Thesis for the Degree of Master of Engineering

Antimicrobial hydrogels based on polyvinyl alcohol and diphlorethohydroxycarmalol (DPHC) derived from brown alga *Ishige okamurae*

: An *in vitro* and *in vivo* study of wound healing and tissue regeneration

By

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Interdisciplinary Program of Biomedical Mechanical & Electrical Engineering

College of Engineering

Pukyong National University

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항균효능을 갖는 창상피복제 제작을 위한 PVA/Diphlorethohydroxycarmalol (DPHC) 하이드로겔의 *in vitro* 및 *in vivo* 연구

Advisor: Prof. Won-Kyo Jung

by

Min-Sung Kim

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List of abbreviations

DPHC	Diphlorethohydroxycarmalol
PVA	Poly (vinyl alcohol)
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
CFU	Colony forming units
NHDF-neo	Normal human dermal fibroblast-neonatal
HaCaT	Human keratinocyte
HPLC	High performance liquid chromatography
UPLC-Q-Tof MS	Ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide
ISO	International Organization for Standardization
G’	Elastic modulus
G”	Loss modulus
PCL	Polycaprolactone
ACE-1	Angiotensin-I-converting enzyme
DMEM	Dulbecco's minimum Eagle's medium

EDTA	Ethylenediaminetetraacetic acid
CHCl3	Chloroform
EtOAc	Ethyl acetate
CPC	Centrifugal partition chromatography
PPVA	5% PVA hydrogel (PPVA)
PVA/DPHC-I	5 wt% PVA hydrogel containing 0.0125 wt% DPHC (PVA/DPHC-I)
PVA/DPHC-II	5 wt% PVA hydrogel containing 0.025 wt% DPHC (PVA/DPHC-II)
PVA/DPHC-III	5 wt% PVA hydrogel containing 0.05 wt% DPHC (PVA/DPHC-III)
PVA/DPHC-IV	5 wt% PVA hydrogel containing 0.1 wt% DPHC (PVA/DPHC-IV)
SEM	Scanning electron microscopy
Wd	Dry weight
Ws	Swollen weight
TGA	Thermogravimetric analysis
FDA	Fluorescein diacetate
CFU	Colony forming units

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Abstract

상처 부위는 박테리아로부터 감염이 되어 질병이나 합병증이 유발되며 상처치유과정에 부정적인 영향을 미친다. 따라서 항균력을 지닌 창상피복제의 활용이 중요한 화제가 되고 있다. 항균효능을 갖는 해양 기능성 소재를 창상피복제에 적용하기 위하여, 해양 갈조류인 *Ishige okamurae* 로부터 Diphlorethohydroxycarmalol (DPHC)을 HPLC와 HPCPC를 이용하여 분리하고 Q-TOF-MS 로 질량을 확인하였다. DPHC 의 항균효능을 알아보기 위해 *S. epidermidis, C. albicans, S.aureus,* 및 *P. aeruginosa* 에 대해 최소 억제 농도(MIC)와 최소 살균 농도 (MBC)를 조사하였다. MIC 는 약 128 μg / mL 이며 MBC 는 약 512 μg / mL 의 결과가 도출되었다. DPHC 는 피부 섬유아세포 (NHDF-neo) 및 각질 형성 세포 (HaCaT)에서 MTT 분석에 의해 세포독성을 나타내지 않았다.

하이드로겔은 화학적, 기계적 및 물리적 특성의 정밀한 제어뿐만 아니라 쉽게 조작할 수 있는 유연성과 같은 많은 이점을 지닌다. 폴리비닐알콜 (PVA)은 친수성,

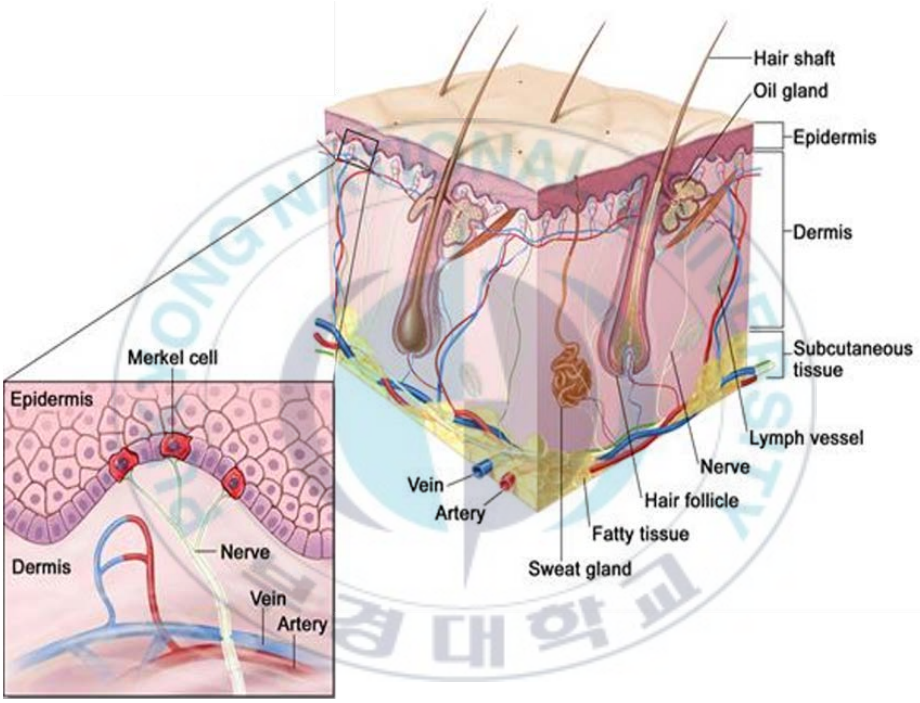
생체 적합성 및 생분해성과 같은 특성으로 인해 약물 전달 시스템 및 다양한 산업 분야에 널리 응용되는 합성 고분자이다. 제작된 PVA/DPHC 하이드로겔의 특성을 조사하기 위해 SEM 이미지, 수분 흡수 팽윤 시험, 약물 방출 시험 및 유변학적 특성을 분석하였다. ASTM E2149 에 의해 그람 양성세균 (*S. aureus*) 및 그람 음성균 (*P. aeruginosa*)에 대하여 PVA/DPHC 하이드로겔의 항균활성을 시험하였다. PVA/DPHC 하이드로겔의 독성시험은 간접 접촉 테스트, 직접 접촉 테스트 및 ICR 생쥐에 대한 독성시험으로 평가되었다. PVA/DPHC 하이드로겔은 *S. aureus* 및 *P. aeruginosa* 에 대해 약 99 %의 생존율을 감소시키는 약물방출 사멸능력을 가졌으며, MTT 분석과 FDA 형광실험에 의해 NHDF-neo 및 HaCaT 에서 어떠한 독성 효과도 나타내지 않았다. 또한, PVA/DPHC 하이드로겔은 ICR 마우스의 비처리군보다 높은 상처 치유 효과를 나타냈다. 이 연구는 PVA/DPHC 하이드로겔이 상처치유에 도움을 주는 창상피복제의 적용 가능성을 제시하고 있다

1. Introduction

1.1. Wound healing

Skin consisted of complex layers such as epidermis, dermis and subcutaneous tissue is the most important barrier protecting the body from external factors to prevent toxins and infection. Skin is the largest organ in the surface area of body and thick average thickness of skin is about 2-3 mm [1]. Epidermis is consisted of outer layer composed to dead cells and the other layers consisted of viable cells such as keratinocytes which form 95% of all epidermal cells, melanocytes which produce the pigment melanin, and Langerhans's cells which are an immune system [2-5]. The secondary layer of skin is called dermis composed of elastin fibers, ground substance and collagen fiber between the epidermis and subcutaneous tissues [2].

Wound is a shape of injury being damaged from dangerous part such as sharp part and rough surface. The wound is defined as damage or loss of the anatomical structure and function of tissue [6]. Wound healing requires interactions between the cells, the extracellular matrix, and the growth factor [7]. The healing adult skin is a complex process containing inflammatory stage, epithelializing stage, proliferate stage and remodeling stage [8]. It is said the length of time that the wound heals is very long. So these days, a number of researchers agonize methods helping wound healing process. As advanced researches, one of methods for wound healing is protecting injured parts from harmful bacteria. Wound sites can be infected with harmful bacteria easily, as well as the main reason protecting bacteria is that they can cause disease or complications [9].



[Figure adapted from the site of Terese Winslow LLC, Medical and Scientific Illustration]

Fig. 1. Anatomy of the skin

1.2. Bacteria

Wound are susceptible to microbial contamination from exogenous and endogenous sources ,and can provide a good environment for the microbial growth and colonization [10, 11]. After microbial colonization in wounds, microorganism can delay and fail to the wound healing by producing various substances such toxins, proteases and pro-inflammatory molecules, and causing an excessive and prolonged inflammatory response [12]. Among harmful micro-organism, Bacteria can be classified the gram-positive and the gram-negative prokaryotic micro-organism [13]. Typically, *Staphylococcus aureus*, a gram-positive bacterium, is the most common pathogen in the human body but is investigated that it can lead immoderately to death by inducing serious diseases such as abscess, folliculitis, necrotizing fasciitis, myositis, osteoarthritis, infectious arthritis and vertebral osteomyelitis [14-18]. Also, *Staphylococcus epidermidis*, a gram-positive bacterium, is less virulent than *Staphylococcus aureus*, but it is a terrible pathogen that causes various inflammation and diseases [19]. *Candida albicans*, a gram-positive bacterium, is usually infected in the human skin, causing inflammation in the skin, mouth and esophagus as well as inducing various candidiasis [20]. *Pseudomonas aeruginosa*, a gram-negative bacterium, is most closely related to human infectious disease [21]. It is widely distributed in nature and is included in the normal bacteria of human intestinal tract, and may be found in skin and armpit which is wet especially in the body. It is opportunistic pathogen in body weaken the resistance and immune system through

disease such as wound, cancer, and so on [22]. Typically, it can cause bedsore, pneumonia, bacteremia, septicemia, and meningitis [23, 24]. So many researchers look into the possibility of aid to heal wounds by protecting from harmful bacteria

1.3. Hydrogel

There are various types of wound dressing applications, but they can be typically divided into three major types classified by passive products, interactive materials containing polymeric films, and bioactive wound dressing materials [25]. Before 1960s, passive products such as gauze for wound dressing was used to have a minimal role as a common cover in wound healing, but they are not suitable for acute and chronic wounds [26]. Bioactive wound dressing such as collagen and chitosan affect directly to wound healing process by delivery of bioactive compounds [27]. Finally, the interactive materials dressing provide a beneficial effect including maintaining of moisture environment and preventing of excessive heat loss on wound site [25]. Among the interactive materials dressing, hydrogel can help fast wound healing by keeping wet condition [28]. Generally, gels are very highly utilized materials found all around our lives. Also, our tissue and organs is composed various gels. Most gels are composed polymers or colloids. They are filled by fluid. Gels are that elastic modulus (G') is greater than loss modulus (G'') [29]. So, even though gels are soft, gels are solid characteristics. Hydrogel is simply gel to interact with water and not to dissolve easily due to crosslinking forms as network of chains[30]. Hydrogels offer many advantages,

such as precise control of chemical, mechanical, and physical properties as well as flexibility to design features that can be easily manipulated for intended applications [31]. Hence earlier mentioned advantages, hydrogels can be applied to biomedical fields containing medical device, drug delivery system, and so on. So, many researchers consistently study fabricating methods and development of hydrogels based many biocompatible resources containing synthetic resource such as polyvinyl alcohol (PVA) and polycaprolactone (PCL) as well as natural resource such as pectin, alginate, and chitosan [32].

1.4. Poly (vinyl alcohol) (PVA)

PVA is a linear synthetic polymer prepared by hydrolysis of poly vinyl acetate as a shown in fig. 2. (A) [33]. PVA is water-soluble and hydrophilic polymer having the numerous hydroxyl groups on the backbone [34]. Particularly, PVA can offer the possibility of attaching monomer such as cell signaling molecules and drugs [35]. Also, PVA has harmlessness and non-toxicity, low temperature crystallization ability, high tensile strength property and high elongation property [36]. Furthermore, PVA is easily physically cross-linked without requiring the use of toxic chemicals by repeated cycles of freezing and thawing [37]. PVA hydrogels have the inner network based crystallites that occur between the hydroxyl groups of PVA during the freezing-thawing cycles in fig. 2. (B), and have forms porous wall containing free water in the inner network [38]. Also, PVA hydrogel can be easily crosslinked and fabricated despite blending other

resource to improve biological ability. So, this advantage has developed constantly PVA hydrogels blended with bioactivity materials to enhance beneficial functions such as the cell adhesion, proliferation and mechanical property in recent studies [39, 40]

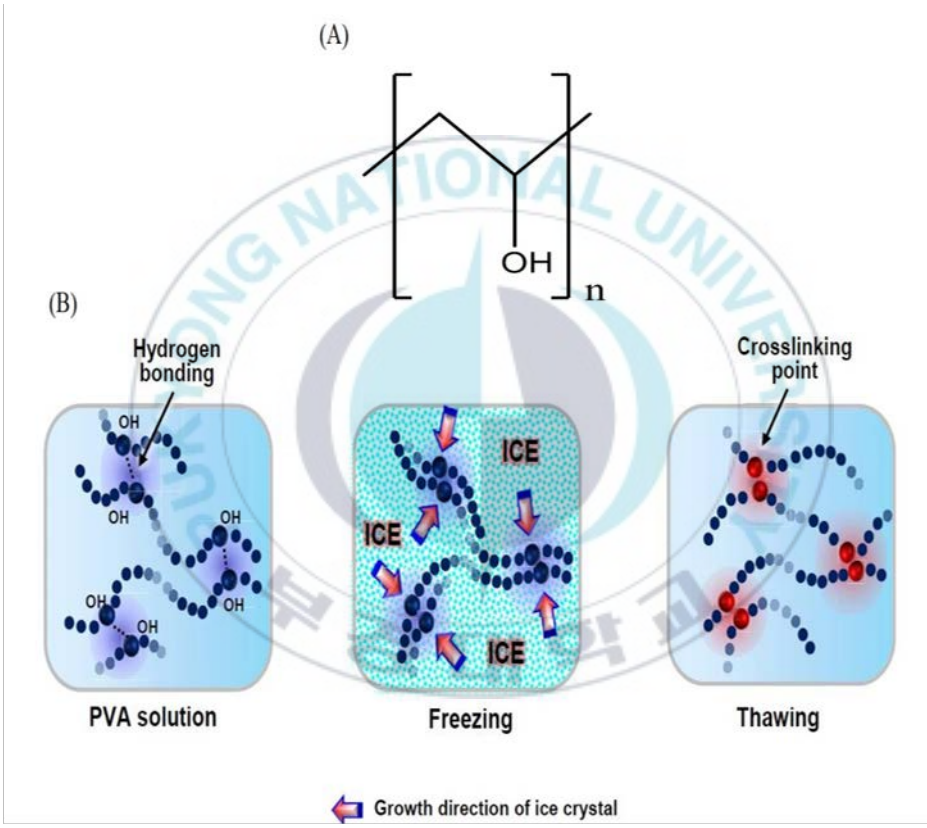


Fig. 2. Chemical structure of poly (vinyl alcohol) (PVA) (A) and diagram of PVA hydrogel formation by freezing thawing methods (B)

1.5. Diphlorethohydroxycarmalol

In this study, diphlorethohydroxycarmalol (DPHC) isolated from *Ishige okamurae*, a brown algae of marine, is investigated as a compound to load in PVA hydrogel in order to have antibacterial effect. First of all, marine have many merits. Marine takes possession about 70% of the Earth's surface [41]. However, there are many things that are yet to be revealed, so the value of the research is even higher. As advanced researches, materials from marine organism have high biocompatibility and bioactive component. Among materials from marine organism, Phlorotannins isolated a marine brown algae are well known as phenolic bioactive agents playing a vital role related to assist human health and nutrition such as anti-proliferative activity, antioxidant activity, anticancer activity, anti-HIV activity, angiotensin-I-converting enzyme (ACE-I) inhibition and so on [42]. Especially, brown algae among marine organism have many bioactive component and many researchers have being studied that. *I. okamurae* have effective functionalities such as anti-oxidant, cytoprotective, nitric oxide inhibitory, and anti-cancer effect [43, 44]. By these functionalities, there are many fields that use *I. okamurae*. Mainly, medicine, health functional food, cosmetics and medical application are corresponded. Also, *I. okamurae* is grown in warm coasts and easily found in Jeju island, Korea [45]. Due to easy harvest, it is commercially valuable. These valuable *I. okamurae* have DPHC as a main compound. The DPHC is consisted of four phloroglucinol units and functional hydroxyl groups and have antioxidant effect, anti-inflammatory effect and inhibitory effect on α-glucosidase and α-amylase [46].

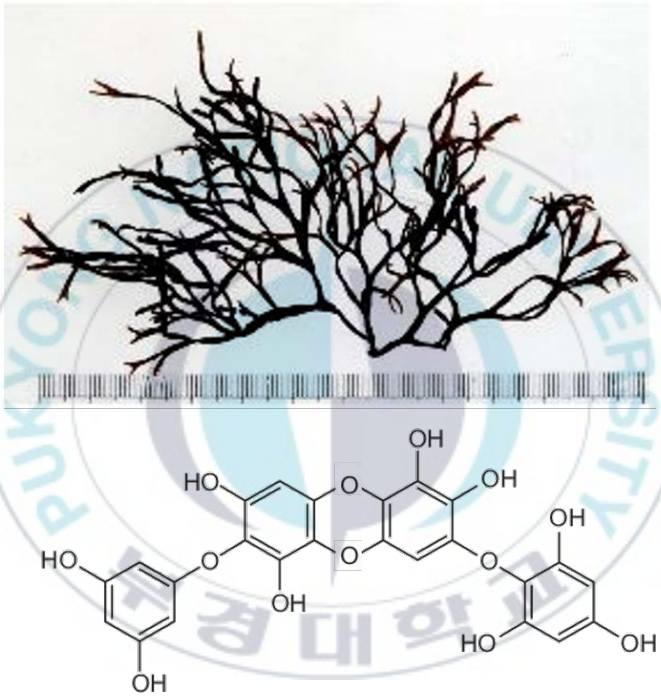


Fig. 3. ***Ishige okamurae*** **and chemical structure of diphlorethohydroxycarmalol (DPHC)**

1.6. Goal of study

This study would like to prove that diphlorethohydroxycarmalol (DPHC) isolated from *Ishige okamurae*, a brown algae of marine, has antibacterial effect by performing minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *S. epidermidis*, *C. albicans*, *S. aureus*, and *P. aeruginosa*. Also this study would like to prove that hydrogel made by blending DPHC and PVA having several properties (hydrophilic, biocompatibility and biodegradability) has anti-bacterial effect and wound healing ability by investigating in vitro experiment including physical characterizations, bacterial inhibition ability and cytotoxicity (in-direct and direct contact), and in vivo experiment including wound closure ability and histological test. Consequently, based on evaluation of characterization having PVA/DPHC hydrogels in vitro and in vivo, this study would like to suggest that PVA/DPHC hydrogels have possibility as wound healing application.

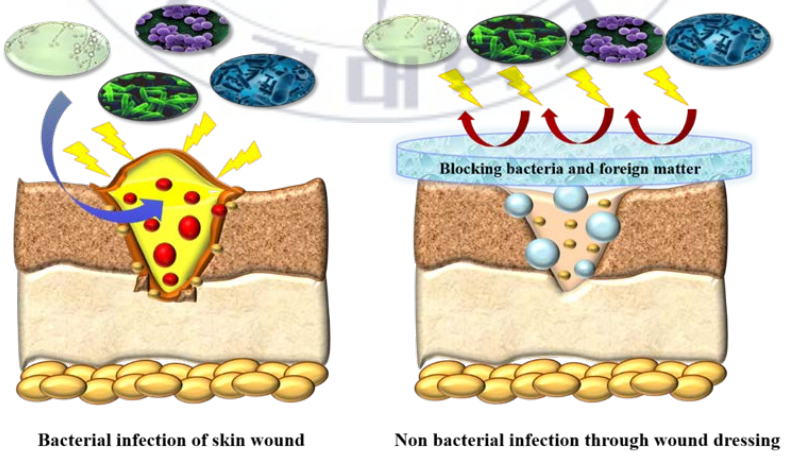


Fig. 4. Diagram of hydrogel having antibacterial effect for Wound dressing

2. Material and method

2.1. Materials

Ishige okamurae was collected from coasts in Jeju island, Korea. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) were purchased from Sigma-Aldrich, USA. Dulbecco's minimum Eagle's medium (DMEM), trypsin/ethylenediaminetetraacetic acid (EDTA), Fetal bovine serum (FBS) and other materials used in cell culture experiment were purchased from GIBCO™, Invitrogen Corporation, USA. Hoechst 33342 were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Fluorescein diacetate (FDA) were purchased from Sigma-Aldrich, USA. All other chemicals and solvents were of analytical grade, and water used in experiment was deionized.

2.2. Extraction, isolation and purification of DPHC

Diphlorethohydroxycarmalol (DPHC) was isolated by *Ishige okamurae* Yendo. Briefly, the dried *Ishige okamurae* was extracted with 70% ethanol and filtered using Whatman filter papers. The filtered extracts was evaporated at 40℃ to remove ethanol completely and freezed-dried under vacuum. The extracts were suspended in distilled water and partitioned with n-Hexane, chloroform (CHCl3), and ethyl acetate (EtOAc) by solvent fractionation methods. The EtOAc fractions, polyphenol-rich components, was separated frations by using two-phase solvent system of centrifugal partition chromatography (CPC) (System Instruments Co, CPC 240). The DPHC-rich fractions obtained from the EtOAc fractions were purified by high performance liquid chromatography (HPLC) using a Thermo Fisher Scientific HPLC system equipped with Ultimate 3000 UV detector (Variable Wavelength Detector, VWD-3400) and Atlantis ® dC18 (3 μm, 4.6×150 mm; Waters). by stepwise elution with acetonitrile–water gradient (UV range: 230 nm, flow rate: 0.2 ml/min). Finally, the molecular weight of the DPHC was identified by an ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-Tof MS) (Bruker, maXis-HD) based metabolomic technique.

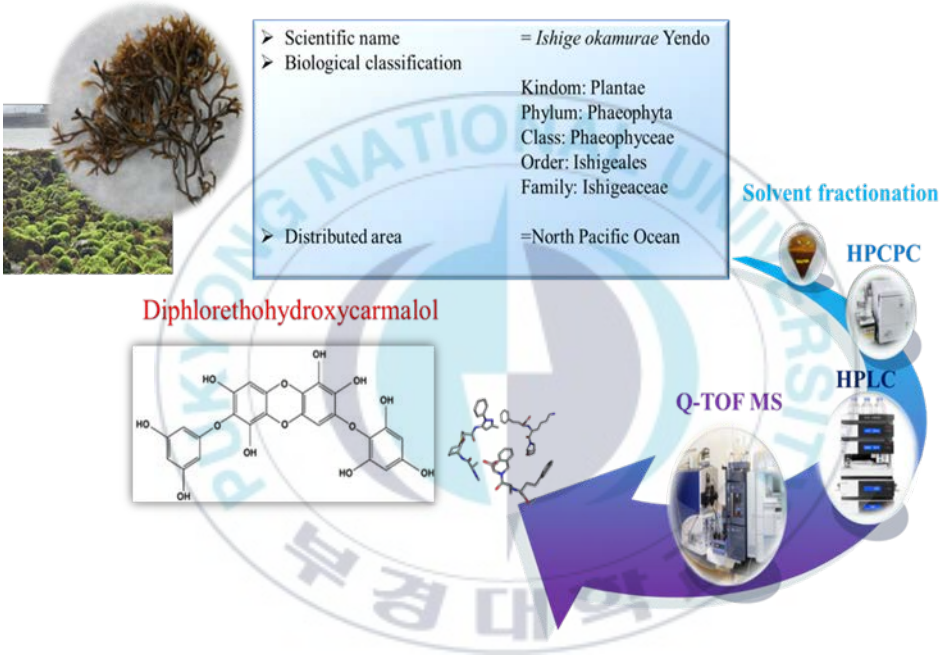


Fig. 5. Isolation and purification process of diphlorethohydroxycarmalol (DPHC) from Ishige okamurae

2.3. In vitro cell experiments

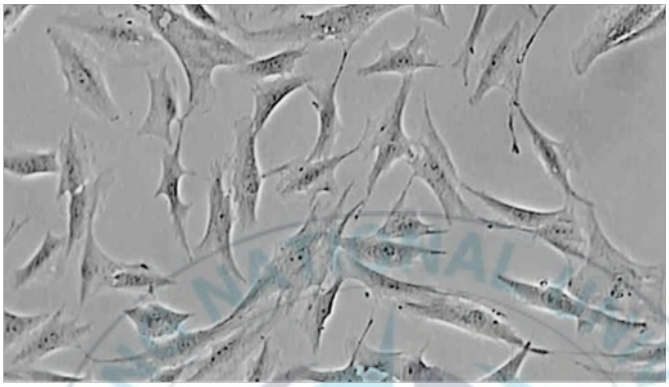
2.3.1. Cell culture

Cell culture experiments were performed by using normal human dermal fibroblast-neonatal (NHDF-neo) and human keratinocyte (HaCaT) in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution in an incubator with a humidified atmosphere of 5% CO2 at 37 °C. To detach NHDF and HaCaT, trypsin/EDTA solutions were added into cell culture plate. After centrifugation, only cell pallets performed by centrifugation were cultured in other sterilized cell culture plate.

2.3.2. Cytotoxicity evaluation

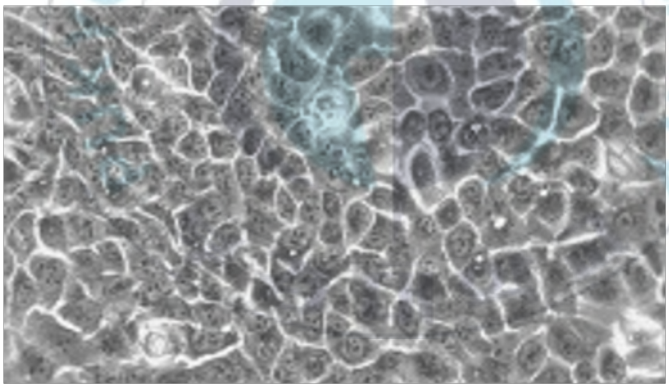
Cell viability was determined by the MTT assay to estimate the reduction of yellow tetrazolium salt in MTT into purple formazan crystals performed by the dehydrogenase enzymes secreted by mitochondria. NHDF-neo and HaCaT cells were seeded in each 96 well plates. The DPHC was treated by concentration in 96 well plate seeded cell. After 24 hours, MTT solution (1 mg/mL in PBS) was treated in 96 well plate seeded cell treated DPHC. After 4 hours, the solution was aspirated and re-incubated with DMSO at 37 °C for 30 min. After 30 min, the absorbance at 570 nm was measured using a microplate reader (Gen 5™ ELISA BioTek, USA) [47, 48].

(A)



[Figure adapted from the site of Promo Cell]

(B)



[Figure adapted from the site of CLS Cell Lines Service]

Fig. 6. Morphology of normal human dermal fibroblast-neonatal (NHDF-neo) (A) and human keratinocyte (HaCaT) (B)

2.4. Antibacterial activity

2.4.1. Bacterial strains and culture conditions

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, *C. albicans* KCTC 7965, *P. aeruginosa* KCTC 1637. 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, C. albicans* and *P. aeruginosa* strains were grown at 37°C in tryptic soy broth (TSB; Difco Inc., Detroit, MI).

2.4.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

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 37°C 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 sub-culturing 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 ≥99.9%.

2.5. Fabrication of hydrogels containing DPHC

PVA hydrogels blended with DPHC were fabricated by a freezing-thawing method. Aqueous 10 wt% PVA solutions were prepared by dissolving PVA powders into 1% PBS solution boiling 70℃ for 2 hours and cooling aqueous 10 wt% PVA solution at room temperature. 0.025 wt%, 0.05 wt%, 0.1 wt%, and 0.2 wt% DPHC solutions were prepared by dissolving DPHC powders into 1% PBS solution at room temperature. PVA solutions and DPHC solutions were mixed at a ratio of 1:1 each depending on each concentrations. After mixing, PVA/DPHC mixtures were stabilized at room temperature in a dark. After stabilizing, each mixture was poured into 24 well plates and underwent repeated freezing-thawing cycles per 3 times, consisting of 18 h freezing at -80 °C and 6 h thawing at room temperature. Consequently, a pure 5 wt% PVA hydrogel (PPVA), 5 wt% PVA hydrogel containing 0.0125 wt% DPHC (PVA/DPHC-

I), 5 wt% PVA hydrogel containing 0.025 wt% DPHC (PVA/DPHC-II), 5 wt% PVA hydrogel containing 0.05 wt% DPHC (PVA/DPHC-III), and 5 wt% PVA hydrogel containing 0.1 wt% DPHC (PVA/DPHC-IV) were fabricated.

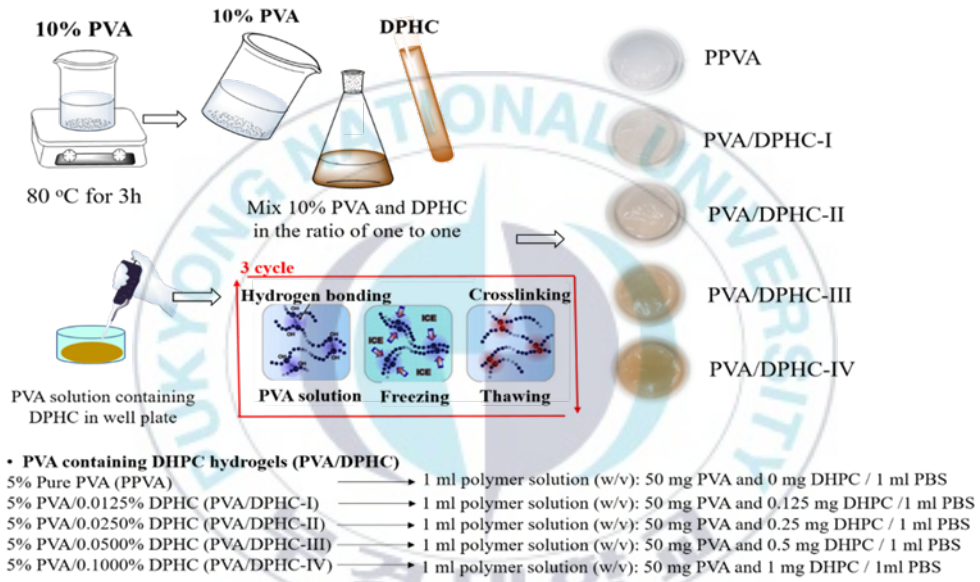


Fig. 7. The schematic fabrication process of PPVA, PVA/DPHC-I, PVA/DPHC-II, PVA/DPHC-III and PVA/DPHC-IV

2.6. Characterization of hydrogels

2.6.1. Microstructural evaluation

The structural morphology of PVA/DPHC hydrogels were examined by magnifying the hydrogel 1,500 and 3,000 diameters using scanning electron microscopy (SEM) (Tescan, Czech, VEGA II LSU) at 5 kV. The porous structures of PVA/DPHC hydrogels were confirmed from the SEM image using image analysis software (Image J, National Institutes of Health, USA).

2.6.2. Water swelling analysis

Dried hydrogels was initially weighed and immersed in DW at 37 °C until reaching the equilibrium state for water-uptake measurements. The dry weight (Wd) of hydrogels was determined by lyophilizing, and the swollen weight (Ws) of hydrogels was determined by blotting the surface water with blotting paper. The swelling ratio of hydrogels was calculated using the following formula:

$$\text{Swelling ratio (\%)} = [((Ws-Wd))/Wd] \times 100$$

2.6.3. Drug release test

The release test of DPHC from hydrogels (PPVA, PVA/DPHC-I, PVA/DPHC-I, PVA/DPHC-II, PVA/DPHC-III, and PVA/DPHC-IV) was performed by Folin-Ciocalteu method. The hydrogel was submerged in 2ml of 1x PBS and incubated at 37° C to maintain a similar condition of human body temperature. At certain time points (1,

2, 4, 6, 12, 24, 48, and 72 hours), the released solutions from hydrogels were calculated the percentage of the released total DPHC contents using the standard curve based the absorbance at 730 nm using a microplate reader (Gen 5™ ELISA BioTek, USA).

2.6.4. Gel fraction analysis

The PVA/DPHC hydrogels were prepared by freezing-drying methods under vacuum and weighted (Wo). The dried hydrogels were soaked in 1x PBS by time. At certain time points (6, 12, 24, 48, and 72 hours), the soaked hydrogels were freezed-dried again to remove the soluble parts. The hydrogels dried after soaking were weighted (We). The Gel fraction ratio of PVA/DPH hydrogels was calculated as follows equation:

Gel fraction (%) = [We/Wo] x 100

2.6.5. Rheological properties

The dynamic mechanical analysis was performed by Discovery HR-2 Hybrid Rheometer with 8 mm parallel plate geometry (TA Instruments, USA). Hydrogels loaded with a static load of 0.05 N at 25 °C and deformed at constant amplitude over a range of frequencies (0.1 Hz–10 Hz) to measure storage modulus (G´).

2.6.6. Thermogravimetric analysis

Thermogravimetric analysis (TGA) of the PVA/DPHC hydrogels were performed using a Pyris 1 TGA analyzer (Perkin-Elmer TGA-7, Waltham, MA, USA) with a scan range from 50 to 700 °C and a constant heating rate of 10 °C/min under continuous nitrogen

2.7. Indirect contact and direct contact test on cell

The cytotoxicity of PVA hydrogels containing DPHC was evaluated on NHDF-neo and HaCaT cell according to International Organization for Standardization (ISO) 10993–5 [49]. The PVA hydrogels containing DPHC sterilized by UV irradiation for 1 hour were immersed in culture medium for 24 hours and 72 hours at 37 °C to obtain extract medium. NHDF-neo and HaCaT cells were seeded in each 96 well plates at adensity of 1×10^4 and incubated with extracted medium and flesh medium for 24 h. Cell viability was determined by MTT assay [50]. Also, direct contact test was evaluated by immersing PVA hydrogels containing DPHC sterilized by UV irradiation on NHDF-neo and HaCaT cell in fresh medium for 24 hours and investigated by fluorescein diacetate (FDA) for live cell and heochst 33342 for nuclear staining.

2.8. Bacterial inhibition test of PVA/DPHC hydrogels

The antimicrobial activity of PVA/ DPHC hydrogels against the Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *P. aeruginosa* was assessed by a viable cell-counting method. Upon appropriate dilution with sterilized 0.9% saline solution, a culture of about 10^5 CFU/mL and 10^7 CFU/mL was prepared and used for antimicrobial testing. The *S. aureus* suspension and the *P. aeruginosa* suspension were incubated in the presence of the fibrous mats at 37 °C. At various exposure times (0, 30, 60, 180, and 360 min) aliquots were taken from the bacterial suspension and several decimal dilutions were made. The several decimal dilutions were quickly spread on the

nutrient agar and incubated at 37 °C for 24 h. The surviving microorganisms were counted by the spread-plate method in triplicate for each experiment. The number of the surviving cells was determined as colony forming units (CFU).

2.9. *In vivo* animal experiments

2.9.1. Wound healing animal models

Male ICR mice were housed under conditions of a light/dark cycles (12/12 hours). ICR mice were investigated wound healing ability after acclimatization period of 7 days [51]. The wound healing ability of the PVA hydrogels containing DPHC was investigated wound closure ability for 14 days after performing scratch wound 5mm in diameter on the 8-week-old ICR mouse. The wounded mice were investigated wound healing ability into 6 mice per group; (1) control group (non- treated), (2) Epi-Derm Silicon Gel Sheeting (1.8 mm thick, Biodermis, Las Vegas, USA), (3) PVA hydrogel group and (4) PVA/DPHC-IV. The wound closure was pictured at 0, 2, 4, 7, and 14 day. The averages of opened wound area were measured by image program and plotted as relative % of original wound.

2.9.2. Histological examination and staining

Tissues were fixed in formalin for 48 hours. The fixed tissues of excised wound site were dehydrated using alcohol and distilled water, embedded in paraffin and cut into 6 μm sections by microtome. The sections were gradually put into the xylene for 20 minutes, 100% alcohol for 5 minutes, 95% alcohol for 2 minutes, 80% alcohol for 2

minutes, distilled water for 5 minutes and hematoxylin to stain nuclear for 5 min. After washing to remove excess hematoxylin in distilled water, the sections were put into eosin to stain extracellular matrix for 2 minutes. In reverse, the sections were gradually put into distilled water for 5 minutes, 80% alcohol for 2 minutes, 95% alcohol for 2 minutes, 100% alcohol for 5 minutes, xylene for 10 minutes and covered finally the section using neutral resin [52]. Photographs were taken with Leica microscope (Leica Microsystems UK Ltd, Milton Keynes, UK).

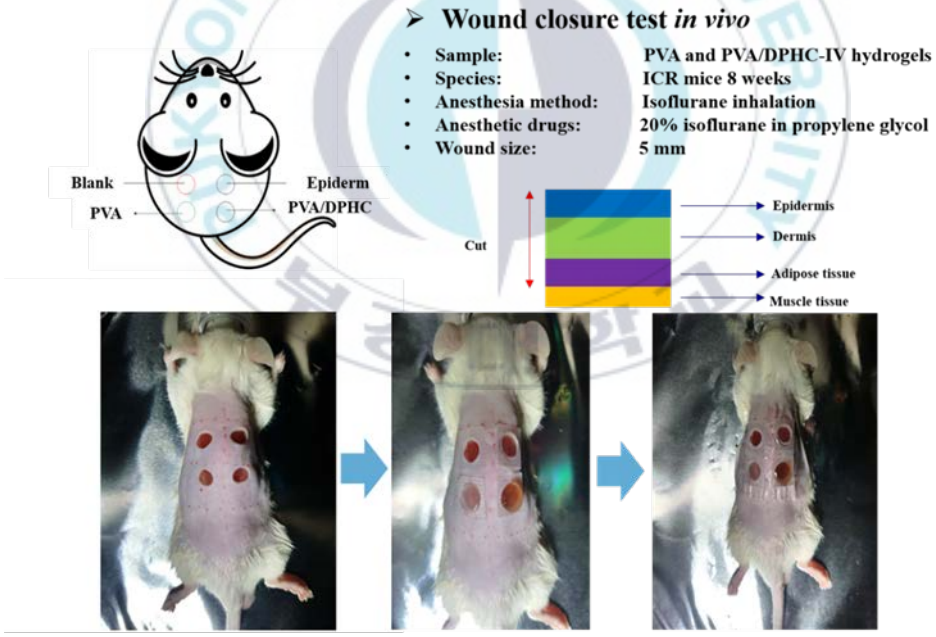


Fig. 8. In vivo experiment diagram of ICR mouse

3. Result

3.1. Characterization of DPHC

3.1.1. Extraction, isolation and purification of DPHC

DPHC derived from brown algae, *Ishige okamurae*, has polar property based hydrogen and hydroxyl group. Ethanol extraction was frequently used to obtain phenolic compounds. Yield of 70% ethanol extraction was 4.7% from *Ishige okamurae*. Also, ethanol extracts was separated the material with different polarity into fractions using different degrees of unsaturation by liquid-liquid solvent fractionation with two liquid having different polarity [53]. Solvent fractionation has advantages that fraction separation can be achieved efficiently and the filtration is easier [54]. As results of fractionation using n-Hexane, CHCl3 and EtOAc with distilled water, Ethanol extracts (100%) was separated 3.5% n-Hexane fraction, 39.5% CHCl3 fraction and 11.8 % EtOAc fraction in fig. 9. It is investigated that EtOAc fraction included rich DPHC by HPLC. So, EtOAc fraction was separated again by using CPC to obtain compound having more rich DPHC. Finally, fraction containing rich DPHC from CPC was purified by HPLC in fig. 10. (A). As a previous study, DPHC has about 512 molecular weight. So, DPHC was identified by evaluating molecular weight of DPHC using ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry in fig. 10. (B).

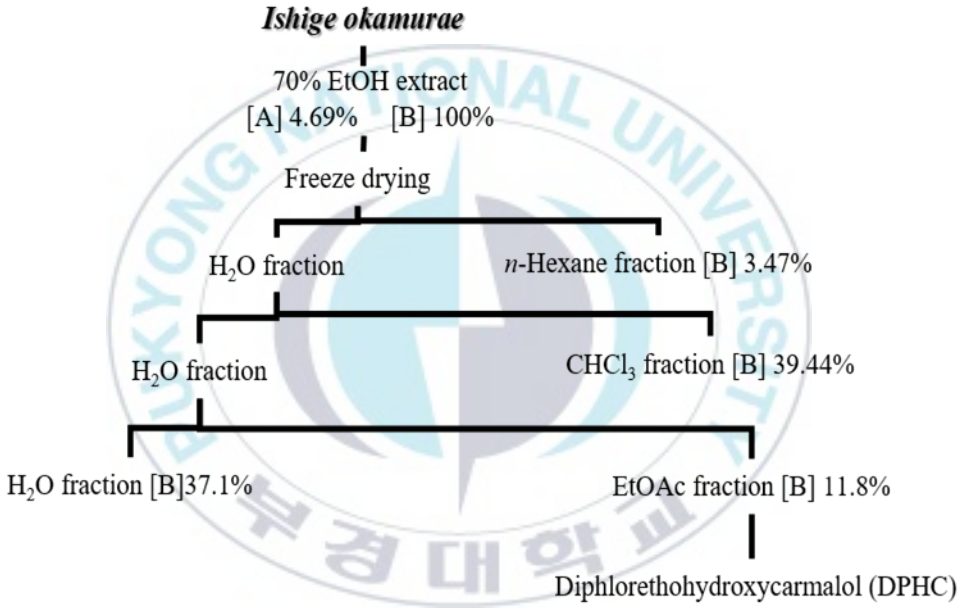
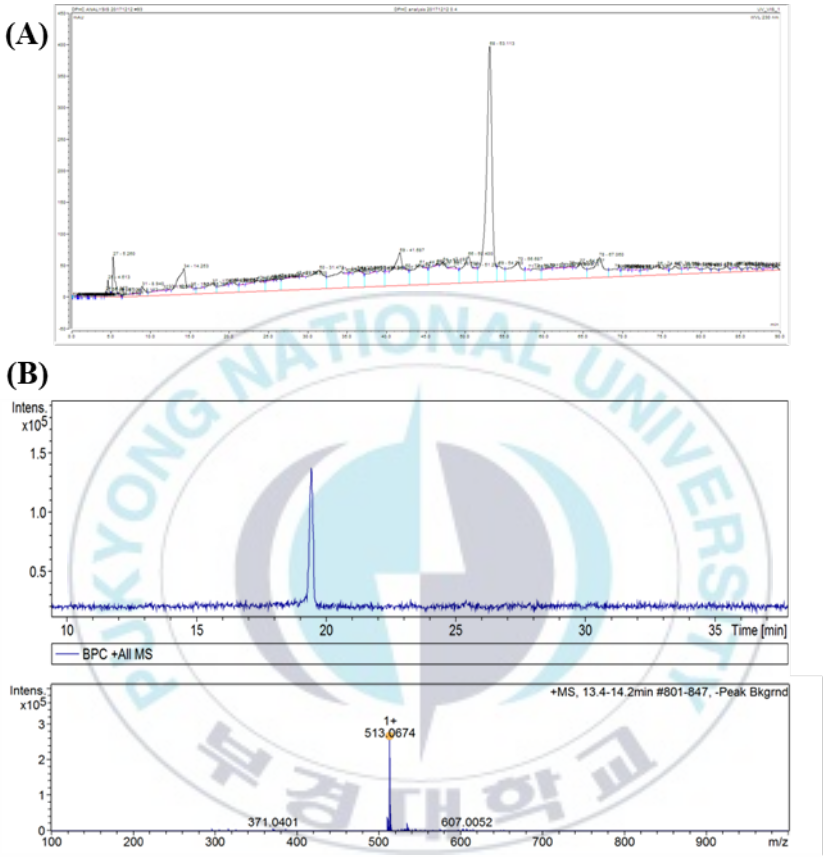


Fig. 9. Yield of extracts and solvent fractionations from *Ishige okamurae*



- M + H = 513.0674 – 1 = 512.0674
- Diphlorethohydroxycarmalol (DPHC) = MW 512.38 g/mol

Fig. 10. Chracterization of diphlorethohydroxycarmalol (DPHC) from high performance liquid chromatography (HPLC) (A) and ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC-Q-Tof MS)

3.1.2. Cytotoxicity of DPHC

To investigate the cytotoxic potential of DPHC in NHDF-neo and HaCaT cells, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to investigate cell viability after 24 hours treatment with DPHC. As a shown in fig. 11. (A), NHDF-neo cells were investigated to show non-cytotoxicity against different DPHC concentrations comparing non-treated DPHC group (0 μg/ml). Also, as a shown in fig. 11. (B), HaCaT cells were investigated to show non-cytotoxicity against different DPHC concentrations (6.25, 12.5, 25, 50, 100 μg/ml).

3.2. The antibacterial activity of DPHC

The DPHC from *Ishige okamurae* exhibited the bacteria inhibition activity against cutaneous pathogens such as the gram positive bacterium *Candida albicans*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and the gram negative bacterium *Pseudomonas aeruginosa*. As a shown in table. 1, the antibacterial activity of the DPHC was quantitatively evaluated by the MIC and MBC assay. The MIC values of the DPHC against cutaneous pathogens were investigated 128 μg/mL against *C. albicans*, 64 μg/mL against *S. epidermidis*, 128 μg/mL against *S. aureus* and 128 μg/mL against *P. aeruginosa*. Also, the MBC values of the DPHC against cutaneous pathogens were investigated 512 μg/mL against *C. albicans*, 128 μg/mL against *S. epidermidis*, 512 μg/mL against *S. aureus* and 256 μg/mL against *P. aeruginosa*.

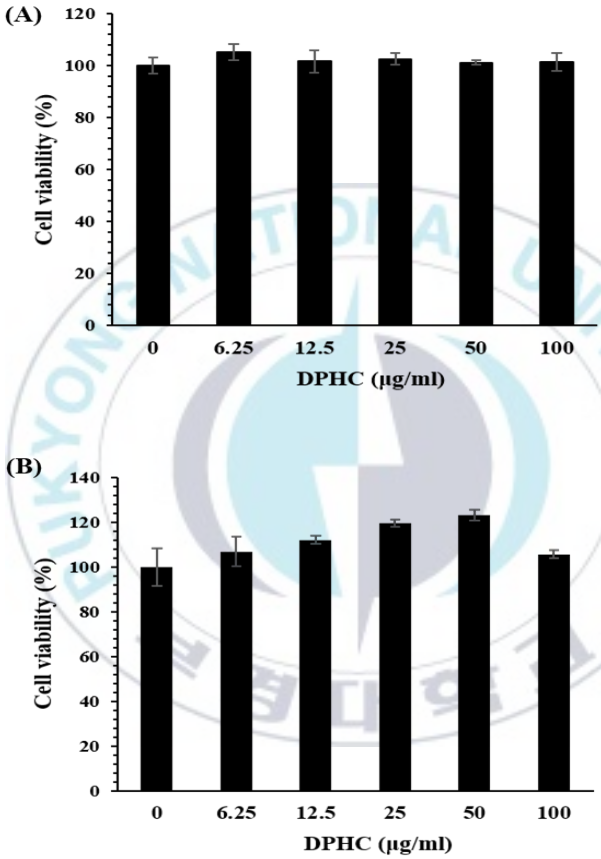


Fig. 11. Cytotoxicity of normal human dermal fibroblast-neonatal (NHDF-neo) (A) and human keratinocyte (HaCaT) (B)

Strains	MIC (μg/mL)	MBC (μg/mL)
	DPHC	DPHC
C. albicans KCTC 7965	128	512
S. epidermidis ATCC 14990	64	128
S. aureus KCTC 1927	128	512
P. aeruginosa KCTC 1637	128	256

Table 1. Comparison DPHC and solvent fraction with antibacterial effect: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

3.3. Physical properties of the PVA/DPHC hydrogels

3.3.1. Hydrogel morphological analysis

The PVA hydrogels were crosslinked by freezing-thawing methods at -80 °C and room temperature in 24 well plate and confirmed in fig. 7 that it successfully maintained a constant morphology even if DPHC was blended in the PVA hydrogel. The preparation condition of PVA/DPHC hydrogels was shown in fig. 7. The PVA/DPHC hydrogels could be confirmed that they showed rich in brown color as a concentration-dependence of DPHC and expected visually that the DPHC was regularly districted in hydrogel. The surface morphologies of the PVA/DPHC hydrogels were investigated by scanning electron microscopy (SEM). As shown in fig. 12. (A) and (B), the morphology of all hydrogels was porous and interconnected. It was analyzed x1500 and x3000 magnification. The porous surface of hydrogels could be confirmed that the pore was larger as a concentration-dependence of DPHC. Whereas the PPVA was shown regularly porous structure, PVA/DPHC-I, PVA/DPHC-II, PVA/DPHC-III, and PVA/DPHC-IV were shown irregularly distributed holes and gradually lager holes as a concentration-dependence of DPHC. As shown in fig. 12. (C), quantification of the pore size were investigated by image analysis software in surface area of hydrogels. The PPVA was consisted generally porous area of 10~30 μm2 size and the PVA/DPHC-IV hydrogel was consisted 40% porous area of 10~30 μm2 size, 20% porous area of 40~60 μm2 size, 20% porous area of 70~90 μm2 size, and 16% porous area of 100~120 μm2 size in total porous area. In short, a hydrogel gradually had a lager pore size as the

concentration of DPHC increased in hydrogel.

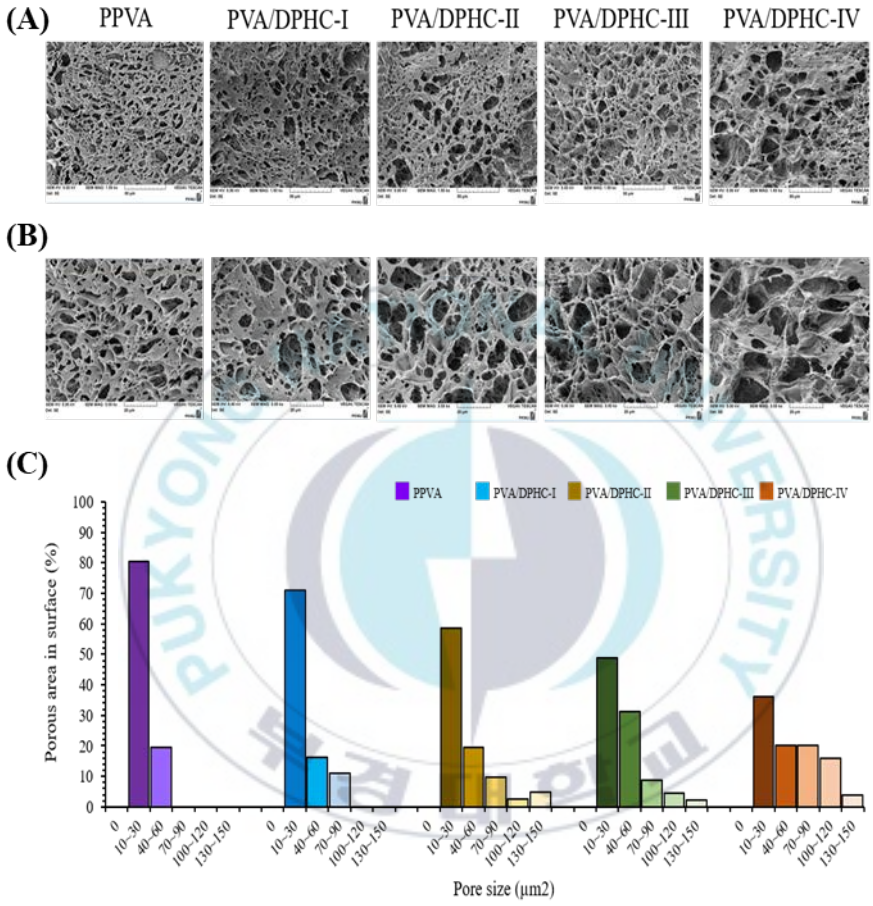


Fig. 12. SEM images of microporous structure of hydrogels containing DPHC in a concentration-dependent manner: (A) scanning electronic micrographs at ×1500 magnification; (B) scanning electronic micrographs at ×3000 magnification; (C) porous area in surface of hydrogels

3.3.2. Rheological properties

The relative mechanical properties of hydrogels were investigated by rheology test, as shown in fig. 13. (A). The storage modulus (G') and the loss modulus (G'') were affected the gelation time of hydrogel. The hydrogel network was collapsed and turned to be a sol state after G″ was higher than G′. This assay investigated the change in viscoelastic property against the changing frequency to test the stability of the mechanical properties. The G' investigated that the PVA/DPHC-IV was about 40 Pa lower than PPVA hydrogels and the G'' investigated that the PVA/DPHC-IV was about 10 Pa higher than PPVA hydrogels. As a dose-dependent DPHC, the G' and G'' values slightly were changed. The higher the DPHC concentration of PVA hydrogels, the lower G'' against frequency could be shown and the higher G'' against frequency could be shown. But the altered difference of G' and G'' values didn't be large dramatically relating to the error value.

3.3.3. Thermogravimetric analysis

The thermal degradation percentage of the DPHC and PVA/DPHC hydrogels was evaluated by thermogravimetric analysis, as shown in fig. 13. (B). The TGA data for DPHC investigated that the starting temperature of the degradation was 50 °C and that the DPHC weight gradually decreased about 48% until 700 °C. The TGA data for PVA/DPHC hydrogels investigated that the degradation of hydrogels was not shown at 50-200 °C and shown high weight loss ratio at 260-400 °C. Especially, the largest gap of weight loss percentage between the PVA/DPHC-I and the PVA DPHC-IV

investigated the gap of about 11% at 350 °C. After 400 °C, the TGA data for PVA/DPHC hydrogels investigated that the weight of the hydrogels gradually decreased. The higher the DPHC concentration of PVA hydrogels, the stronger stability against fever could be shown

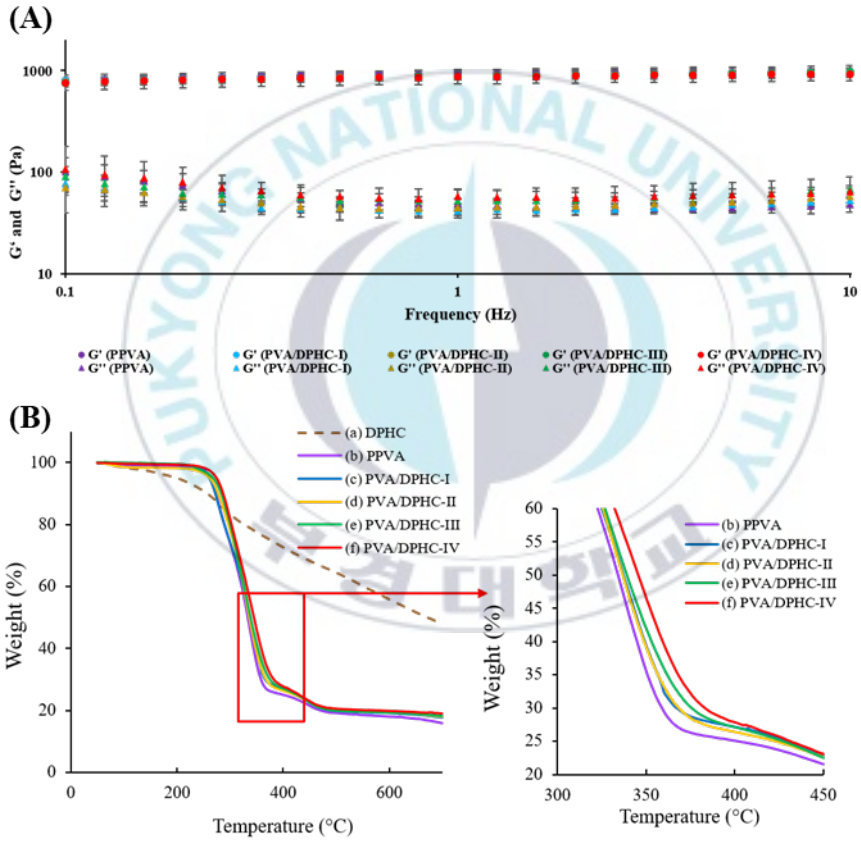


Fig. 13. Physical properties of the PVA/DPHC hydrogels: (A) rheological mechanical property analysis; (B) thermogravimetric analysis

3.3.4. The swelling

The timed water absorption percentage was shown in fig. 14. (B). All of the hydrogels showed fast absorption ability for 6 hours, and gradually increased after 6 hours. Also, after 24 hours, they did not show conspicuously the difference in the degree of water absorption percentage. As the concentration of DPHC in hydrogels increases, the swelling behavior of PVA/ DPHC hydrogels was showed higher abilities as compared to PPVA hydrogels. There were 600 wt% in PPVA hydrogels, 680 wt% in PVA/DPHC hydrogels-I, 725wt% in PVA/DPHC hydrogels-II, 700 wt% in PVA/DPHC hydrogels-III and 930 wt% in PVA/DPHC hydrogels-IV at 72 hours as compared dried hydrogels. In case of PVA/DPHC hydrogel-IV, it was confirmed that the water absorption percentage was increased approximately 1.5 times as compared with PPVA hydrogel at 72 hours. Other hydrogels increased the water absorption percentage as the concentration of DPHC in hydrogels increases.

3.3.5. Drug release analysis

Drug delivery characterization are very vital for drug carriers and should be evaluated. The released behavior of DPHC from PVA/DPHC hydrogels was shown in fig. 14. (A). All of the hydrogels showed fast release ability for 6 hours, and gradually increased after 6 hours. Also, after 24 hours, they did not show conspicuously the difference in the degree of DPHC released percentage. The higher the DPHC concentration of the soaking solution, the more DPHC could be loaded. The in virto release behavior of

DPHC from PVA/DPHC hydrogels was controlled by the loaded amount of the DPHC. The amount of the released DPHC was shown that the PVA/DPHC hydrogel-IV is 2 times higher than the PVA/DPHC-I at 12 hours.

3.3.6. Gel fraction analysis

The timed gel fraction of hydrogels was shown in fig. 14. (C). All of the hydrogels showed fast gel fraction for 6 hours, and gradually increased after 6 hours. As the concentration of DPHC in hydrogels increases, the gel fraction of PVA/ DPHC hydrogels was showed higher weight loss abilities as compared to PPVA hydrogels. The PVA/DPHC-IV was 3% more lose than PPVA hydrogel at 6 hours and 72 hours. As a dose-dependent DPHC, the gel fraction of PVA/DPHC hydrogel against solution slightly were changed. The higher the DPHC concentration of PVA hydrogels, the lower weight loss ability could be shown.

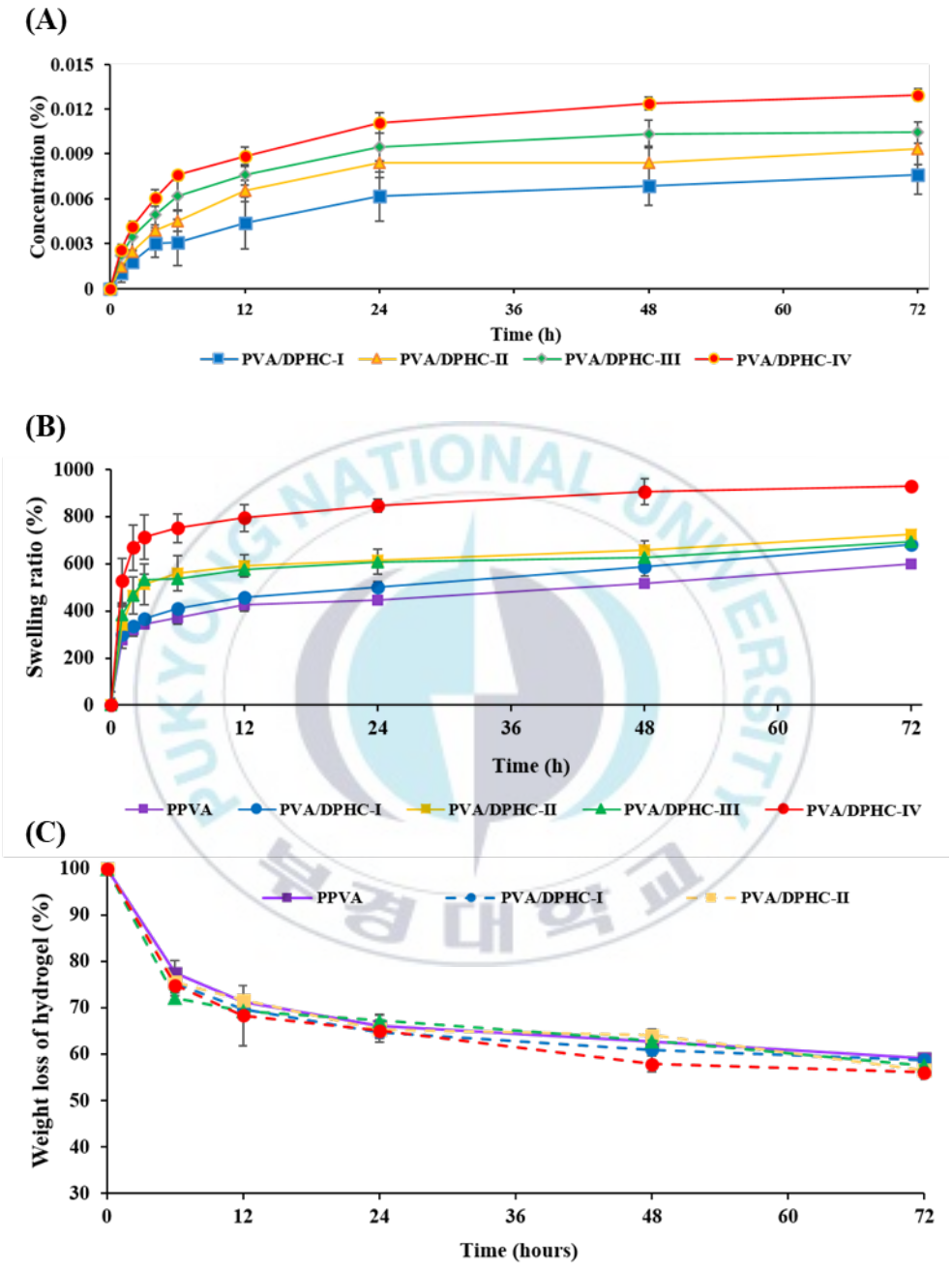


Fig. 14. Physical properties of the PVA/DPHC hydrogels: (A) DPHC release analysis; (B) the swelling analysis of hydrogel; (C) gel fraction analysis of hydrogel

3.4. Indirect contact and direct contact test on cell

The cytotoxicity of the extractability and leachability of hydrogels were confirmed by indirect and direct contact test on NHDF-neo and HaCaT cell according to ISO 10993-5. In order to investigate cell viability against low concentration and high concentration of the extracts, all hydrogels were immersed for 1 day and 3 days in culture media. As a shown in fig. 15. (C), the cell viability against the extracts was investigated numerical values by MTT assay at 540 nm absorbance. The NHDF-neo and HaCaT cell viability of the control was set 100%, and the NHDF-neo and HaCaT cell viabilities of the extracts of all hydrogel groups were not investigated below 70% non-toxic limited values according to ISO 10993-5. As a shown in fig. 15. (A) and (B), the NHDF-neo and HaCaT cell viabilities contacted hydrogels directly were investigated by Hoechst 33342 and FDA assay indicating to visible data. The cell viability was monitored by observation in bright field as well with FDA fluorescent reagent to stain only living cells comparing with hoechst 33342 staining nucleic acid of cell spreading widely on cell plate in fluorescence microscopy. The NHDF-neo and HaCaT cells contacted directly hydrogels were generally alive comparing Hoechst staining and FDA staining.

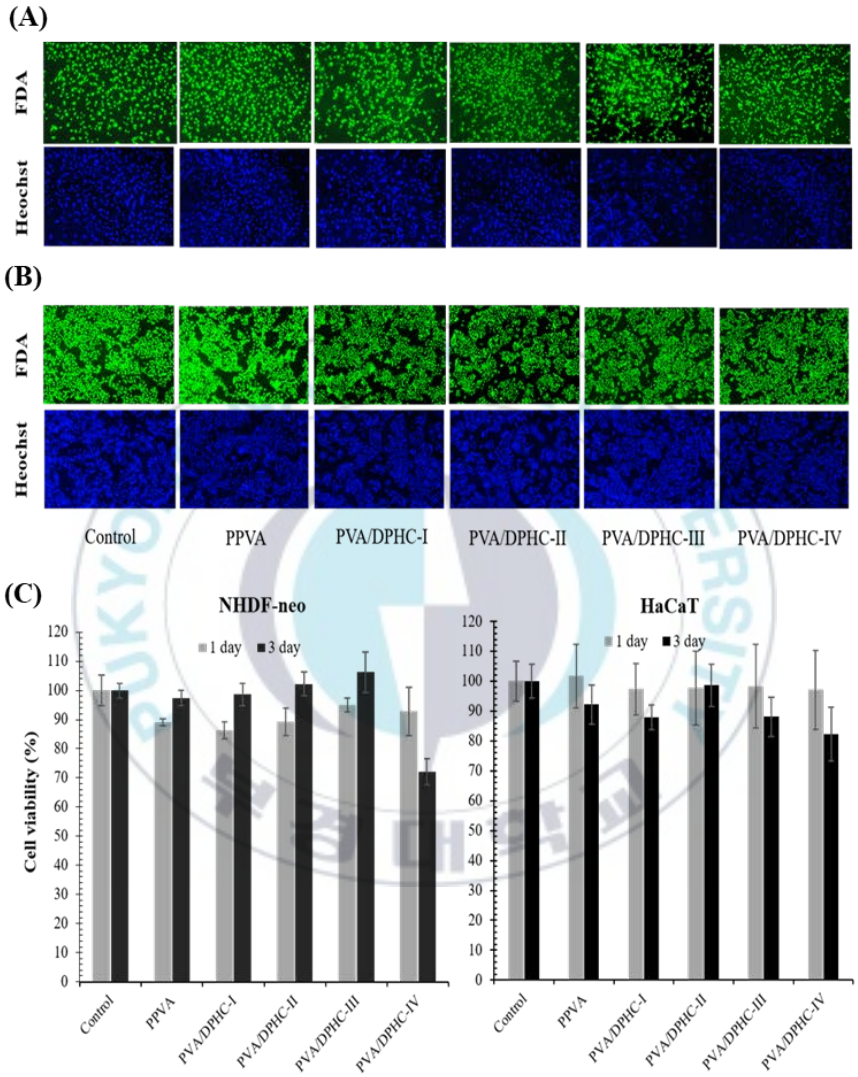


Fig. 15. Cytotoxicity assay of PVA/DPHC hydrogels was carried out using NHDF-neo and HaCaT : (A) direct contact test on NHDF-neo; (B) direct contact test on HaCaT; (C) in-direct contact test

3.5. Bacterial inhibition test of PVA/DPHC hydrogels

Antibacterial activity of hydrogels against the Gram positive bacterium *S. aureus* and the Gram negative bacterium *P. aeruginosa* was investigated by ASTM e2149. ASTM e2149 was suitable to evaluate release-killing ability of hydrogel [55]. Bacterial inhibition activity was evaluated by counting the viable microorganisms present after incubating hydrogels for 0, 30, 60, 180, 360 minutes in bacteria suspension. Fig. 16. (A) and (B) and fig. 17. (A) and (B) were shown the bacterial viability for each 5 log bacteria normal density and 7 log bacteria high density of *S. aureus* and *P. aeruginosa* against the control, PPVA and PVA/DPHC-IV.

As a shown in fig. 16. (A), the control and PPVA were not shown bacterial reduction ability against *S. aureus* in 5 logs for 360 minutes, whereas the bacterial reduction ability of the PVA/DPHC-IV against *S. aureus* was investigated about 90% bacterial reduction at 60 minutes and almost bacteria killed after 180 minutes in 5 logs. Also, as a shown in fig. 16. (B), the control and PPVA were not shown bacterial reduction ability against *S. aureus* in 7 logs for 360 minutes, whereas the bacterial reduction ability of the PVA/DPHC-IV against *S. aureus* was investigated about 99% bacterial reduction for 360 minutes in 7 logs.

As a shown in fig. 17. (A), the control and PPVA were not shown bacterial reduction ability against *P. aeruginosa* in 5 logs for 360 minutes, whereas the bacterial reduction ability of the PVA/DPHC-IV against *P. aeruginosa* was investigated about 90% bacterial reduction at 180 minutes and almost bacteria killed at 360 minutes in 5 logs.

Also, as a shown in fig. 17. (B), the control and PPVA were not shown bacterial reduction ability against *P. aeruginosa* in 7 logs for 360 minutes, whereas the bacterial reduction ability of the PVA/DPHC-IV against *P. aeruginosa* was investigated about 99% bacterial reduction for 360 minutes in 7 logs.

Fig. 17. (C) was visual data counting the viable gram positive bacterium *S. aureus* and gram negative bacterium *P. aeruginosa* present after incubating in 5 log bacteria density.

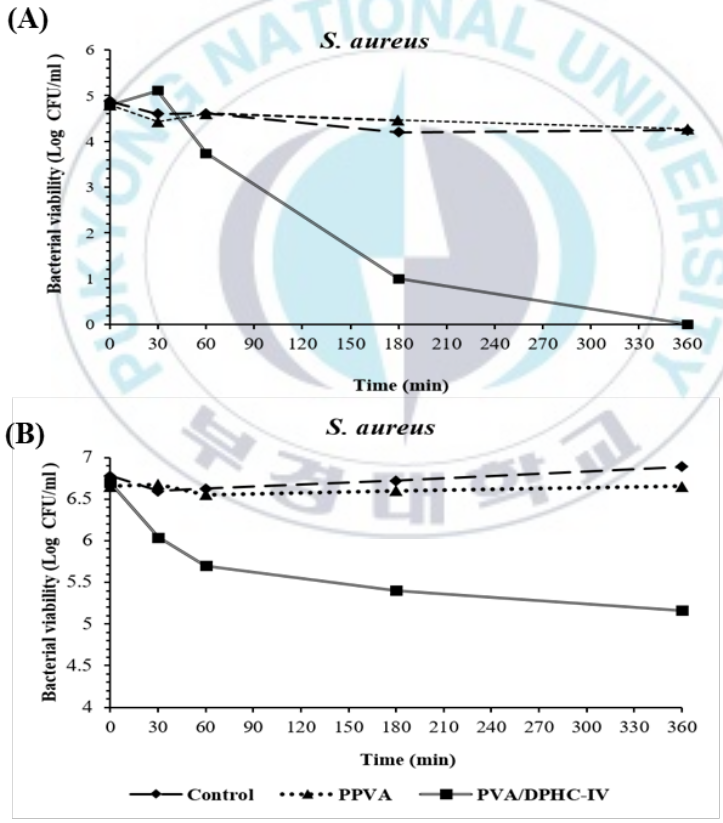


Fig. 16. The anti-bacterial activity of PPVA and PVA/DPHC-IV: (A) S. aureus viability (5 Log CFU/ml); (B) S. aureus viability (7 Log CFU/ml)

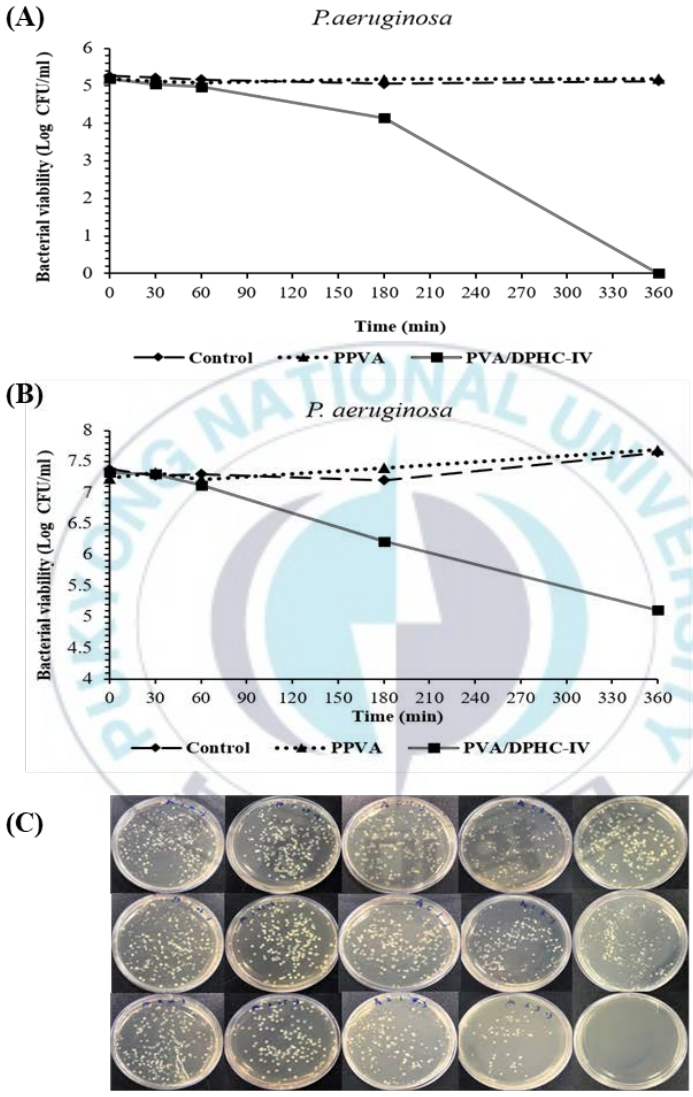


Fig. 17. The anti-bacterial activity of PPVA and PVA/DPHC-IV: (A) P. aeruginosa viability (5 Log CFU/ml); (B) P. aeruginosa viability (7 Log CFU/ml); (C) visual data to count the P. aeruginosa viability (5 Log CFU/ml)

3.6. *In vivo* experiment

3.6.1. Wound closure ability

To investigate the wound healing ability of the PVA hydrogels containing DPHC *in vivo*, the 8-week-old ICR mice were created skin incisions 5mm in diameter on the back of mice. The wound closure was pictured at 0, 2, 4, 7, and 14 day. Comparing control group, Epi-Derm silicon gel sheeting, PPVA and PVA/DPHC-IV, wound areas of all groups were reduced to the wound center performing the growth of new epidermis in all wound lesions. But the wound closure abilities of all groups relatively were different per 0, 2, 4, 7, and 14 day as a shown in fig. 18. (B). The control group were shown a low wound closure ability cured about 15% for 4 days, whereas the control groups were shown a wound closure ability cured about 60% at 7days. The PVA groups were shown a wound closure ability cured about 25% for 4 days, and the control groups were shown a wound closure ability cured about 65% at 7days. Whereas, The PVA/DPHC-IV groups and Epi-Derm groups were shown a high wound closure ability cured about 35% for 4 days, and the PVA/DPHC-IV groups and Epi-Derm groups were shown a high wound closure ability cured about 75% and 85% at 7days. Finally, all treated groups were investigated that their wounds almost were closured at 14 days. Consequentially, the wound closure ability was resulted that Epi-Derm and PVA/DPHC-IV had a high wound closure ability comparing with control groups.

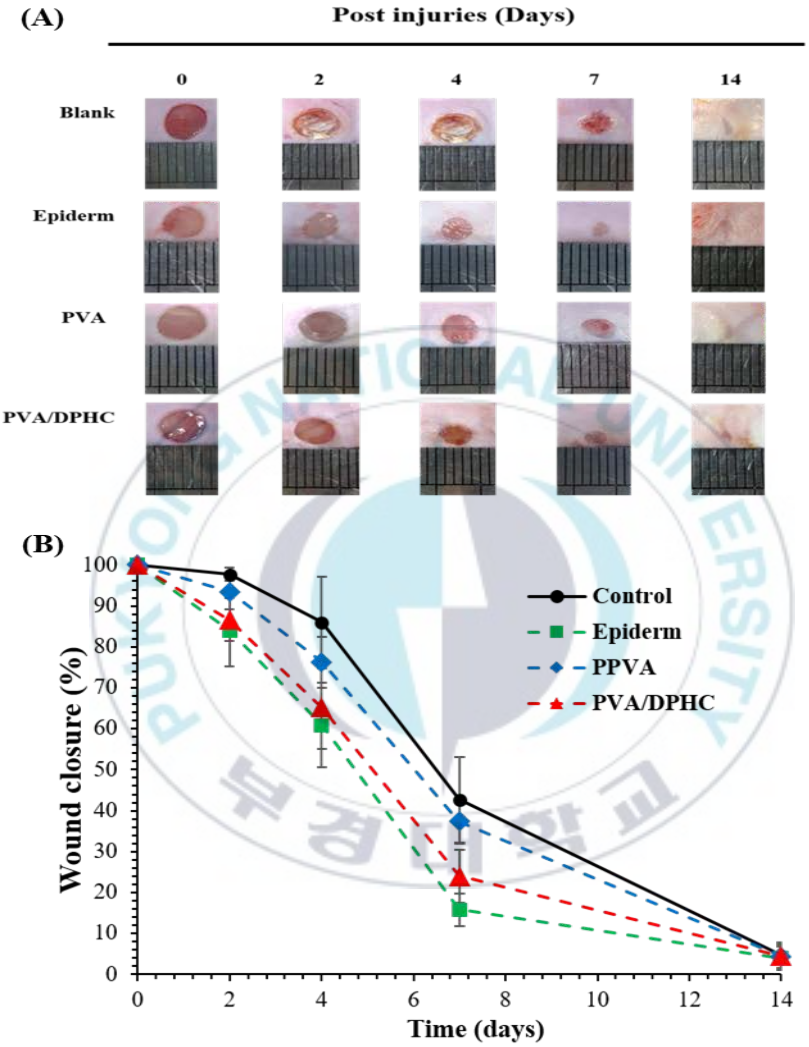


Fig. 18. The wound closure ability of male ICR mice (8 weeks of age) having a full-thickness 5 mm excisional wounding: (A) The wound closure was pictured at 0, 2, 4, 7, and 14 day; (B) Graph of the averages of opened wound area measured by image program and plotted as relative % of original wound

3.6.2. Histological property

Histology of wound areas cured for 14 days was shown in fig. 19 and it was shown that representative images of H&E stained histopathological sections of granulation/healing tissues of normal, control, Epi-Derm, PVA and PVA/DPHC-IV groups treated on the back of ICR mouse (40x magnification and scale bar 50 μm). Comparing with normal tissue of ICR mouse, all treated groups were investigated to perform re-epithelialization by observing features of wound healing such as sebaceous gland, hair follicle, fibroblast and keratinocyte. Generally all treated groups distinctly were shown proliferation and differentiation of the epidermis and dermis. Epi-Derm groups were shown a mature sebaceous gland, whereas the control, PVA, and PVA/DPHC-IV groups were shown an immature sebaceous gland. But PVA/DPHC-IV group was shown a numerous hair follicle comparing with other groups. Consequentially, although the control and PVA groups were investigated a good re-epithelialization effect, they were investigated less features of wound healing such as sebaceous gland and hair follicle. Also, Epi-Derm groups were investigated to perform rapidly mature sebaceous gland and PVA/DPHC-IV group was investigated to perform rapidly hair follicle.

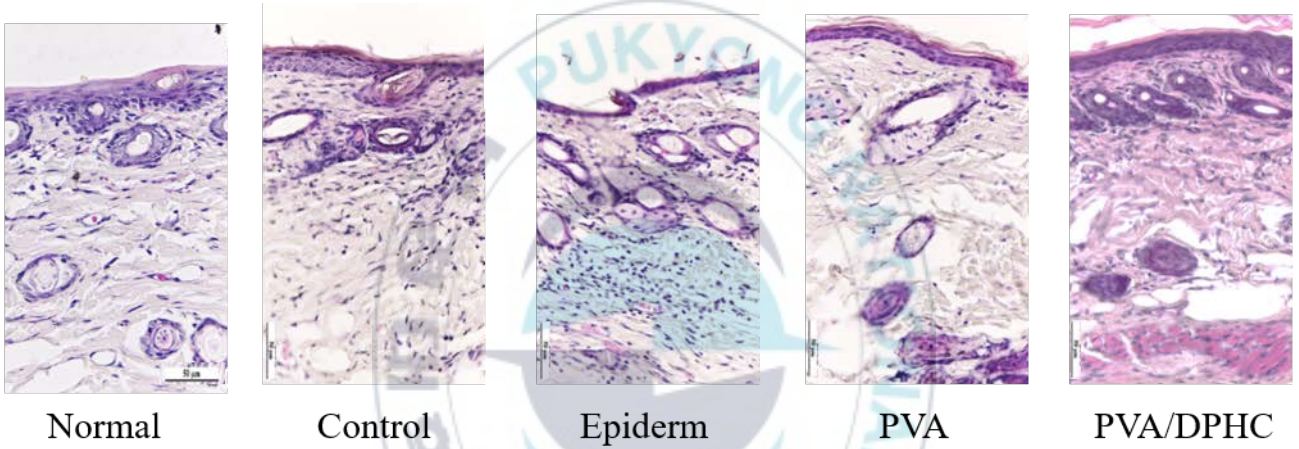


Fig. 19. Representative images of H&E stained histopathological sections of granulation/healing tissues of control, Epiderm, PVA and PVA/DPHC hydrogel treated mouse on days 14 post-wounding (40x magnification and scale bar 50 µm)

4. Discussion

Phlorotannins isolated a marine brown algae are well known as phenolic bioactive agents playing a vital role related to assist human health and nutrition such as antioxidant activity, anticancer activity, anti-HIV activity, angiotensin-I-converting enzyme (ACE-I) inhibition and so on. The isolated and characterized phlorotannins from marine brown algae are representative compounds such as phloroglucinol, eckol, dieckol, fucodiphloroethol G, phlorofucofuroeckol A, 7-phloroeckol, and 6,6′-bieckol [56]. These phenolic compounds have a potential antimicrobial ability based their ability to denature proteins, characterization of compound classified as surface-active agents [57]. The MIC value for eckol from a brown algae, *Echklonia cava*, investigates antibacterial activity in range of 125-250 μg/ml against the gram-positive bacterium *S. aureus [58]*. Also, the MIC value for dieckol investiges antibacterial activity in range of 32-64 μg/ml against the gram-positive bacterium *S. aureus* [59]. As a similar, the DPHC has the antibacterial activity in range 64-128 μg/ml against the gram positive bacterium *C. albicans*, *S. epidermidis* and *S. aureus*. Also, the DPHC has the antibacterial activity in range 128 μg/ml against the gram negative bacterium *P. aeruginosa*. As a result, the DPHC has a potential to be used as ingredient having a good antibacterial effects against the gram-positive and negative bacterium.

One of the main purpose of wound dressing application is to protect the wound cites from external factor because wounds are easily infected and contaminated by external factors. Bacteria, one of the external factors, causes complications by permeating into

wound. Thus, antibacterial research consistently was performed for wound dressing. As a wound dressing application, hydrogels offer many advantages, such as precise control of chemical, mechanical, and physical properties as well as flexibility to design features that can be easily manipulated for intended applications. Among hydrogels, PVA hydrogel can be crosslinked physically undamaging other materials added to the PVA hydrogel as well as indicates fast wound healing by keeping wet condition. So this study utilizes the PVA hydrogel to contain the DPHC stably because the DPHC consisted of four phloroglucinol units and functional hydroxyl groups could be easily broken a linking chain by factors such as heat, pressure, ion charge, organic solvent and so on [60]. The PVA hydrogel can be fabricated and crosslinked by the freezing-thawing methods [40]. The more abundant the PVA hydrogels have crystallites that occur between the hydroxyl groups of PVA during the freezing-thawing method, the higher the PVA hydrogels have high mechanical strength [61]. But investigating different microstructural evaluation as a dose-dependent DPHC contained the PVA hydrogels in fig. 12 was that the DPHC was slightly interrupted to perform crystallites by interfering with the encounter of hydroxyl groups [62]. The porosity of the PVA hydrogel containing DPHC investigated larger comparing PPVA as a dose-dependent DPHC for such a reason. The rheological characterization was determined to indicate mechanical properties of physically cross-linked hydrogels relating G' rheological value [29]. Rheological properties expectably investigated lower G' value as a dose-dependent DPHC due to lager porosity and interruption to perform crystallites. The more crosslinking PVA hydrogel was performed, the less water-content PVA hydrogel

was investigated [63]. Thus, due to increase space where water can be contained by having lager porosity, water swelling ability was improved as a dose-dependent DPHC of the PVA hydrogels. Also, the course of degradable ability and chain scission in the PVA hydrogel is due to oxidative degradation from the presence of residual oxygen [64]. So, gel fraction ability of the PVA hydrogel containing DPHC was investigated higher ratio as a dose-dependent DPHC due to have greater effect from increased water content. Whereas, organic structure having aromatic ring of phloroglucinol unit has good thermal degradation behaviors and flame-retarding performances [65]. Although the mechanical properties is lower depending the DPHC amount, the thermal degradation ratio was decreased by increasing the amount of DPHC loaded in the PVA hydrogels. The DPHC released amounts were increased as a dose-dependent DPHC in the PVA hydrogels containing DPHC. So, the PVA hydrogels were controlled amount of DPHC needed for wound by measuring amount of DPHC loaded in the PVA hydrogels.

The PVA hydrogels containing DPHC was investigated cytotoxicity and antibacterial activity through *in vitro* test to present possibility prior to apply for the human wound. During the skin regeneration process, fibroblasts performed to produce extracellular-matrix components and stimulate angiogenesis, myo-fibroblast proliferation and activation [66]. Keratinocytes are typical cells constituting the epidermis and these produce pro-inflammatory mediators to prevent invasion of pathogens [67]. For such a reason, The PVA hydrogel containing DPHC was investigated that the hydrogels did

not affect fibroblast and keratinocyte that perform many biological functions in the skin and blood during the skin regeneration process. Although the hydrogels did not affect cells, they affect bacterial grow and death. When the antibacterial effect of the hydrogels was estimated by the ASTM E2149 antimicrobial test, the bacterial reduction ability of the PVA/DPHC-IV against *S. aureus* and *P. aeruginosa* was investigated about 99% bacterial reduction related to the released amounts of hydrogels for 360 minutes.

In this study, animal experiments were performed to investigate in depth the effect and stability of hydrogels. An effect observed *in vitro* experiment cannot be assured that it definitely is observed *in vivo* experiment. Thus, *in vivo* experiments were performed on genetically similar mouse for human. In the skin, re-epithelialization is most important in wound healing process because of covering the wound, restoring functions and reconstitution of an epithelium by the migration of epidermal cells [68]. As a shown in fig. 18, PVA/DPHC-IV was investigated a rapid re-epithelialization effect comparing to the control and PPVA groups. According to previous study, the DPHC had anti-inflammatory effect reducing strongly the production of interleukin 6 (IL-6) through downregulation of the NF-κB and Jak2-STAT5 pathway and upregulation of SOCS1 [69]. Also, the DPHC had anti-oxidant effect relating radical scavenging activity [70]. The antioxidant activity in wound dressing has been enhanced on the wound healing process due to their regulation of the overproduction of ROS as inflammatory mediator under pathological conditions. So, the DPHC having anti-

oxidant and anti-inflammatory activities could enhance the wound healing process by controlling inflammation period and regulation of the overproduction of ROS. Moreover, PVA/DPHC-IV IV group was investigated to perform rapidly hair follicle because the DPHC have effect performing to increase prostaglandin (PG) E2 involved in hair growth by stimulating COX expression in keratinocyte [71]. These results suggest that the DPHC was applied to the fabricated of PVA hydrogel without loss of antimicrobial activity and wound healing activity for use as wound dressing.

5. Conclusion

In this study, we could isolate and purify diphlorethohydroxycarmalol (DPHC), phlorotannin compound derived from brown alga *Ishige okamurae*, by performing EtOH extraction, solvent fractionation, HPLC and HPCPC. First of all, the antibacterial activity of DPHC has not been revealed until now but this study demonstrates the fact that DPHC has antibacterial effect helping wound healing process against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans* and *Pseudomonas aeruginosa* from MIC and MBC test. To use wound dressing application protecting the wound cites from external factor, polyvinyl alcohol (PVA), biocompatible polymer, hydrogel containing DPHC could be fabricated by performing freezing-thawing method. Also, PVA/DPHC hydrogels could be demonstrated antibacterial ability and wound healing ability by performing microstructural evaluation, rheological test, thermogravimetric analysis, swelling test, release test, gel fraction test, cytotoxicity test, bacteria-killing test, histological test and wound closure test in vivo. Based data in this study, this study can be suggested that PVA/DPHC hydrogels have possibility of wound dressing by bio-application.

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