**Thesis for the Degree of Master of Engineering**

# *In vitro* Antimicrobial Activity against Cutaneous Pathogens and Anti-inflammatory Effect of *Ishige okamurae*


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# *In vitro* Antimicrobial Activity against Cutaneous Pathogens and Anti-inflammatory Effect of *Ishige okamurae*

# (패 추출물의 피부 병원균에 대한 항균효과 및 HaCaT cell 에서 항염증 효과)

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# 패 추출물의 피부 병원균에 대한 항균효과 및 HaCaT cell 에서 항염증 효과

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## 요 약

해양에 서식하는 생물은 육상생물과 구별되는 여러 가지 화합물과 생리활성 물질을 함유하는 것으로 밝혀져 왔다. 최근에 해양생물 중 해조류가 가지고 있는 다양한 생리활성 물질에 대한 연구가 주목을 받고 있다. 이에 해조류 유래의 생리활성 물질이 가지고 있는 항산화, 항염증, 항혈액응고 및 면역력 증강 등에 대한 다양한 연구가 진행되고 있다. 하지만, 해조류 유래 물질의 병원성 미생물에 대한 항미생물 활성에 대한 연구는 상대적으로 미미한 상황이다. 이에 본 연구에서는 phlorotannin, fucoxanthin 및 fucose-containing polysaccharides 등의 생리활성 물질을 다량으로 함유하고 있다고 알려진 갈조류에 속하는 패(*Ishige okamurae*)를 이용하여 피부 병원성 미생물에 대한 항미생물 효과와 항염증 효과를 조사하였다.

본 연구에서는 패를 에탄올로 추출하여 동결건조 한 후 각종 유기용매를 이용하여 극성별로 분획하고 이들 분획 추출물을 이용하여

항균 활성을 측정 하였다. 이중, EtoAc 추출물과 *n*-hexane 추출물이 피부 병원균들에 대해 가장 뛰어난 항균효과를 나타내었다. 그 중 *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans* 및 *Propionibacterium acnes* 와 같은 미생물에 대한 MIC 값은 128-512 μg/mL 로 나타났으며, *Pseudomonas aeruginosa* 는 MIC 값이 64 μg/mL 로 가장 좋은 효과가 나타났다.

이들 피부 병원미생물에 의한 감염증 치료에 사용되는 항생제 등의 약품이 개발되어 효과적으로 사용되고 있으나, 이들 약제들의 오남용에 의한 내성균이 증가하고 있다고 보고되고 있다. 특히, *P. aeruginosa* 의 경우 다수의 항생제들에 내성을 보여 새로운 항생물질의 탐색 및 대체 치료방법이 필요하다. 본 연구에서 피부 병원미생물 중에서 다수의 항생제에 대한 강한 내성을 나타내는 *P. aeruginosa* 균주들을 대상으로 본 연구에서 높은 항균효과를 나타내었던 패의 *n*-hexane 분획물과 EtoAc 분획물에서 순수 분리한 화합물 diphlorethohydroxycarmalol (DPHC)를 이용하여 기존의 항생제와의 병용에 의한 항균효과를 조사하였다. 실험결과, 패의 *n*-hexane 분획물과 DPHC 는 항생제와 병용 사용하였을 때 보다 강한 항균 활성을 나타내는 것으로 분석되었다. 이 결과는 다양한 항생제에 대한 내성을 획득하여 항생제 치료요법이 어려운

*P. aeruginosa* 감염증 치료에 패 유래 추출물 또는 화합물이 유용하게 적용될 수 있다는 것을 의미한다.

또한, 피부조직에 대한 패 추출물의 활용과 패 추출물의 독성을 파악하기 위하여 각질 형성 세포인 인간 유래 HaCaT cell 을 이용하여 세포독성 실험을 실시하였고, LPS 로 염증을 유도한 HaCaT cell 에서 패 추출물의 항염증 효과에 대해서도 조사하였다. 그 결과, 패 추출물(200 μg/mL)은 HaCaT cell 에 대한 세포독성을 나타내지 않았다. 그리고 이 농도에서 패 추출물은 염증 관련 지표물질들 중의 하나인 nitric oxide (NO)의 생성을 약 20% 억제시키는 항염증 활성도 나타내는 것으로 분석되었다.

이상의 결과를 종합해 보면, 패 추출물은 피부 병원균들에 대한 뛰어난 항균 활성뿐만 아니라 LPS 로 염증이 유도된 인간 유래 각질 형성 세포에서 대한 항염증 효과도 가지고 있는 것으로 나타내었다. 이러한 향후, 패 추출물은 다양한 피부 질환 치료제 및 신약개발에 이용될 수 있을 것으로 기대된다.

# 1. Introduction

Opportunistic pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Propionibacterium acnes* are often associated with cutaneous pathogens. These pathogenic bacteria are often involved in the development of abnormal follicular keratinization and inflammation known acne vulgaris (Yamaguchi et al., 2009). Generally, topical therapeutic option for these cutaneous pathogens is antibiotic treatment to kill the microbes. However, irrational use of such antibiotics resulted in drug-resistant pathogens, treatment failures and fatal outcomes in various infectious diseases (Jo et al., 2005).

Surveillance of *P. aeruginosa* infections has revealed trends of increasing antimicrobial resistance, including carbapenem resistance and multidrug resistance (Driscoll et al., 2007). Ceftazidime, Carbapenem, or piperacillin resistance rates increased up to 50% in intensive care unit patients and the multidrug resistance rate, which is resistant to three or more antibiotics, reached 30% (El and Alhajhusain, 2009; Song and Joo, 2010). Because of the lack of antibiotics effective against multidrug-resistant *P. aeruginosa*, Colistin that has not been used in clinical practice for some time due to toxicity has reappeared and started to be used again (Li et al,. 2006; Michalopoulos and Karatza, 2010). However, new toxicity occurs in more than

3% of patients with the use of colistin, and because of the incompleteness of the drug, multidrug-resistant *P. aeruginosa* has emerged as a clinically significant problem (Paul et al,. 2010; Song and Joo, 2010).

In the present scenario of multiple drug resistance to human pathogenic organisms, this has necessitated a search for new antimicrobial substances from other natural sources (Ahmad and Beg, 2001). The Importance as a source of novel bioactive substances is growing rapidly and researchers have revealed that marine algae originated compounds exhibit various biological activities (Wijesekara et al., 2010). Although there are various physiologically active substances in marine algae, there is not much research on the influence of marine algae against cutaneous pathogens. Hence, the present study was aimed at exploiting bioactive compounds of the *Ishige okamurae* against cutaneous pathogens.

*I. okamurae*, a kind of brown algae with narrow fronds, thick cortical layer and acute apices, belongs to the family of Ishigeaceae and grows on rocks in the upper and middle intertidal zone on rough open coasts (Cho et al., 2005). Phlorotannin, a polyphenolic component of *I. okamurae*, is known to have antiplasmin inhibitory activity and antiallergic activity (Lim et al., 2002; Nakayama et al., 1989). Other physiologically active substances in *I. okamurae* are known to contain fucoxanthin, taurine, and fucose-containing polysaccharides (Kim, 2009). They are rich in alginic

acid, an indigestible polysaccharide that has not been degraded by digestive enzymes in the body (Jang et al., 2011).

The object of this study is to evaluate an antimicrobial activity of *I. okamurae* against cutaneous pathogens and an anti-inflammatory effect of *I. okamurae* in an inflammation model that the inflammation was induced by lipopolysaccharide (LPS) in HaCaT cells of human keratinocyte cell line.

# 2. Materials and Methods

## 2.1. Preparation of materials

### 2.1.1. Raw materials and extraction

The fresh *I. okamurae* collected from the coastal area of Jeju Island, Korea. As pre-treatment process, *I. okamurae* was washed three times with water to remove salt and epiphytes. The dried *I. okamurae* was ground and then finely powdered with a blender (HMF-1000A; Hanil Electronics, Seoul, Korea). The dried sample (1.0 kg) was extracted with 70% EtOH (ethanol; 10.0 L × 3 times) at 70°C for 3 hrs in triplicate. And then, the solvent was evaporated a using rotary evaporator (Eyela Co., Tokyo, Japan) at 40°C *in vacuo*. The combined crude EtOH extract was suspended in 10% ethanol and fractionated with Hexane (*n*-hexane; 1.0 L × 3 times), dichloromethane, EtoAc (ethyl acetate; 1.0 L × 3 times) and water solution in order, according to relative polarities. Each fraction was concentrated using the evaporator under vacuum at 45°C.

### 2.1.2. Isolation and identification of phlorotannins from *I. okamurae*

Since the EtoAc fraction of *I. okamurae* exhibited the strong antibacterial activities, the active compounds were extracted from the EtoAc fraction using a silica gel and Sephadex LH-20 column chromatography. The active compound was finally purified by reverse-phase high performance liquid chromatography (RP-HPLC) on Atlantis dC18 column (3 μm , 4.6 x 150 mm, Waters, Milford, USA) with a linear gradient of acetonitrile (0-100% v/v, 100min). Elution peaks were detected to a wavelength range of 230nm. Identification of active compound molecular weight was through quadrupole time-of-flight mass spectrometry (Q-TOF MS)

## 2.2. Evaluation of antimicrobial activity

### 2.2.1. Strains and culture conditions

As shown in Table 1, the test microbial strains were obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea) and the American Type Culture Collection (ATCC; Manassas, VA, USA); *S. aureus* KCTC 1927, *S. epidermidis* ATCC 14990, *P. aeruginosa* KCTC 1637, *C. albicans* KCTC 7965, *P. acnes* KCTC 3314. Four of *P. aeruginosa* clinical isolates were provided by the

Gyeongsang National University Hospital (Jinju, Korea), a member of the National Biobank of Korea. *S. aureus*, *S. epidermidis* and *P. aeruginosa* strains were grown aerobically at 37°C in tryptic soy broth (TSB; Difco Inc., Detroit, MI). *C. albicans* was cultivated in TSB (Difco Inc.) and incubated at 25°C. *P. acnes* strains were anaerobically cultivated in brain heart infusion broth (BHI; Difco Inc.) supplemented with 1.0% glucose, and incubated at 37°C for 24 h in a $CO_2$ incubator (NAPCO 5400; General Laboratory Supply, Pasadena, TX, USA), in a 10% $CO_2$ humidified atmosphere.

**Table 1. List of microbial strains used in this study**

| Strains | Medium | Culture condition | Source |
|---|---|---|---|
| ***Staphylococcus aureus*** **KCTC 1927** | Tryptic soy broth | 37°C, 24 h under aerobic condition | Korea Collection for Type Cultures (KCTC) |
| ***Staphylococcus epidermidis*** **ATCC 14990** | Tryptic soy broth | 37°C, 24 h under aerobic condition | American Type Collection (ATCC) |
| ***Propionibacterium acnes*** **KCTC 3314** | Brain heart infusion broth | 37°C, 24 h under anaerobic condition | Korea Collection for Type Cultures (KCTC) |
| ***Candida albicans*** **KCTC 7965** | Tryptic soy broth | 25°C, 24 h under aerobic condition | Korea Collection for Type Cultures (KCTC) |
| ***Pseudomonas aeruginosa*** **KCTC 1637** | Tryptic soy broth | 37°C, 24 h under aerobic condition | Korea Collection for Type Cultures (KCTC) |
| ***P. aeruginosa*** **isolate 366** | Tryptic soy broth | 37°C, 24 h under aerobic condition | Gyeongsang National University Hospital |
| ***P. aeruginosa*** **isolate 4068** | Tryptic soy broth | 37°C, 24 h under aerobic condition | Gyeongsang National University Hospital |
| ***P. aeruginosa*** **isolate 4135** | Tryptic soy broth | 37°C, 24 h under aerobic condition | Gyeongsang National University Hospital |
| ***P. aeruginosa*** **isolate 4561** | Tryptic soy broth | 37°C, 24 h under aerobic condition | Gyeongsang National University Hospital |

### 2.2.2. Measurement of minimum inhibitory concentration and minimum bactericidal concentration

Minimum inhibitory concentration (MIC) is the method of evaluating the antimicrobial activity quantitatively. It is defined as the lowest concentration of crude extract that inhibit the visible growth of a microorganism after 20-24 h of incubation at 25℃ for *C. albicans* and at 37℃ for bacterial strains (Grierson and Afolayan, 1999). The experiment procedures were followed by the guideline of Clinical and Laboratory Standards Institute (CLSI, 2012). MIC assay was performed using serial two-fold dilution method with Mueller–Hinton broth (MHB; Difco Inc.) and 96-well microtiter plates (with clear flat bottoms). Once suspension culture had done, MIC values were determined by reading the plates visually. This test was repeated three times.

Minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium (Amyes et al., 1996). It can be determined from broth dilution minimum inhibitory concentration (MIC) tests by subculturing to agar plates that do not contain the test agent. The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by $\geq$99.9%..

### 2.2.3. Antibiotic susceptibility test

Antibiotic susceptibility test (AST) is used to determine whether an organism is susceptible or resistant to an antimicrobial agent (Jenkins and Schuetz, 2012). As shown in Table 2, the antibiotic resistance of test strains was confirmed against 4 kinds of commercial antibiotics by MIC assay. An antibiotic was serially diluted and then the bacterial growth was visually checked.

### 2.2.4. Determination of fractional inhibitory concentration

Fractional inhibitory concentration (FIC) assay is widely used for evaluation of *in vitro* synergy for multiple agents (Odds, 2003). This test method assesses interaction between antimicrobial agents by exposing bacteria to varying concentrations of the antimicrobials drugs (Hsieh et al., 1993). In this study, the synergy effect between marine algal extract and antibiotics against pathogens of human skin was evaluated. FIC index was calculated using the following formula:

$FIC_A = MIC_A$ in combination / $MIC_A$,
$FIC_B = MIC_B$ in combination / $MIC_B$,
FIC index = $FIC_A + FIC_B$

The interaction was defined as synergistic if the FIC index was <1; additive if the FIC index was 1.0; sub-additive if the FIC index was between 1.0 and 2.0; indifferent if the FIC index was 2, and antagonistic if the FIC index >2. Synergy was further sub-classified as marked (FIC index, ≤0.50) and weak (FIC index, between 0.50 and 1.0) (Lee et al., 2014; Kim et al., 2016).

**Table 2. List of antibiotics used in this study**

| Antibiotic | Company | Solubility | Application |
|---|---|---|---|
| Amikacin | Sigma-Aldrich | $H_2O$ | Antibacterial agent |
| Ceftazidime | | DMSO [1], $H_2O$ | |
| Ciprofloxacin | | 2M-HCl | |
| Meropenem | | DMSO, $H_2O$ | |

[1] DMSO stands for dimethyl sulfoxide.

## 2.3. Investigation of anti-inflammatory activity

### 2.3.1. Cell culture

Human HaCaT were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Germany) supplemented with 1% antibiotic-antimycotic solution (Gibco BRL) and 10% fetal bovine serum (Gibco BRL) at 37°C and in a 5% $CO_2$ atmosphere. For experiments, the cells were harvested by trypsin-EDTA (Gibco BRL) treatment and seeded into 96-well microplates (Greiner, Germany) at a density of 40,000 cells/$cm^2$.

### 2.3.2. Cytotoxicity assessment by MTT assay

The cell viability was evaluated by MTT assay, a method based on the reduction of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The medium containing HaCaT cells was cultured into a 96-well plate at a density of 1 x $10^5$ cells/mL. The plate was incubated overnight and treated with 100 μL of DMEM medium containing different concentrations of chitosan and oligo-chitosan (25, 50, 100 and 200 μg/mL). After 24 h of incubation, MTT solution (1 mg/mL) was added to each well and the plate was incubated for another 4 h at 37ºC. The blue formazan

salt was dissolved in dimethyl sulfoxide (DMSO) and then optical density was measured at 540 nm with a GENios microplate reader (Tecan Austria GmbH, Salzburg, Austria). The optical density of formazan formed by untreated cells was taken as 100% of viability.

### 2.3.3. Measurement of nitric oxide production

Nitric oxide (NO) levels in the culture supernatants were determined by measuring nitrite by Griess reaction (Sun et al., 2003). The HaCaT cells were seeded in 96-well plates with 1X105 cells/well and allowed to adhere for 2h at 37°C. The cells were incubated for 24h with or without 1 μg/mL of LPS (Sigma Chemical Co., St. Louis, MO, U.S.A.) in the absence or presence of the *I. okamurae* extracts. As a parameter of NO synthesis, the nitrite concentration was measured by the Griess reaction using the supernatant of the HaCaT cells as previously described. Briefly, 100 μL of the cell culture supernatant were reacted with 100 μL of Griess reagent (0.1% naphtylethylenediamine dihydrochloride and 1% sulfanilamide in 5% $H_3PO_4$). NO production in the culture medium was measured by Griess reaction (Sun et al., 2003). The absorbance of the mixture was measured with an Infinite F200 pro-microplate reader (TECAN, Männedorf, Switzerland) at 540 nm.

## 2.4 Statistical analysis

Analyses were performed in triplicate, and data were averaged over the three measurements. The standard deviation was also calculated. Multiple comparisons were evaluated by two-way analysis of variance using SPSS ver. 23.0 statistical software (SPSS Inc., Chicago, USA). Significant differences between means were determined using Duncan's multiple range tests. A $P<0.05$ was considered significant.

# 3. Results and Discussion

## 3.1. Determination of MIC and MBC of *I. okamurae*

The ethanolic extract of *I. okamurae* exhibited an antibacterial activity against cutaneous pathogens, suggesting that the extract contains antibacterial substances. To further investigate the antimicrobial activity against the pathogens of human skin, the ethanolic extract of *I. okamurae* was fractioned into *n*-hexane (Hexane), chloroform ($CHCl_3$), ethyl acetate (EtoAc) and $H_2O$ soluble fraction (Fig.1). The antibacterial activity of *I. okamurae* fractions was quantitatively evaluated by the MIC assay (Table 3). The MIC values of *I. okamurae* fractions were in the range of 64 to 2,048 μg/mL against cutaneous pathogens tested in this study. Among these, EtoAc and Hexane fractions showed strong antibacterial activity in the MICs range of 64 to 512 μg/mL. However, $H_2O$ fractions exhibit weak antibacterial activity ranging from 512 to 2,048 μg/mL against cutaneous pathogens. These results suggested that a substance with antimicrobial activity against the pathogens of human skin was abundantly present in the EtoAc and Hexane soluble fractions.

In order to evaluate the bactericidal effect of *I. okamurae*, the MBC assay was performed (Table 4). The MBC values of *I. okamurae* fractions were in the range of

512 to above 2,048 μg/mL against cutaneous pathogens. The MBC values of *I. okamurae* fractions were 2-folds increased compared to those of MIC values. Similar patterns between MIC and MBC values were reported by several studies (Eom et al., 2016; Kim et al., 2016). Considering both MIC and MBC results, Hexane and EtoAc fractions of *I. okamurae* exhibited the highest antibacterial activity against cutaneous pathogens. Interestingly, fractions of *I. okamurae* exhibited strong antibacterial activity *against P. aeruginosa* strains. In the current study, there are few studies on natural substances having antimicrobial activity against *P. aeruginosa* strains.

In addition, these results strongly indicated that *I. okamurae* will be a potential candidate to develop an alternative therapeutic agent for the treatment against skin borne pathogens and associated infections.

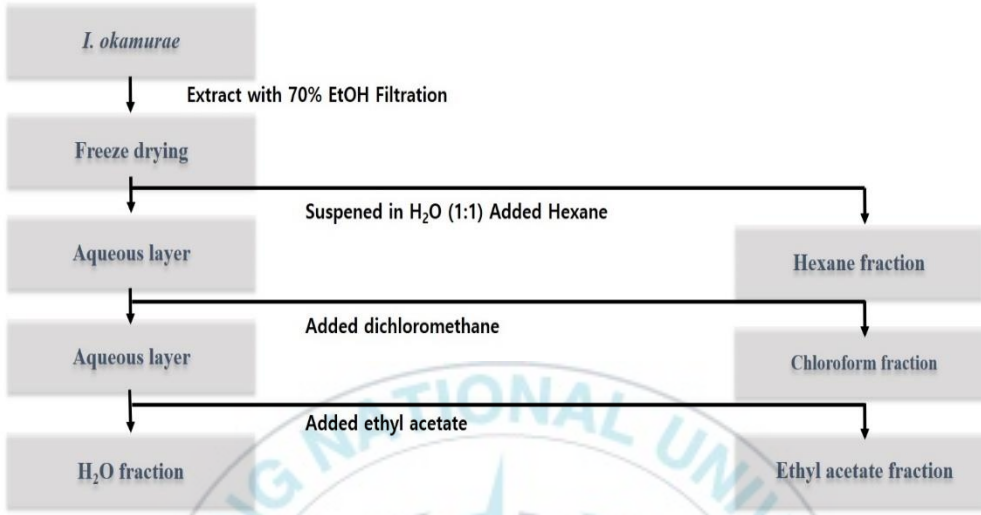



**Fig. 1. Scheme of extraction and fractionation.**

**Table 3. Minimum inhibitory concentration of fractions from *Ishige okamurae* against cutaneous pathogens.**

| Strains | MIC (μg/mL) | | | | |
|---|---|---|---|---|---|
| | **EtOH** | **Hexane** | **$CHCl_3$** | **EtoAc** | **$H_2O$** |
| ***Staphylococcus aureus* KCTC 1927** | 512 | 512 | 512 | 256 | 1,024 |
| ***Staphylococcus epidermidis* ATCC 14990** | 512 | 512 | 1,024 | 128 | 512 |
| ***Propionibacterium acnes* KCTC 3314** | 512 | 256 | 1,024 | 512 | 2,048 |
| ***Candida albicans* KCTC 7965** | 512 | 128 | 512 | 512 | 2,048 |
| ***Pseudomonas aeruginosa* KCTC 1637** | 256 | 64 | 256 | 128 | 1,024 |
| ***P. aeruginosa* isolate 366** | 256 | 128 | 512 | 128 | 1,024 |
| ***P. aeruginosa* isolate 4068** | 256 | 64 | 256 | 128 | 1,024 |
| ***P. aeruginosa* isolate 4135** | 512 | 128 | 512 | 128 | 1,024 |
| ***P. aeruginosa* isolate 4561** | 512 | 128 | 512 | 512 | 1,024 |

· EtOH, ethanol extracts; Hexane, *n*-hexane-soluble fraction; $CHCl_3$, dichloromethane; EtoAc, ethyl acetate-soluble fraction; $H_2O$ water-soluble fraction.

**Table 4. Minimum bactericidal concentration of *Ishige okamurae* fractions against cutaneous pathogens.**

| Strains | MBC (μg/mL) | | | | |
|---|---|---|---|---|---|
| | **EtOH** | **Hexane** | **$CHCl_3$** | **EtoAc** | **$H_2O$** |
| ***Staphylococcus aureus* KCTC 1927** | 2,048 | 1,024 | 2,048 | 512 | >2,048 |
| ***Staphylococcus epidermidis* ATCC 14990** | 2,048 | 2,048 | 2,048 | 512 | >2,048 |
| ***Propionibacterium acnes* KCTC 3314** | 2,048 | 1,024 | >2,048 | 2,048 | >2,048 |
| ***Candida albicans* KCTC 7965** | 2,048 | 512 | 2,048 | 2,048 | >2,048 |
| ***Pseudomonas aeruginosa* KCTC 1637** | 1,024 | 256 | 2,048 | 512 | >2,048 |
| ***P. aeruginosa* isolate 366** | 1,024 | 256 | 2,048 | 1,024 | >2,048 |
| ***P. aeruginosa* isolate 4068** | 1,024 | 256 | 1,024 | 1,024 | >2,048 |
| ***P. aeruginosa* isolate 4135** | 2,048 | 512 | 2,048 | 1,024 | >2,048 |
| ***P. aeruginosa* isolate 4561** | 2,048 | 512 | 2,048 | 2,048 | >2,048 |

· EtOH, ethanol extracts; Hexane, *n*-hexane-soluble fraction; $CHCl_3$, dichloromethane; EtoAc, ethyl acetate-soluble fraction; $H_2O$ water-soluble fraction.

## 3.2. Antibiotics resistance of cutaneous pathogens

Antibiotics such as amikacin, ceftazidime, ciprofloxacin and meropenem have been extensively used to treat diseases caused mainly by cutaneous pathogens (Kang et al., 2011). However, continued use of these antibiotics caused antibiotic-resistance and the emergence of multidrug resistant bacteria (Unemo and Nicholas, 2012). In this study, the antibiotic susceptibility was assessed by the MIC assay. The antibiotic resistant profile was determined based on the analysis MIC breakpoint (EUCAST).

Among the stains tested in this study, MICs of all strains against amikacin were in the range of the acceptable MIC breakpoint values ranging from 4 to 8 μg/mL indicating susceptibility to the test agent (Table 6). In addition, the MIC of *S. aureus*, *S. epidermidis* and *P. acnes* were showed susceptibility against all four antibiotics used in this study. However, *P. aeruginosa* strains exhibited resistant against ceftazidime, ciprofloxacin and meropenem. The MIC of *P. aeruginosa* strains against ceftazidime is 8 μg/mL, which was over range of MIC breakpoint range 1 to 4 μg/mL. The MIC of *P. aeruginosa* strains against ciprofloxacin and meropenem is 16 μg/mL, which was over range of MIC breakpoint range 2 to 8 μg/mL.

These antibiotic resistant profiles against *P. aeruginosa* are almost similar with the previous results (Bonomo and Szabo, 2006). To solve this problems, antibiotic combination therapy has been studied and clinically used (Lister et al., 2006).

**Table 5. Minimum inhibitory concentrations of antibiotics against cutaneous pathogens**

| Strains | MIC (μg/mL) | | | |
|---|---|---|---|---|
| | Amikacin | Ceftazidime | Ciprofloxacin | Meropenem |
| ***Staphylococcus aureus*** **KCTC 1927** | 1 | 2 | 0.5 | 1 |
| ***Staphylococcus epidermidis*** **ACTC 14990** | 1 | 4 | 4 | 1 |
| ***Propionibacterium acnes*** **KCTC 3314** | 4 | 0.5 | 4 | 4 |
| ***Pseudomonas aeruginosa*** **KCTC1637** | 1 | 8 | 8 | 16 |
| ***Pseudomonas aeruginosa*** **isolated 366** | 2 | 4 | 4 | 8 |
| ***Pseudomonas aeruginosa*** **isolated 4068** | 1 | 16 | 16 | 16 |
| ***Pseudomonas aeruginosa*** **isolated 4135** | 1 | 8 | 16 | 8 |
| ***Pseudomonas aeruginosa*** **isolated 4561** | 2 | 16 | 16 | 16 |
| **MIC Breakpoint** | 4-8 [a)] | 1-4 [a)] | 2-8 [a)] | 8 [a)] |

[a)] Reference : EUCAST : European committee on antimicrobial susceptibility testing.

## 3.3. Synergistic effect between Hexane fraction of *I. okamurae* and antibiotics against cutaneous pathogens

Since many antibiotic resistance and multidrug resistant bacteria have been reported, not only new antibiotics or therapies but also developing new drug or alternative therapies in combination with antibacterial materials derived from natural product are required (Nascimento et al., 2000; Eom et al., 2016). Based on previous results, the Hexane fraction of *I. okamurae* was presented the highest antibacterial activity against *P. aeruginosa* among the fractions, hence, Hexane fraction was chosen for further studies. The synergistic interaction of Hexane fraction of *I. okamurae* and antibiotics was tested against *P. aeruginosa* strains by the checkerboard method using FIC assay as stated in materials and methods (Hsieh et al., 1993; Meletiadis et al., 2010).

As shown in Table 7, the MICs of ceftazidime, ciprofloxacin and meropenem against *P. aeruginosa* strains ranged from 4 to 16 μg/mL. However, the MICs of against *P. aeruginosa* strains were dramatically decreased in combination with Hexane fraction of *I. okamurae*. The MICs of ceftazidime against *P. aeruginosa* KCTC 1637 and isolate 4135 strains were reduced, up to 2 μg/mL when applied in combination with Hexane fraction. In the same way, *P. aeruginosa* isolate 4068 and 4561 strains were reduced up to 4 μg/mL. The MICs decreased two fold in the combination of Hexane fraction. In addition, the MICs of ciprofloxacin against *P.*

*aeruginosa* strains were reduced two to three fold in combination with Hexane fraction and the median ∑FIC indices were ranged from 0.50 to 0.63. The MICs of meropenem were ranged from 0.63 to 0.75 indicate that weak synergistic antimicrobial effect in combination with Hexane fraction. The MICs of meropenem decreased two fold in combination with Hexane fraction. As a result, the Hexane fraction of *I. okamurae* with antibiotics resulted in a weak synergistic effect against *P. aeruginosa* strains. Among Hexane-soluble compound, it is believed to contain a substance indicated synergistic antimicrobial effect with antibiotics. For more effective result, experiments are underway to isolate single compound from the Hexane fraction of *I. okamurae*. It has been previously reported that pure compound isolated from Hexane extract of seaweed (*Halimeda gracilis*) that exhibit strong antibacterial effect (Hendri et al., 2017). The results of the study, it is expected to have alternative therapeutic ingredient against antibiotic-resistant *P. aeruginosa*.

**Table 6. Fractional Inhibitory concentration of antibiotics in combination with Hexane fraction of *Ishige okamurae* against cutaneous pathogens**

| Strains | Test compound | MIC (μg/mL) | $\sum FIC_{max}$ [a)] | $\sum FIC_{min}$ [b)] | Median $\sum FIC$ [c)] | Minimum concentration for observing synergy |
|---|---|---|---|---|---|---|
| *P. aeruginosa* KCTC 1637 | Hexane | 64 | 1.06 | 0.31 | 0.57 | 8 |
| | Ceftazidime | 8 | | | | 2 |
| | Hexane | 64 | 1.06 | 0.52 | 0.63 | 8 |
| | Meropenem | 16 | | | | 4 |
| *P. aeruginosa* isolate 4068 | Hexane | 64 | 1.00 | 0.31 | 0.50 | 4 |
| | Ceftazidime | 16 | | | | 4 |
| | Hexane | 64 | 1.06 | 0.38 | 0.57 | 8 |
| | Ciprofloxacin | 16 | | | | 4 |
| | Hexane | 64 | 1.50 | 0.52 | 0.75 | 16 |
| | Meropenem | 16 | | | | 4 |

[a)] $\sum FIC_{max}$, maximum FIC; [b)] $\sum FIC_{min}$, minimum FIC; [c)] $\sum FIC_{med}$, medium FIC

The FIC index indicated synergistic effect: <0.5, marked synergy; 0.5 to <1.0, weak synergy; 1.0, additive; >1.0 to <2.0, subadditivie; 2.0, indifferent; >2.0, antagonistic.

**Table 6. Continued**

| Strains | Test compound | MIC (μg/mL) | $\sum FIC_{max}$ [a)] | $\sum FIC_{min}$ [b)] | Median $\sum FIC$ [c)] | Minimum concentration for observing synergy |
|---|---|---|---|---|---|---|
| ***P. aeruginosa*** **isolate 4135** | **Hexane** | **128** | **1.13** | **0.38** | **0.63** | **16** |
| | **Ceftazidime** | **8** | | | | **2** |
| | **Hexane** | **128** | **1.25** | **0.31** | **0.63** | **16** |
| | **Ciprofloxacin** | **16** | | | | **4** |
| ***P. aeruginosa*** **isolate 4561** | **Hexane** | **128** | **1.00** | **0.38** | **0.52** | **8** |
| | **Ceftazidime** | **16** | | | | **4** |
| | **Hexane** | **128** | **1.06** | **0.38** | **0.50** | **8** |
| | **Ciprofloxacin** | **16** | | | | **2** |
| | **Hexane** | **128** | **1.13** | **0.50** | **0.75** | **32** |
| | **Meropenem** | **16** | | | | **8** |

[a)] $\sum FIC_{max}$, maximum FIC; [b)] $\sum FIC_{min}$, minimum FIC; [c)] $\sum FIC_{med}$, medium FIC

The FIC index indicated synergistic effect: <0.5, marked synergy; 0.5 to <1.0, weak synergy; 1.0, additive; >1.0 to <2.0, subadditivie; 2.0, indifferent; >2.0, antagonistic.

## 3.4. Isolation and effect of diphlorethohydroxycarmalol from *I. okamurae*

Several studies suggest that marine blown algae has various biologically active components specifically, phenols and polyphenols as secondary metabolites and these are responsible for their biological activities (Okada et al., 2004; Kim et al., 2006; Holdt and Karaan, 2011). The previous reports on *I. okamurae* have revealed that it contains abundant to phlorotannin derivaties with various bioactivites, such as plasmin inhibitory and antiallergic activity (Nakayama et al., 1989; Lim et al., 2002), antioxidant (Kim and Kim, 2013), anti-inflammatory (Kim et al, 2009). However, there have been no reports reagrding the antimicrobial activities of isolated phlorotannins from *I. okamurae* against cutaneous pathogens. Hence, this study attempted to isolate phlorotannins from *I. okamurae* and evaluated its antimicrobial effects against human skin pathogens. For this purpose, the EtoAc fraction of *I. okamurae* was subjeted to further purification inorder to isolate the active compounds (Fig.4).

The single compound was extracted from EtoAc fraction was using Sephadex LH-20 column chromatography. The compound were applied to reverse-phase high performance liquid chromatography (Fig.5). The isolated compound molecular weight were identified through quadrupole time-of-flight mass spectrometry (Q-TOF

MS) (Fig.6). The active compound of EtoAc fraction was identified as diphlorethohydroxycarmalol (DPHC) based on comparison data and previous stduy (Li and Glombitza, 1991). DPHC, a kind of phlorotannin, have been reported on physiologically active including antioxidant properties and anti-obesity effect (Park et al., 2013). Nonetheless, no studies on the antibacterial efffect of the phlorotannin from *I. okamurae* have been reported.

Hence, MIC and MBC assay was conducted to investigate the antimicrobial activity of the DPHC of *I. okamurae* EtoAc fraction against the cutaneous pathogens (Table 5). The MIC values of DPHC from *I. okamurae* were in the range of 64 to 512 μg/mL against cutaneous pathogens tested in this study. It has been previously reported that the purified phlorotannins from EtoAc fraction of *Eisenia bicyclis* demonstrated antimicrobial activity with MIC value ranging from 32 to 256 μg/mL against acne-related bacteria (Lee et al., 2014). Compared with these, it was clear that DPHC showed strong antimicrobial activity with MIC value ranging from 64 to 256 μg/mL against cutaneous pathogens.

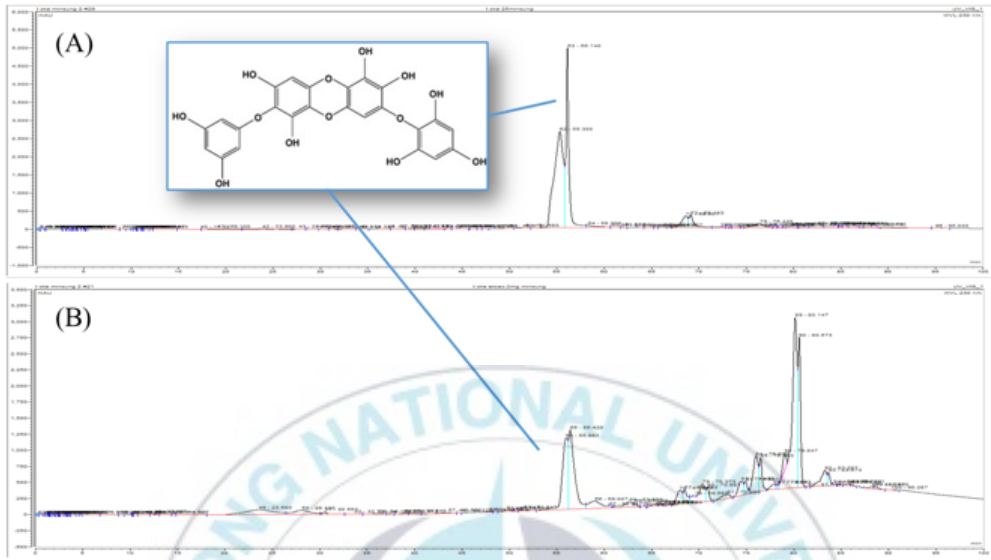
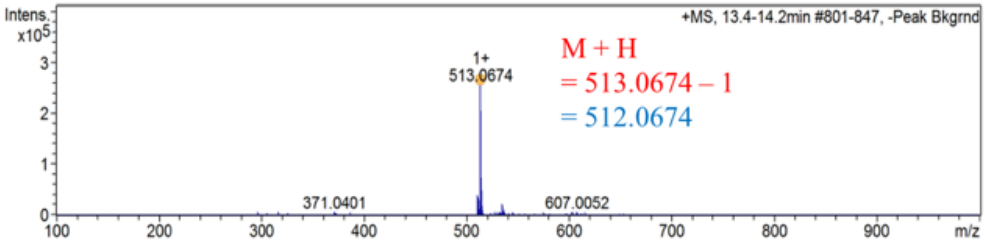



**Fig. 2. High performance chromatography raw data: (A) DPHC; Diphlorethohydroxycarmalol (B) *Ishige okamurae* EtoAc fraction.**




| Meas. m/z | # | Ion Formula | m/z | err [ppm] | mSigma | # mSigma | Score | rdb | e⁻ Conf | N-Rule |
|---|---|---|---|---|---|---|---|---|---|---|
| 513.0674 | 1 | C24H17O13 | 513.0664 | -2 | 7.9 | 1 | 85.62 | 16.5 | even | ok |
| | 2 | C25H13N4O9 | 513.0677 | 0.6 | 19 | 2 | 100 | 21.5 | even | ok |
| | 3 | C22H5N14O3 | 513.0664 | -2 | 22.5 | 3 | 47.6 | 27.5 | even | ok |

➢Diphlorethohydroxycarmalol (DPHC)

$= C_{24}H_{16}O_{13}$

= MW 512.38 g/mol

**Fig. 3. Identification of DPHC; Diphlorethohydroxycarmalol molecular weight through quadrupole time-of-flight mass spectrometry (Q-TOF MS)**

**Table 7. MIC and MBC determination for diphlorethohydroxycarmalol from *Ishige okamurae* against cutaneous pathogens.**

| Strains | MIC (μg/mL) | MBC (μg/mL) |
|---|---|---|
| ***Staphylococcus aureus* KCTC 1927** | 128 | 512 |
| ***Staphylococcus epidermidis* ATCC 14990** | 64 | 256 |
| ***Propionibacterium acnes* KCTC 3314** | 256 | 1,024 |
| ***Candida albicans* KCTC 7965** | 128 | 512 |
| ***Pseudomonas aeruginosa* KCTC 1637** | 64 | 256 |
| ***P. aeruginosa* isolate 366** | 64 | 256 |
| ***P. aeruginosa* isolate 4068** | 64 | 256 |
| ***P. aeruginosa* isolate 4135** | 128 | 512 |
| ***P. aeruginosa* isolate 4561** | 128 | 512 |

## 3.5. Synergistic effect between diphlorethohydroxycarmalol and antibiotics against cutaneous pathogens

Since many antibiotic resistance and multidrug resistant bacteria have been reported, not only new antibiotics or therapies but also developing new drug or alternative therapies in combination with antibacterial materials derived from natural product are required (Nascimento et al., 2000; Eom et al., 2016). Based on previous results, a combination of DPHC and antibiotics against *P. aeruginosa* strains was tested by the checkerboard method using FIC assay as stated in Materials and Methods (Hsieh et al., 1993; Meletiadis et al., 2010). Comparing the combination with DPHC and antibiotics, the combination with Hexane fraction and antibiotics was assessed using same methods.

As shown in Table 7, the MICs of ceftazidime against *P. aeruginosa* strains was reduced from 4 to 1 μg/mL when administered combination with DPHC. In all test strains, the median ∑FIC indices were from 0.50 to 0.63 indicate that weak synergistic antimicrobial effect between ceftazidime and DPHC from *I. okamurae*. The MICs of ciprofloxacin also indicated weak synergistic antimicrobial effect when administered combination with DPHC. The MICs of ciprofloxacin were reduced from 8 to 4 μg/mL and the median ∑FIC indices were ranged from 0.52 to 0.57. Both MICs of ceftazidime and MICs of ciprofloxacin were decreased two to three fold in the combination with DPHC from *I. okamurae*. However, the synergy effect between

meropenem and DPHC was relatively weaker that median ∑FIC indices from 0.63 to 1.03 against *P. aeruginosa* strains comparing with ceftazidime and ciprofloxacin. The MICs of meropenem against *P. aeruginosa* KCTC 1637, isolate 4065 and 4561 strains were reduced, up to 8 μg/mL when in combination with DPHC.

It has been reported that marine substances including *E. bicyclis* and *S. serratifolium* exhibited synergy effects against *P. acnes* in combination with antibiotics (Eom et al., 2011; Kim et al., 2016). However, synergy effects of marine natural products with antibiotics against *P. aeruginosa* have not been reported. Comparing the previous studies that antibiotic combination synergy effect such as β-lactam and aminoglycoside was exhibited partial synergy effect (Pai, 2010), *I. okamurae* has a potential to restore antimicrobial activity of old antibiotics against *P. aeruginosa*. It was anticipated *that I. okamurae* would be a good candidate in the treatment of antibiotic-resistant *P. aeruginosa* infection.

**Table 8. Fractional Inhibitory concentration of antibiotics in combination with DPHC against cutaneous pathogens**

| Strains | Test compound | MIC (μg/mL) | $\sum FIC_{max}$ [a)] | $\sum FIC_{min}$ [b)] | Median $\sum FIC$ [c)] | Minimum concentration for observing synergy |
|---|---|---|---|---|---|---|
| *P. aeruginosa* KCTC 1637 | DPHC* | 64 | 1.03 | 0.50 | 0.63 | 2 |
| | Ceftazidime | 8 | | | | 2 |
| | DPHC | 64 | 1.50 | 1.00 | 1.03 | 8 |
| | Meropenem | 16 | | | | 8 |
| *P. aeruginosa* isolate 4068 | DPHC | 64 | 1.06 | 0.38 | 0.50 | 16 |
| | Ceftazidime | 16 | | | | 4 |
| | DPHC | 64 | 1.13 | 0.50 | 0.57 | 16 |
| | Ciprofloxacin | 16 | | | | 8 |
| | DPHC | 64 | 1.25 | 0.52 | 1.00 | 16 |
| | Meropenem | 16 | | | | 8 |

DPHC* Diphlorethohydroxycarmalol,

[a)] $\sum FIC_{max}$, maximum FIC; [b)] $\sum FIC_{min}$, minimum FIC; [c)] $\sum FIC_{med}$, medium FIC

The FIC index indicated synergistic effect: <0.5, marked synergy; 0.5 to <1.0, weak synergy; 1.0, additive; >1.0 to <2.0, subadditivie; 2.0, indifferent; >2.0, antagonistic.

**Table 8. Continued**

| Strains | Test compound | MIC (μg/mL) | $\sum FIC_{max}$ [a)] | $\sum FIC_{min}$ [b)] | Median $\sum FIC$ [c)] | Minimum concentration for observing synergy |
|---|---|---|---|---|---|---|
| *P. aeruginosa* isolate 4135 | DPHC | 128 | 1.13 | 0.38 | 0.50 | 16 |
| | Ceftazidime | 8 | | | | 1 |
| | DPHC | 128 | 1.06 | 0.25 | 0.52 | 16 |
| | Ciprofloxacin | 16 | | | | 4 |
| *P. aeruginosa* isolate 4561 | DPHC | 128 | 1.00 | 0.52 | 0.63 | 4 |
| | Ceftazidime | 16 | | | | 2 |
| | DPHC | 128 | 1.06 | 0.50 | 0.52 | 4 |
| | Ciprofloxacin | 16 | | | | 4 |
| | DPHC | 128 | 1.50 | 0.52 | 0.63 | 32 |
| | Meropenem | 16 | | | | 8 |

DPHC* Diphlorethohydroxycarmalol,

[a)] $\sum FIC_{max}$, maximum FIC; [b)] $\sum FIC_{min}$, minimum FIC; [c)] $\sum FIC_{med}$, medium FIC

The FIC index indicated synergistic effect: <0.5, marked synergy; 0.5 to <1.0, weak synergy; 1.0, additive; >1.0 to <2.0, subadditivie; 2.0, indifferent; >2.0, antagonistic.

## 3.6. Inhibitory effect of *I. okamurae* extracts on LPS-induced NO production in HaCaT cells

Nitric oxide (NO) is an important intracellular and intercellular signaling molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in cardiovascular, nervous and immunological system (Aktan, 2004). NO, an inflammatory marker, is synthesized from arginine by nitric oxide synthase (NOS) that increased by the inducible nitric oxide synthase (iNOS), subsequently resulting in cytotoxicity and tissue damage (Kim et al., 1998; Kinaci et al., 2012). The expression of iNOS is induced by lipopolysaccharide (LPS) and an excess of NO is related to LPS-induced tissue damages (MacMicking et al., 1997). As an indicator of LPS-induced NO production, nitrite accumulation in the cultured media was determined by the Griess method (Sun et al., 2003). Here, it was assessed an inhibitory effect of *I. okamurae* against LPS-induced inflammation in HaCaT cells. As shown in Fig.6, *I. okamurae* extracts showed an ability to reduce the NO production (about 20-30%) compared to control. NO production was monitored in HaCaT cells in the presence of various concentrations ranging 50 to 200 μg/mL of the *I. okamurae* extracts. The *I. okamurae* extracts inhibited NO production in a concentration-dependent manner. The 200 μg/mL of *I. okamurae* extracts showed 10% more effective than 50 μg/mL. This result indicates that the *I. okamurae* extracts possessed anti-inflammatory effect in HaCaT cells. These anti-inflammatory profiles

of *I. okamurae* are almost similar with the previous studies (Kim et al., 2008). Hence, these findings suggest that the inhibition of NO production by extracts might be due to the suppression of NO-induced iNOS transcription. Furthermore, inflammation not only plays a role in the inflammatory diseases, but also in the progression of cancer (Balkwill et al., 2005). Hence it is speculated that *I. okamurae* extracts with anti-inflammatory activity may effectively prevent the development of inflammatory bowel disease and colorectal cancers.

Describing in more detail, the NO production was reduced into about 20% at the concentration in 200 μg/mL of *I. okamurae* extracts exhibiting no cell cytotoxicity compared to control in HaCaT cells. In conclusion, these results demonstrate that *I. okamurae* extracts suppress NO production in LPS-stimulated human keratinocytes without side effect. Further studies are required to search mechanism and cytokines of *I. okamurae* in LPS-stimulated human keratinocytes.

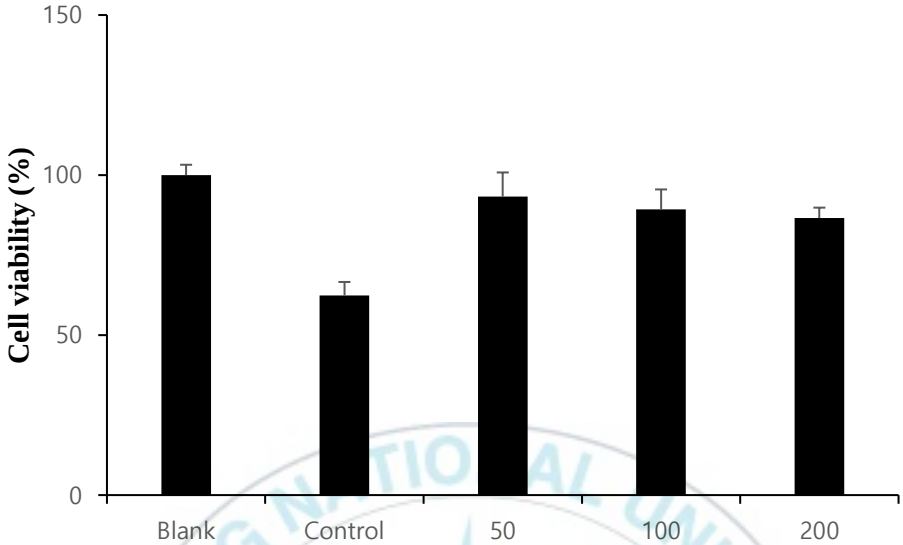



**Fig. 4. Effect of *Ishige okamurae* extracts (μg/mL) on cell viability in LPS-induced HaCaT cells.**

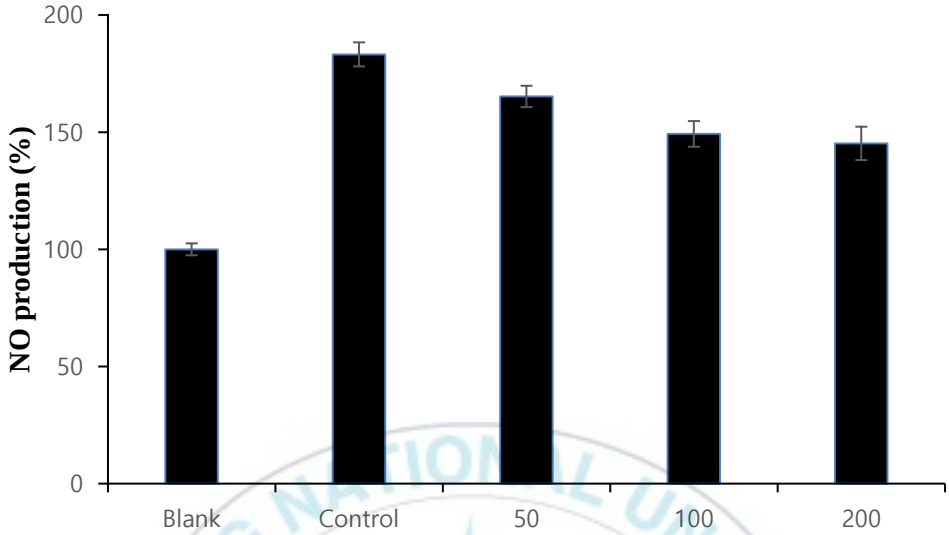



**Fig. 5. Effect of *Ishige okamurae* extracts (μg/mL) on nitric oxide (NO) production in LPS-induced HaCaT cells.**

# Conclusion

Opportunistic pathogens such as *P. acnes, S. epidermidis, S. aureus* and *P. aeruginosa* and *C. albicans* are often associated with pathogenic infections. These pathogens can cause various diseases from mild pain to severe morbidity due to the infection in skin tissue. The currently available therapeutic option for these skin pathogens is an antibiotic treatment. However, irrational use of such antibiotics resulted in drug-resistant pathogens, organ damage and immune hypersensitivity. The objective of this study was to discover an alternative antimicrobial agent with lower side effect from marine algae. It has been reported that *I. okamurae* exhibits various biological activities due to the presence of various pharmalogically active substances. However, there are no reports on the antimicrobial activity of *I. okamurae* against cutaneous pathogens. In this study, the ethanolic extract of *I. okamurae* was fractionated into several solvent-soluble fractions and the antimicrobial activity of *I. okamurae* fractions were determined by MIC and MBC assays. Among them, the EtoAc and Hexane-soluble fractions exhibited the strong antimicrobial activity against the pathogens of human skin with MIC values ranging 128 μg/mL to 1,024 μg/mL. To investigate the more effective antimicrobial effect of *I. okamurae*, this study attempted to isolate diphlorethohydroxycarmalol (DPHC) from *I. okamurae* EtOAc fraction.

The antibiotic susceptibility test revealed that cutaneous pathogens exhibited antibiotic resistant against commercial antibiotics. Therefore, the synergistic interaction of DPHC with commercial antibiotics against cutaneous pathogens was determined by checkerboard assay method based on FIC assay. In addition, the combination with Hexane fractions of *I. okamuare* and antibiotics was tested using same method. DPHC and the Hexane fraction were exhibited partial synergistic effect on antibiotics such as ceftazidime, ciprofloxacin and meropenem. Thus, *I. okamurae* can be a potential source of natural product to be used as an effective therapy against skin pathogens. In order to investigate an anti-inflammatory effect of *I. okamurae* extracts, NO, an inflammatory marker was induced in HaCaT cells by LPS (lipopolysaccharide). The 200 μg/mL of *I. okamurae* extracts showed an ability to reduce the NO production by 20% compared to control. The extracts of *I. okamurae* suppressed the NO production in a concentration-dependent manner.

Taken together, the results of the present study suggested that the *I. okamurae* extracts and its isolated compound showed promising antimicrobial activity against antibiotic resistant human skin pathogens with the ability to reduce the inflammation induced by LPS in human keratinocyte cells. Due to less cytotoxicity and potential antimicrobial activity, *I. okamurae* based antimicrobial therapy may possibly control the development of skin borne pathogens and its infections.

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