

ANTIBACTERIAL ACTIVITY OF THE PHLOROTANNINS DIECKOL AND PHLOROFUCOFUROECKOL-A FROM *ECKLONIA CAVA* AGAINST *PROPIONIBACTERIUM ACNES*

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Abstract: Methanol extract of the marine brown alga *Ecklonia cava* has been shown to exhibit antimicrobial activity against *Propionibacterium acnes*. To identify the compounds conferring this antimicrobial activity, methanol extract was fractionated using silica-gel column chromatography by increasing solvent polarity, and the antimicrobial activities of each fraction were determined in terms of the minimum inhibitory concentration. Hexane, diethyl ether, acetone, ethyl acetate, acetonitrile, methanol, and water fractions were generated, with the acetone fraction exhibiting the greatest antibacterial activity (minimum inhibitory concentration = 156 µg/mL). The acetone fraction was further purified by Sephadex LH-20 column chromatography and reverse-phase HPLC using an Alltima C18 column with an H₂O-MeOH linear gradient from 30 to 100%. Two peaks were eluted at 18.1 (P1) and 23.8 (P2) min, both of which exhibited marked antibacterial activity (minimum inhibitory concentration = 39 µg/mL for both). The structures of these compounds were determined on the basis of ¹H and ¹³C nuclear magnetic resonance data and, through comparison with published data, can be phloroglucinol derivatives dieckol (P1) and phlorofucofuroeckol-A (P2). These results suggest that phlorotannins derived from *E. cava* have antimicrobial activity against *P. acnes*, and so may be useful as natural additives in anti-acne cosmetics and pharmaceutical products.

Keywords: dieckol, minimum inhibitory concentration, natural additives, phlorofucofuroeckol-A, phlorotannins.

Resumen: Se conoce que el extracto de metanol del alga parda marina *Ecklonia cava* presenta actividad antimicrobiana contra *Propionibacterium acnes*. Para identificar los compuestos que confieren esta actividad antimicrobiana se fraccionó el extracto de metanol, usando cromatografía en columna de sílica gel, mediante el aumento de la polaridad del disolvente. La actividad antimicrobiana de cada fracción se determinó en términos de la concentración mínima inhibitoria. Las fracciones de hexano, éter dietílico, acetona, acetato de etilo, acetonitrilo, metanol y agua fueron producidas. La fracción de acetona presentó la mayor actividad antibacteriana (concentración mínima inhibitoria = 156 µg/mL). Con base en este resultado, la fracción de acetona se purificó por cromatografía en columna en LH-20 Sephadex y HPLC de fase inversa usando una columna Alltima C18 con un gradiente lineal de H₂O-MeOH de 30 a 100%. Dos picos se eluyeron a 18.1 (P1) y 23.8 (P2) min, ambos tuvieron actividad antibacteriana marcada (concentración mínima inhibitoria = 39 µg/mL para ambos). La estructura de estos compuestos se determinó sobre la base de ¹H y ¹³C de resonancia magnética nuclear y, mediante la comparación con valores publicados, éstos pueden ser derivados de floroglucinol diecol (P1) y florofucofuroeckol-A (P2). Los resultados sugieren que florotaninos derivados de *E. cava* tienen actividad antimicrobiana frente a *P. acnes*, y por lo tanto, pueden ser útiles como aditivos naturales en los cosméticos anti-acné y los productos farmacéuticos.

Palabras clave: aditivos naturales, concentración mínima inhibitoria, diecol, florofucofuroeckol-A, florotaninos.

Acne is a chronic inflammatory condition whose clinical presentation can range from a mild comedonal form to severe cystic acne of the face, chest, and back. Factors that

contribute to the development of acne include hormonal imbalance, bacterial infection, stress, food, or spoiled cosmetic application (Zu *et al.*, 2010; Park *et al.*, 2004). *Propionibac-*

terium acnes is a gram-positive, anaerobic microorganism, which has been recognized as a key factor in the development of inflammatory acne, due to its ability to metabolize sebaceous triglycerides into fatty acids, which attract neutrophils (Jappe, 2003).

Antibiotics are regularly prescribed for the treatment of acne vulgaris; however, antibiotic resistance is increasingly common in many dermatologic infections. The development of antibiotic resistance is multifactorial, involving host characteristics, the mechanism of antibiotic activity, how the antibacterial is used, and environmental factors (Eady, 1998; Swanson, 2003). To address the growing problem of antibiotic resistance, medicinal plants have been investigated as alternative treatments (Chomnawang *et al.*, 2005; Choi *et al.*, 2011).

A previous study of methanol extracts of *Ecklonia cava* showed considerable antibacterial activity against microorganisms frequently involved in acne inflammation, such as *Propionibacterium acnes*, with minimal side effects (Choi *et al.*, 2011). Of the 57 species of seaweed screened for their potential antimicrobial, the methanol extracts of *E. cava* exhibited the highest activity against *P. acnes*, showing 0.31 mg/mL of the minimum inhibitory concentration (MIC) value. However, the active compounds were not identified. In this study, we examined crude extracts of *E. cava* by solvent polarity, gel chromatography, and HPLC with the aim of isolating and identifying the active compounds.

Materials and methods

Algae. The edible marine brown alga *Ecklonia cava* Kjellman was collected from the coast of Jeju Island, South Korea, in 2009 and washed three times with tap water to remove salt. Algae were air-dried for five days at room temperature using an electric fan and ground in a mill and passed through a 500 mesh sieve. The sieved material was stored at -20 °C until use.

Extraction. The sieved powder (1 kg) was suspended in 80% methanol (3 L) for two days to extract soluble compounds. The extract was then filtered through Toyo No. 2 filter paper under reduced pressure. This extraction procedure was repeated three times and the extracts were combined. The combined 80% methanol extract was concentrated and dried

to a dark-green residue (54.9 g) under reduced pressure in a vacuum evaporator.

Purification of antimicrobial compounds. To fractionate according to polarity, the dried extract was then suspended in a 5 mL aliquot of hexane, subjected to silica gel chromatography (3.6 cm i.d. × 40 cm, Silica Gel 60, 15–40 µm, Merck, Germany), and eluted successively with 300 mL of different solvents: hexane, diethyl ether, acetone, ethyl acetate, acetonitrile, methanol, and water. The acetone fraction (2.87 g), which exhibited the greatest antibacterial activity, was further purified by Sephadex LH-20 chromatography (1.5 cm i.d. × 50 cm, Sigma, St. Louis, MO) with 1 L of methanol to yield four sub-fractions containing the desired active compounds. Each 10 mL fraction was collected at a flow rate of 1 mL/min. Of these, sub-fraction 66–75 (583.2 mg), which exhibited the most potent antibacterial activity, was subjected to reverse-phase HPLC purification using a Waters 600 HPLC system (Waters, Milford, MA, USA) and an Alltima C18 column (10 mm i.d. × 250 mm, Alltech Inc., IL, USA) with 5 µm particles. Elution was performed at a flow rate of 4.0 mL/min using a H₂O–MeOH linear gradient, from 30 to 100% for 20 min, and maintained for 20 min. The UV detector was set at 290 nm. The two peaks (P1 and P2), which exhibited the most potent antibacterial activity, were isolated. Each eluted compound was dried under a stream of nitrogen gas obtained from a N₂ generator (G4010E, Domnick Hunter, England; less than 100 ppm oxygen content).

Nuclear Magnetic Resonance (NMR). Two compounds of P1 and P2 were analyzed using NMR. ¹H NMR (400 MHz) and ¹³C NMR (100 MHz) spectra were recorded on a JNM-ECP 400 NMR spectrometer (JEOL, Tokyo, Japan), using a DMSO-d6 solvent peak (2.50 ppm in ¹H and 39.5 ppm in ¹³C NMR) as an internal reference standard. MS spectra were obtained on a JMS-700 spectrometer (JEOL, Japan). The structures of the purified compounds were identified and confirmed to be identical to the spectral data in Nakamura *et al.* (1996), Fukuyama *et al.* (1990), and Kang *et al.* (2003).

Culture of microorganism. *Propionibacterium acnes* (KCTC 3314) was obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, South Korea) and was incubated in CM 1135 brain heart infusion (BHI) agar (Oxoid Ltd.,

Table 1. MIC of the solvent polarity fractionate against *Propionibacterium acnes* tested by broth microdilution assay. The results are shown as average values from three separate experiments (MIC in µg/mL) and “-” is no inhibition at 10 mg/ml.

Minimum inhibitory concentration (µg/mL)							
Silica gel chromatography							
Crude methanol	Hexane	Diethyl ether	Acetone	Ethyl acetate	Acetonitrile	Methanol	Water
<i>P. acnes</i>	312	-	-	156	> 625	> 625	312

Hampshire, England) containing 1% glucose at 37 °C for 72 h under anaerobic conditions using the Bactron Anaerobic Chamber™ system (SHEL LAB, USA). Gas conditions were H₂ 5%, CO₂ 5%, and N₂ 90%.

Determination of MIC value. Antimicrobial activity was determined by broth microdilution assay, following the guideline of the National Committee for Clinical and Laboratory Standards (NCCLS) for anaerobic bacteria M11-A6 (NCCLS, 2004) in a 96 U-shaped well microplate. *Propionibacterium acnes* inocula were prepared from 24 h broth cultures, and suspensions adjusted to 0.5 McFarland solution turbidity. Samples were first diluted to the highest concentration (10 mg/mL) to be tested, followed by serial twofold dilutions to generate a concentration range of 19.5 to 10 mg/mL. All MIC tests were performed using three independent replicates.

Results

Isolation of antibacterial fractions. Methanol extract of *Ecklonia cava* showed considerable antibacterial activity. To identify compounds conferring antimicrobial activity, the methanol extract of *E. cava* (54.9 g) were further fractionated by silica-gel column chromatography using increasing solvent polarity. The yield of each solvent fraction was hexane (0.16 g), diethyl ether (2.95 g), acetone (3.34 g), ethyl

Table 2. MICs and purification factors of *Ecklonia cava* fractions against *Propionibacterium acnes* by broth microdilution assay. The results are shown as average values from three separate experiments (MIC in µg/mL) and “-” is not determined.

Extract and fraction type	MIC (µg/mL)	Purification factor	Weight	Yield (%)
<i>E. cava</i> powder	-	-	1 Kg	100
Methanol extract	312	1	54.9 g	5.49
Acetone fraction (Silica gel chromatography)	156	2	2.87 g	2.87×10 ⁻¹
Fraction No. 66-75 (Sephadex gel chromatography)	78	4	583.2 mg	5.832×10 ⁻²
Dieckol (HPLC)	39	8	9.2 mg	9.2×10 ⁻⁴
Phlorofucofuroeckol-A (HPLC)	39	8	6.4 mg	6.4×10 ⁻⁴

acetate (0.49 g), acetonitrile (1.58 g), methanol (38.56 g) and water (4.32 g). The acetone fraction exhibited the greatest activity against *Propionibacterium acnes* as indicated by the MIC (Table 1). To isolate the compounds conferring antibacterial activity, the acetone fraction was further purified by Sephadex LH-20 gel column chromatography using methanol as the eluent, with each fraction separated into 10

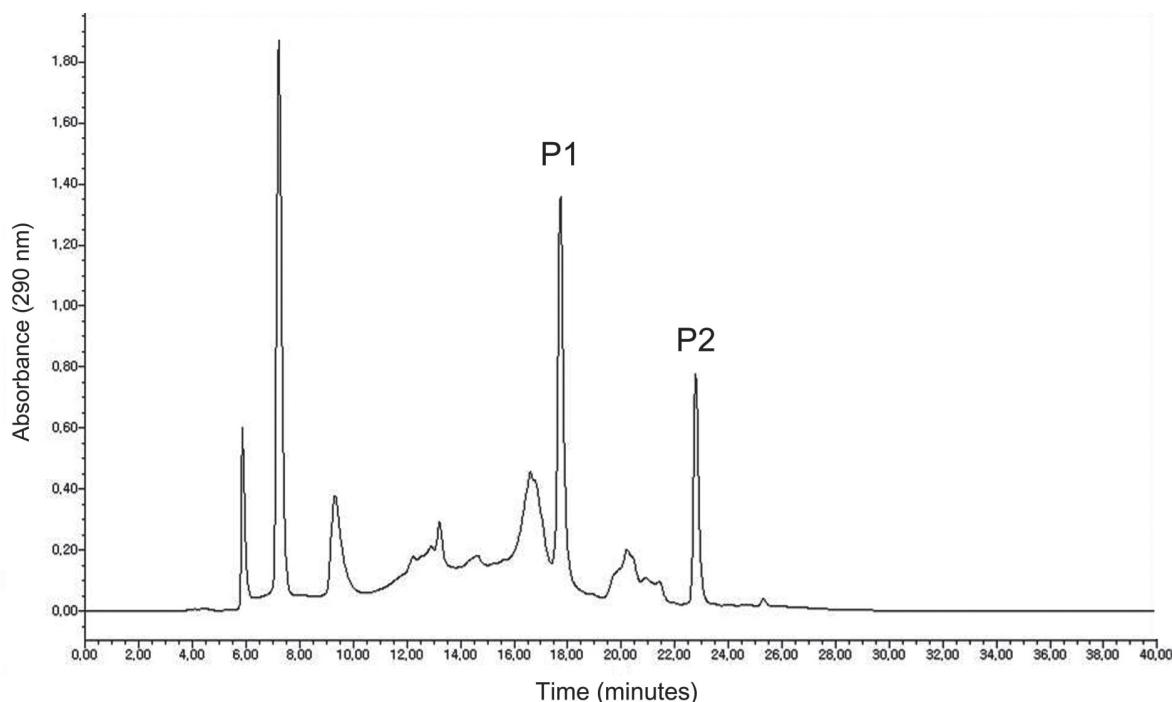


Figure 1. HPLC chromatogram of fraction Nos. 66-75, isolated by Sephadex gel chromatography. Elution was performed at a flow rate of 4.0 mL/min using a H₂O-MeOH linear gradient from 30 to 100% for 20 min, and maintained for 20 min. The UV detector was set at 290 nm.

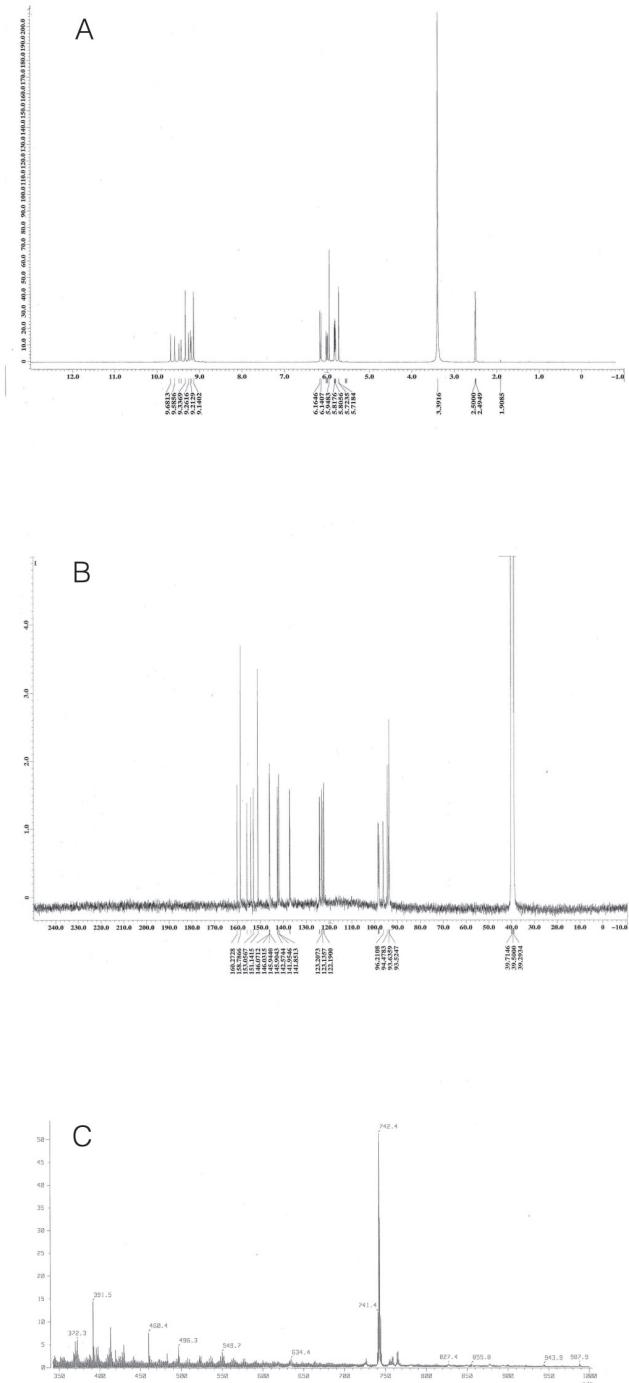


Figure 2. A) ¹H NMR spectrum, B) ¹³C NMR spectrum, and C) LRFAB-MS spectrum of peak 1 isolated by reverse-phase, high-performance liquid chromatographic separation.

mL aliquots. Fraction 66-75 showed the greatest antibacterial activity and was therefore, collected (583.2 mg) for further isolation.

The active fraction was subjected to HPLC using an Alltima C18 reverse-phase column (Alltech Inc., IL, USA). Elution was performed using a linear gradient from 30 to 100%

methanol for 20 min and maintained for 20 min, yielding two peaks at t_R (retention time) = 18 min (P1; 9.2 mg) and t_R = 24 min (P2; 6.4 mg). Both fractions showed marked activity against *Propionibacterium acnes* (Table 2).

Identification of antibacterial compounds. The purified compounds were analyzed in a Low Resolution Mass Spectrometer JMS-700 spectrometer (JEOL, Japan). The two peaks were identified as dieckol (P1) and phlorofucofuroeckol-A (P2) on the basis of NMR and mass spectrophotometer data, as well as through comparison with previously reported values (Figures 1-3). NMR readouts for compounds P1 and P2 were as follows:

P1 (dieckol).- light brown powder; ¹H NMR (DMSO-*d*₆, 400 MHz) δ 9.68 (1H, s, OH-9), 9.58 (1H, s, OH-9’), 9.48 (1H, s, OH-4’), 9.43 (1H, s, OH-4), 9.33 (2H, s, OH-3”,5”) , 9.26 (1H, s, OH-2’), 9.21 (1H, s, OH-2), 9.19 (1H, s, OH-7”), 9.14 (2H, s, OH-3”,5’), 6.16 (1H, s, H-3”), 6.14 (1H, s, H-3), 6.02 (1H, d, J = 3.0 Hz, H-8), 5.98 (1H, d, J = 2.7 Hz, H-8’), 5.94 (1H, s, H-2”,6”), 5.82 (1H, d, J = 2.7 Hz, H-6), 5.81 (1H, d, J = 2.7 Hz, H-600), 5.79 (1H, t, J = 2.0 Hz, H-4’), 5.72 (2H, d, J = 2.0 Hz, H-2”,6’); ¹³C NMR (DMSO-*d*₆, 100 MHz) δ 122.1 (s, C-1), 146.0 (s, C-2), 98.2 (d, C-3), 141.8 (s, C-4), 123.1 (s, C-4a), 142.3 (s, C-5a), 93.5 (d, C-6), 154.2 (s, C-7), 98.0 (d, C-8), 145.9 (s, C-9), 123.2 (s, C-9a), 137.2 (s, C-10a), 160.2 (s, C-1’), 93.6 (d, C-2’), 158.7 (s, C-3’), 96.2 (d, C-4’), 158.7 (s, C-5’), 93.6 (d, C-6’), 122.2 (s, C-1’), 145.9 (s, C-2”), 98.2 (d, C-3”), 141.9 (s, C-4”), 123.1 (s, C-4a”), 142.5 (s, C-5a”), 93.8 (d, C-6”), 153.0 (s, C-7”), 98.4 (d, C-8”), 146.0 (s, C-9”), 122.5 (s, C-9a”), 137.0 (s, C-10a”), 155.8 (s, C-1’”), 94.4 (d, C-2’”), 151.1 (s, C-3’”), 124.0 (s, C-4’”), 151.1 (s, C-5’”), 94.4 (d, C-6’”); LRFAB-MS m/z 742.36 [M]⁺ (Figure 2).

P2 (phlorofucofuroeckol-A).- light brown powder; ¹H NMR (DMSO-*d*₆, 400 MHz) δ 10.13 (1H, s, OH-14), 9.85 (1H, s, OH-4), 9.85 (1H, s, OH-10), 9.43 (1H, s, OH-2), 9.20 (2H, s, OH-3”,5”), 9.17 (2H, s, OH-3”,5’), 8.21 (1H, s, OH-8), 6.72 (1H, s, H-13), 6.43 (1H, s, H-9), 6.29 (1H, s, H-3), 5.84 (2H, t, J = 2.0 Hz, H-4”,4’), 5.76 (2H, d, J = 2.0 Hz, H-2”,6’), 5.72 (2H, d, J = 2.0 Hz, H-2”,6’); ¹³C NMR (DMSO-*d*₆, 100 MHz) δ 122.5 (s, C-1), 146.9 (s, C-2), 98.2 (d, C-3), 141.9 (s, C-4), 122.5 (s, C-4a), 133.9 (s, C-5a), 103.1 (s, C-6), 103.3 (s, C-7), 146.4 (s, D-8), 99.0 (d, C-9), 150.3 (s, C-10), 120.0 (s, C-11), 149.4 (s, C-11a), 150.7 (s, C-12a), 94.8 (d, C-13), 144.7 (s, C-14), 126.3 (s, C-14a), 136.8 (s, C-15a), 160.2 (s, C-1’), 93.7 (s, C-2’), 158.8 (s, C-3’), 96.3 (d, C-4’), 158.9 (s, C-5’), 93.7 (s, C-6’), 159.9 (s, C-1’”), 93.4 (d, C-2’”), 158.8 (s, C-3’”), 96.4 (d, C-4’”), 158.8 (s, C-5’”), 93.4 (d, C-6’”); LRFAB-MS m/z 602.38 [M+H]⁺ (Figure 3).

Antibacterial activity. The MIC of the *Ecklonia cava* methanol extract against *Propionibacterium acnes* was 312 μ g/mL; the MIC of the acetone fraction isolated by silica gel chro-

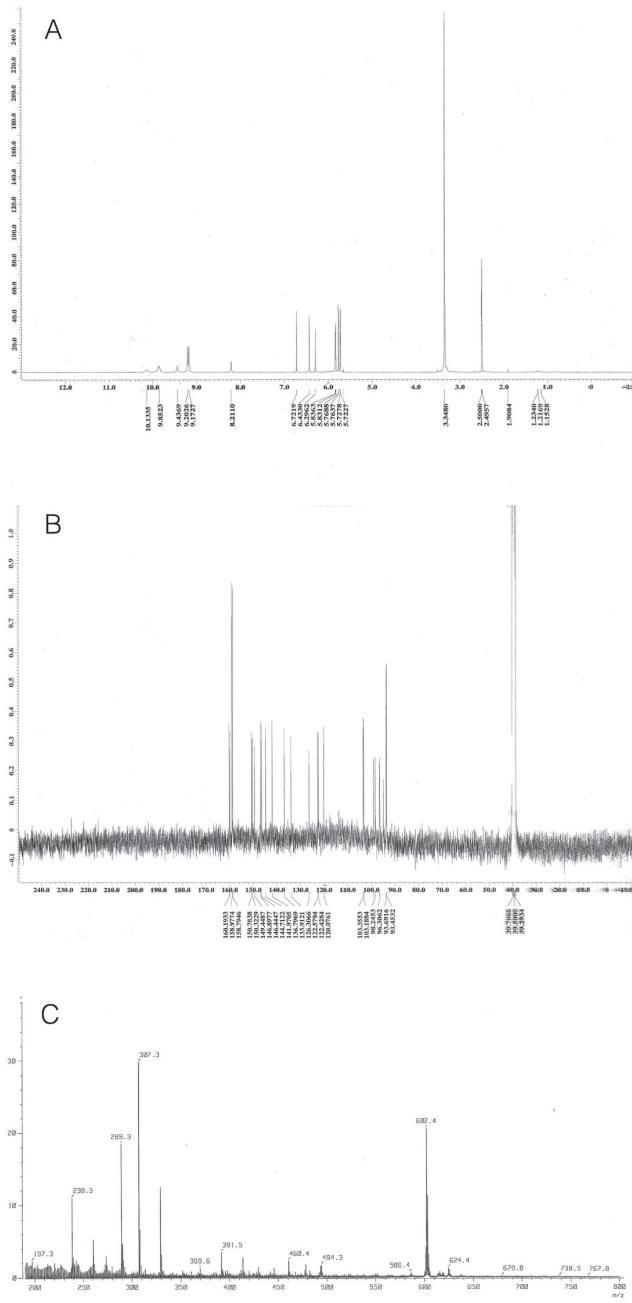


Figure 3. A) ^1H NMR spectrum, B) ^{13}C NMR spectrum, and C) LRFAB-MS spectrum of peak 2 isolated by reverse-phase, high-performance liquid chromatographic separation.

matography was 156 $\mu\text{g/mL}$. The MIC of fraction No. 66-75 purified by Sephadex gel column chromatography was 78 $\mu\text{g/mL}$. The MICs for purified dieckol and phlorofucoxanthin-*A* against *P. acnes* were both 39 $\mu\text{g/mL}$ (Tables 1, 2). The yields of dieckol and phlorofucoxanthin-*A* from *Ecklonia cava* were $9.2 \times 10^{-4}\%$ and $6.4 \times 10^{-4}\%$, respectively (Table 2).

Discussion

Ecklonia cava is a large, perennial brown alga (Laminariaceae) that is widely distributed throughout the subtidal regions of South Korea. Because of the economic and ecological importance of this species, it has been studied extensively (Bolton and Anderson, 1994; Wijesinghe and Jeon, 2012). *Ecklonia cava* has been used as a source of fucoidan, fucan sulfate, and phlorotannins, which have known anti-tumor, anticoagulant, antioxidant, and antithrombin properties (Heo *et al.*, 2005; Ahn *et al.*, 2007; Eom *et al.*, 2012; Wijesinghe and Jeon, 2012). Furthermore, *E. cava* has long been used throughout China and Korea in traditional herbal medicines for the treatment of cancer and inflammation (Xu *et al.*, 2002).

Among the active compounds isolated from *Ecklonia cava*, phlorotannins are the most important. Chemical analysis of *E. cava* revealed that phlorotannins, oligomeric polyphenols of the phloroglucinol class compounds, are the main constituents responsible for the biological activity of *E. cava*; they include eckol, dieckol, bieckol and phlorofucofuroeckol-A (Eom *et al.*, 2012). Structurally, phlorotannins are oligomers and polymers of 1,3,5-trihydroxybenzene (phloroglucinol), which are formed via the polyketide pathway (Ragan and Glombitza, 1986; Waterman and Mole, 1994). They have shown broad therapeutic potential, including anti-diabetes, antioxidation, radiation protection, anti-cancer, anti-HIV, and anti-allergenic activities (Li *et al.*, 2011).

In our previous study, *Ecklonia cava* was used as the starting material to identify compounds exhibiting unique antibacterial activities against microorganisms frequently involved in acne inflammation, such as *Propionibacterium acnes* (Choi *et al.*, 2011). In this study, we further fractioned the *E. cava* methanol extract using silica gel chromatography, Sephadex gel column chromatography, and HPLC. Fractions were then analyzed by NMR and the active components identified. Based on the Low Resolution Fast Atom Bombardment-Mass Spectrometry (LRFAB-MS) data, and through comparison of ^1H and ^{13}C NMR results in Nak-

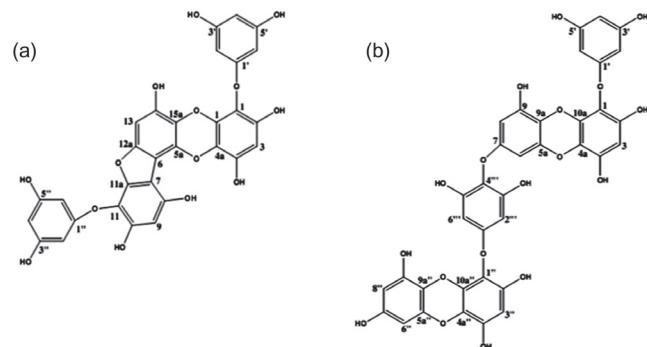


Figure 4. Chemical structures of (A) dieckol, and (B) phlorofuco-furoeckol-A.

mura *et al.* (1996), Fukuyama *et al.* (1990), and Kang *et al.* (2003), the active compounds were identified as dieckol and phlorofucoxanthin.

Dieckol and phlorofucoxanthin are phlorotannin derivatives of brown algae. The antibacterial activities of phlorotannins against a range of pathogenic bacteria have been demonstrated (Eom *et al.*, 2012). They showed antimicrobial effect against food-borne pathogenic bacteria, antibiotic resistance bacteria, and human tinea pedis fungus. Although many studies have examined the antimicrobial activities of phlorotannins isolated from marine algae, we focused specifically on the isolation and identification of antimicrobial compounds active against acne-causing bacteria. The gram-positive bacterium *Propionibacterium acnes* ATCC 6919 was selected based upon its ability to cause acne. The MICs of dieckol and phlorofucoxanthin against *P. acnes* were both 39 µg/mL.

In addition to its antibacterial properties, dieckol purified from *Ecklonia cava* has been shown to exhibit fungicidal activity (MIC = 148 mg/mL) against *Trichophyton rubrum*, a causative agent of dermatophytic nail infections in humans (Lee *et al.*, 2010). Dieckol isolated from *E. stolonifera* exhibits strong anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity, with MICs of 32-64 µg/mL (Lee *et al.*, 2008).

Phlorofucoxanthin purified from *Eisenia bicyclis* inhibits MRSA at concentrations > 32-64 µg/mL (Eom, 2012). Phlorofucoxanthin purified from *E. kurome* exhibits bactericidal activity against *Campylobacter jejuni*, *Escherichia coli*, *Salmonella enteritidis*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus* at concentrations > 800.0 µg/mL (Nagayama *et al.*, 2002).

Structurally, phlorotannins are oligomers and polymers of 1,3,5-trihydroxybenzene (phloroglucinol), which are formed via the polyketide pathway (Ragan and Glombitza, 1986; Waterman and Mole, 1994). Although the relationship between the structure and antimicrobial activity of phlorotannins is poorly understood, the physiological activity may depend on the degree of polymerization of phlorotannin derivatives (Ragan and Glombitza, 1986; Eom *et al.*, 2012). It has been reported that eckol, dieckol, bieckol, and phlorofucoxanthin are the most abundant phlorotannin compounds in *Ecklonia cava* (Li *et al.*, 2011). In this study, two phlorotannins, dieckol and phlorofucoxanthin, representing 9.2×10⁻⁴ and 6.4×10⁻⁴ of the methanol extract, respectively, were shown to exhibit antibacterial activity.

Conclusions

The data suggest that phlorotannins have pronounced antimicrobial activity against acne-causing bacteria. Moreover, no cytotoxic effects were observed following oral administration of phlorotannins at a dosage of 170–1,500 mg/kg/day for 14 days in mice (Nagayama *et al.*, 2002). Together, these

results suggest that the phlorotannins dieckol and phlorofucoxanthin isolated from *Ecklonia cava* could be useful antibiotic agents for both cosmetic and pharmaceutical applications.

Acknowledgments

This research was supported by the Korea Small and Medium Business Administration (Grants No.00036130), Republic of Korea, and was also supported by the Global Healthcare Industry RIS Center, Ministry of Knowledge Economy, Republic of Korea. KKC was also supported by Gyeongnam National University of Science and Technology Grant 2012, Republic of Korea, and Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012-006683). We are grateful to reviewers for helpful comments.

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Received: June 17th, 2013

Accepted: October 12th, 2013