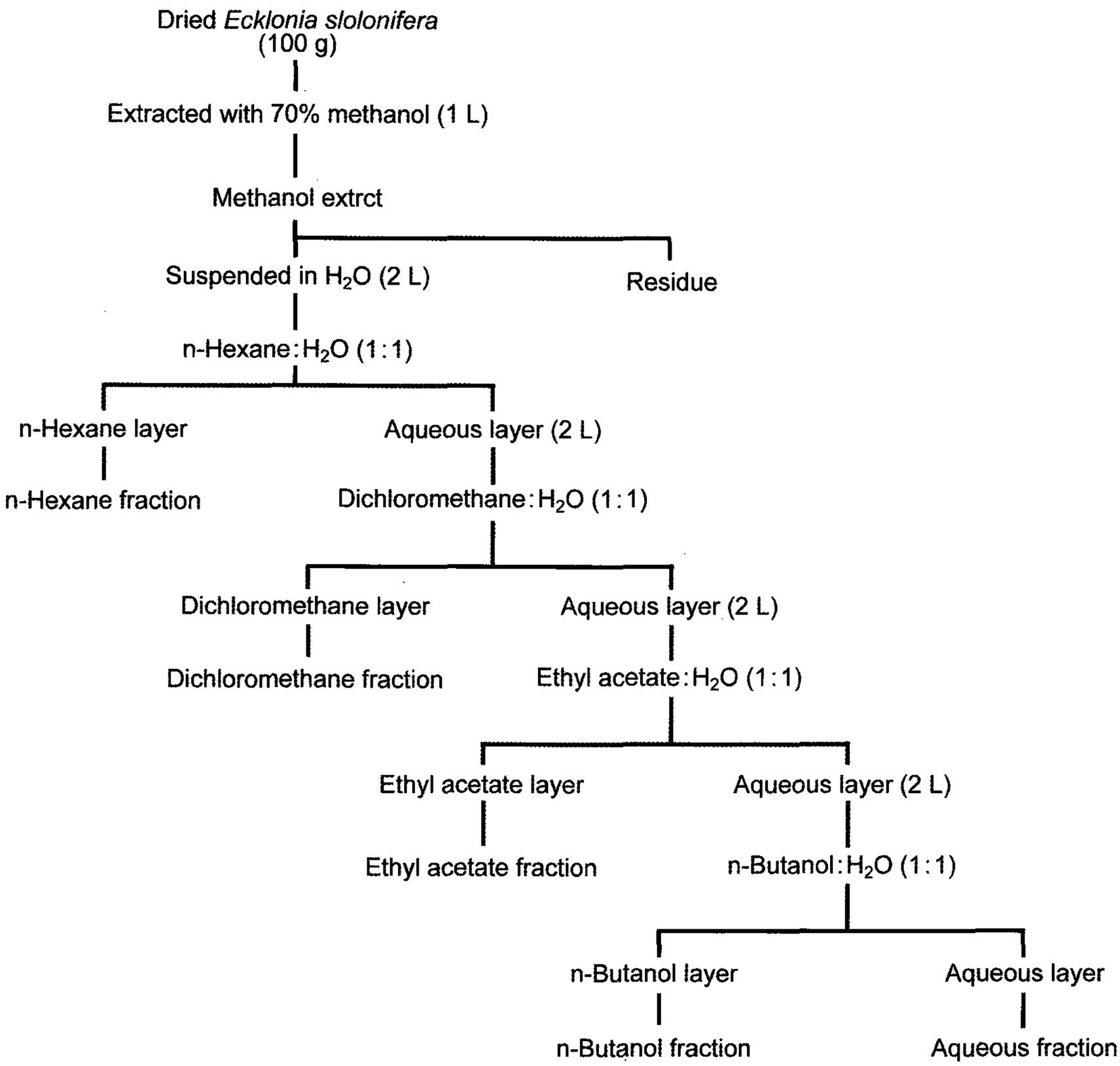


Fig. 1. Scheme of extraction and liquid-liquid solvent fraction. The powered sample (100 g) was extracted with 1 L of 70% methanol twice at 80°C for 3 hr and filtered. The combined filtrate was concentrated by rotary evaporation at 40°C. A liquid/liquid solvent fractionation was performed according to relative polarity.

by the same volume of hexane, dimethylchloride, ethyl acetate, and n-butanol in that order. After shaking vigorously and allowing the two phases to settle, the lower (aqueous) phase was removed. Then, additional 2 L of hexane was added into the previous aqueous phase, shaken, allowed to equilibrate, and then removed the aqueous phase. This step was repeated six times. The six hexane phases were combined and evaporated with a rotary evaporator. Other organic solvents were also fractionated in the order of polarity and evaporated as descried above. The scheme of extraction and solvent fractionation was illustrated in Fig. 1. Finally, hexane, dicholromethane, ethyl acetate, buthanol, and water extract were obtained. The concentration of extracts was adjusted to 100 mg/mL by dissolving in dimethyl sulfoxide and used for further study.

**Evaluation of antibacterial activity**

The antibacterial activity was evaluated by a growth inhibition assay. One ml of bacterial culture containing approximately $10^5$ CFU/mL was spread on MHA plate and a paper disc (8 mm in diameter) containing 10 mg of each extract was then placed in the plate. After incubating 24 hr at 37°C, the diameter of inhibition zone was measured.

**Measurement of minimum inhibitory concentration**

Measurement of minimum inhibitory concentration (MIC) of the extract and vancomycin was determined by the two-fold serial dilution method in MHB as described by the National Committee for Clinical Laboratory Standards (1997). MIC was defined as the lowest concentration of crude extract that inhibited the visual growth after incubation at 37°C for 24 hr and was performed in triplicates.

**Bactericidal activity against MRSA**

In order to evaluate bactericidal activity of *E. stolonifera* extract against MRSA, the hexane fraction (600 μg/mL) was suspended into MHB inoculated by a MRSA strain (KCCM 40510), which was adjusted to an estimated cell density of about $10^4$ CFU/mL. Then, MRSA was cultured at 37°C. The bactericidal activity against MRSA was evaluated by determining the viable cell count method. Samples were collected at indicated times for colony counting.

## Results and Discussion

**Antimicrobial activity**

In preparing medicinal and physio-functional materials, it is common to soak them in alcohol or organic solvent. To screen natural resources exhibiting an antibacterial activity against MRSA, which is the most problematic gram-positive bacterium in public health, we previously prepared methanol extracts of various plants as described above. Antimicrobial activity of methanol extracts against MRSA was qualitatively assessed by measuring inhibition zones (Table 1). The extract from *E. stolonifera* only showed antimicrobial activity against both MRSA strains tested in this study strains, suggesting that *E. stolonifera* contains an antibacterial substance against drug-resistant *S. aureus*, MRSA. Also, the extract exhibits almost similar antibacterial activity against food-borne pathogenic *S. aureus* used as control.

To perform more detailed investigation on antibacterial activity, the methanol extract of *E. stolonifera* was further fractioned with organic solvents such as hexane, dimethylchloride, ethyl acetate, and n-butanol as illustrated in Fig. 1. The antimicrobial activity of hexane, dichloromethane, ethyl acetate, butanol, and water fractions from the methanol

Table 1. Antibacterial activity of methanol extracts from various plants against *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA). 10 mg of methanol extract from various samples was loaded onto a disk (8 mm in diameter). Data are the averages of duplicate experiments. -, no detected antibacterial activity

| Medicinal plants and seaweeds tested in this study | Zone of inhibition (mm) | | |
|---|---|---|---|
| | *S. aureus* (KCTC 1927) | MRSA (KCCM40510) | MRSA (KCCM40511) |
| *Ecklonia stolonifera* | 11 | 12 | 11 |
| *Zostera marina* | - | - | - |
| *Undaria pinnatifida* | - | - | - |
| *Ecklonia cava* | - | - | - |
| *Angelica gigas* | - | - | - |
| *Aurantii nobilis pericarpium* | - | - | - |
| *Camellia sinensis* | - | - | - |
| *Carthamus tinctorius* | - | - | - |
| *Cnidium officinale* | - | 8 | - |
| *Cuscuta japonica* | - | - | - |
| *Hovenia dulcis* | 11 | - | 9.5 |

extract was evaluated by measuring inhibition zones. Among them, the hexane fraction showed the strongest antibacterial activity, which was even higher than that of the methanol extract (Table 2). Other fractions also showed antibacterial activity against MRSA strains, even though the activity was less than that of hexane fraction. No significant activity, however, was observed in water fraction (Table 2). Considering these results, we supposed that a hydrophobic substance(s) from *E. stolonifera* exhibited antibacterial activity against MRSA and others.

**Measurement of MIC value**

To evaluate quantitatively antibacterial activity of *E. stolonifera* against MRSA and other bacteria related in food borne and spillage, its MIC value was determined by the two-fold serial dilution method (Table 3). The methanol extract and solvent fractions showed antibacterial activity against all Gram-positive bacteria including MRSA and Gram-negative bacteria, whereas vancomycin as a positive control had no antibacterial effect against Gram-negative bacteria as well known. MIC values of the methanol extract and solvent fractions against MRSA were in the ranges of 500-900 μg/mL (Table 3). Among them, the hexane fraction showed the strongest antibacterial activity against MRSA strains tested with MIC ranging from 500 to 600 μg/mL. The difference MIC values between two MRSA strains indicated that the mechanism of drug resistance was different. The fraction also exhibited the strongest antibacterial activity against other bacterial strains including Gram-negative bacteria, suggesting that the crude extract also contains other antibacterial substances against Gram-negative bacteria and others.

It is well known that vancomycin interferes with cell wall synthesis, as does penicillin, eventually leading to lysis of cell (Barna and Williams, 1984). As the result, this antibiotic is effective only Gram-positive bacteria not Gram-negative. Considering these results, we supposed that *E. stolonifera* contains a novel anti-MRSA substance and its anti-MRSA mechanism differs from that of vancomycin since vancomycin was not effective against Gram-negative bacteria.

**Bactericidal activity of *E. stolonifera* extract against MRSA**

*E. stolonifera* extract showed an antibacterial activity against MRSA as described above. We investigated whether the extract contains bacterio-static or bactericidal activity against MRSA. As shown Fig. 2, the hexane fraction of *E. stolonifera* extract exhibited a bactericidal activity, indicating that the fraction contains a bactericidal substance

Table 2. Antibacterial activity of solvent extracts of *Ecklonia stolonifera* against *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA). Methanol extract and other solvent fractions were obtain as described in Materials and Methods. 10 mg of each sample was loaded onto a disk (8 mm in diameter). Data are the averages of duplicate experiments. -, no detected antibacterial activity

| Profile of solvent extract | Zone of inhibition (mm) | | |
|---|---|---|---|
| | *S. aureus* (KCTC 1927) | MRSA (KCCM 40510) | MRSA (KCCM 40511) |
| Methanol extract | 11 | 12 | 11 |
| Hexane fraction | 18 | 20 | 10 |
| $CH_2Cl_2$ fraction | 11 | 10 | 10 |
| Ethyl acetate fraction | 12 | 11 | 10 |
| Butanol fraction | 9 | 11 | 10 |
| Water fraction | - | - | - |

Table 3. Antibacterial activity of solvent extracts of *Ecklonia stolonifera*. [a]MIC (Minimum inhibitory concentration) of each solvent extract and vancomycin was determined by the two-fold serial dilution method in Mueller Hinton broth. [b]MRSA, methicillin resistant *Staphylococcus aureus*. [c]NA, not active

| Strains | MIC[a] (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | Methanol extract | Hexane fraction | $CH_2Cl_2$ fraction | Ethyl acetate fraction | Butanol fraction | Vancomycin |
| MRSA[b] (KCCM40510) | 800 | 600 | 800 | 700 | 800 | 2 |
| MRSA (KCCM 40511) | 900 | 500 | 700 | 700 | 700 | 2 |
| *Staphylococcus aureus* (KCTC 1927) | 900 | 500 | 600 | 700 | 800 | 0.5 |
| *Bacillus subtilis* (KCTC 1028) | 900 | 600 | 800 | 700 | 800 | 0.5 |
| *Escherichia coli* (KCTC 1682) | 900 | 500 | 700 | 700 | 600 | NA[c] |
| *Klebsiella pneumonia* (KCTC 2242) | 900 | 600 | 800 | 600 | 600 | NA |
| *Salmonella typhimurium* (KCTC 1925) | 900 | 500 | 700 | 500 | 700 | NA |
| *Vibrio parahaemolyticus* (KCTC 2729) | 900 | 600 | 800 | 600 | 800 | NA |

against MRSA (Fig. 2). In the presence of the hexane fraction (600 μg/mL), MRSA cells were gradually reduced and no cells observed after the treatment of 18 hr.

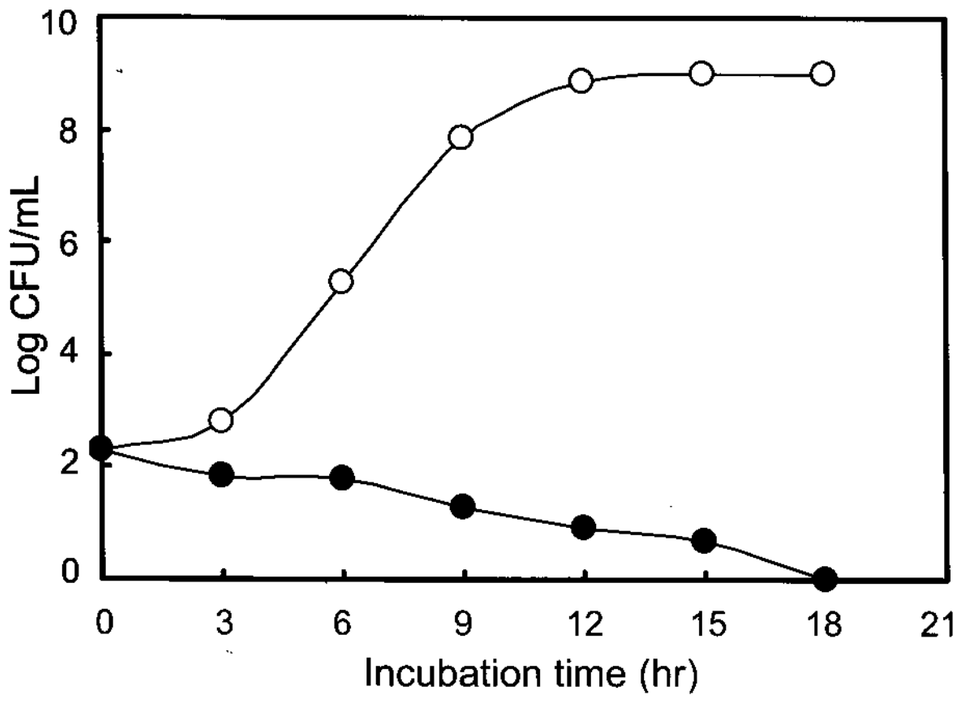


Fig. 2. Bactericidal activity of *Ecklonia stolonifera* extract against methicillin resistant *Staphylococcus aureus* (MRSA). MRSA cells (KCCM 40510) were obtained from the culture grown to exponential phase in Mueller Hinton broth at 37°C and inoculated at a concentration of about $10^2$ cfu/mL in a fresh Mueller Hinton broth containing hexane fraction of *E. stolonifera* extract (600 μg/mL). Then, MRSA was cultured at 37°C. The bactericidal activity against MRSA was evaluated by determining the viable cell count method. Data are the averages of duplicate experiments. ○, control; ●, in the presence of 600 μg/mL of hexane fraction.

In this paper, we report antibacterial properties of *E. stolonifera* extract against MRSA and other bacteria related in food borne and spillage. The results suggested the possible use of *E. stolonifera* extract in controlling MRSA and food safety. We are planning to perform experiments to isolate single active novel components from the extract and to verify their chemical structures. These efforts may provide useful data and clue to further investigate to develop a novel antibiotic against MRSA.

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