

## Evaluation of the Antibacterial Activity of Rhapontigenin Produced from Rhapontin by Biotransformation Against *Propionibacterium acnes*

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**Biotransformation is often used to improve chemical activity. We evaluated the antimicrobial activity of rhapontigenin, converted from rhapontin after treatment with Pectinex. Rhapontigenin showed 4–16 times higher antimicrobial activity than rhapontin. The activity was higher against Gram-positive strains than Gram-negative strains. Minimum inhibitory concentrations (MICs) of rhapontigenin, retinol, and five antibiotics were determined by the microbroth dilution method for antibiotic-sensitive and -resistant *Propionibacterium acnes*. We also investigated the *in vitro* antibacterial activity of rhapontigenin in combination with antibiotic against antibiotic-resistant *P. acnes*. The antibiotic combination effect against resistant *P. acnes* was studied by the checkerboard method. The combination formulations (rhapontigenin and clindamycin, retinol and clindamycin) showed synergistic effects on the inhibition of the growth of clindamycin-resistant *P. acnes*. It is predictable that the combination of antibiotics with rhapontigenin is helpful to treat acne caused by antibiotic-resistant *P. acnes*. The antibacterial activity of rhapontigenin was enhanced by biotransformation.**

**Keywords:** Rhapontigenin, *P. acnes*, biotransformation, checkerboard test

Rhapontin (rhaponticin), which has a stilbene moiety, is a major component of *Rheum undulatum* root extract. Rhapontin glucoside is hydrolyzed into rhapontigenin, the aglycone of rhapontin, by glucosidase. In a previous study, rhapontigenin showed antioxidant activity by scavenging reactive oxygen species (ROS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [33]. The scavenging effect of rhapontigenin was greater than rhapontin.

Rhapontigenin also protected the membrane lipid peroxidation and cellular DNA damage induced by H<sub>2</sub>O<sub>2</sub>. Another study reported rhapontigenin had anticancer activity [29].

Enzymatic conversion of prodrugs improves bioavailability [13], whereas biotransformation of enzymes with glucosides into aglycones improves their biological activities [14, 18, 20]. Rhapontigenin, converted from rhaponticin by intestinal microflora, showed improved antithrombic and antiallergic activities [11, 21]. Phenolic compounds have antimicrobial activity [7, 23, 28], which may be improved by removing glucosides through biotransformation [26].

*Propionibacterium acnes* belongs to the human cutaneous normal flora and is a major etiologic agent of acne vulgaris [32]. Retinoids such as retinoic acid, retinol, and retinaldehyde are most commonly used to treat acne and to prevent skin aging because of their antioxidant activity [22]. However, their main side effect is skin irritation [3]. Clindamycin is the most frequently used topical anti-*P. acnes* agent. However, extensive use of the antibiotic has caused the occurrence of widespread resistance in cutaneous *Propionibacterium* [19]. One strategy to prevent antibiotic resistance is the use of combination antibiotic therapy [30].

The aim of the present study was to evaluate the *in vitro* antimicrobial activity of rhapontigenin produced from rhapontin by biotransformation and to investigate the synergistic effects of rhapontigenin combined with clindamycin against clindamycin-resistant *P. acnes*.

### MATERIALS AND METHODS

#### Bacterial Strains

Gram-negative strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, and Gram-positive strains *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212, were used. Anaerobic Gram-positive *Propionibacterium acnes* KCTC 3314 was obtained from the Korean Collection for Type Culture (Daejeon, Korea). *P. acnes* CCARM 9010 (clindamycin-resistant strain) was

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obtained from the Culture Collection of Antimicrobial Resistant Microbes (Seoul, Korea). *P. acnes* strains were cultured in GAM broth (Nissui, Tokyo, Japan) at 37°C for 48 h in an anaerobic gas-generating pouch (GasPak EZ; Becton, Dickinson and Company, Sparks, MD, U.S.A.). Other strains were cultured in Mueller–Hinton broth (Difco, U.S.A.) at 37°C for 18–24 h.

#### Antibiotics and Chemicals

Clindamycin, penicillin G, chloramphenicol, erythromycin, and retinol were purchased from Sigma (St. Louis, MO, U.S.A.). Ampicillin was purchased from USB (Cleveland, OH, U.S.A.). FM4-64 was purchased from Molecular Probe (Eugene, OR, U.S.A.).

#### Preparation of Rhapontigenin

The root of *Rheum undulatum* (48 kg) was extracted with methanol (160 l). The extract was distilled at 75°C and suspended in 40 l of water. Ethylacetate (80 l) was added and mixed for 30 min, and then left for 2 h at room temperature. The water layer was separated and left for 16 h at room temperature, followed by filtration. The filtered material was washed with water (10 l) and ethylacetate (10 l) and vacuum dried for 30 h at 50°C, which yielded 1 kg of crude rhapontin. Rhapontin (30 g) was dissolved in dimethylsulfoxide (DMSO), and then 1.5 l of acetate buffer (pH 4.0) and 100 ml of Pectinex (Novozymes, Denmark) were added and the reaction was incubated for 2 h at 55°C with shaking at 100 rpm. Water (150 ml) was added and the reaction mixture was left overnight at 4°C, followed by filtration. The filtered material was vacuum dried for 30 h at 50°C, yielding 11.8 g of rhapontigenin. The bioconversion process of rhapontin to rhapontigenin is shown in Fig. 1. Rhapontin and rhapontigenin were identified using HPLC (LCQ Advantage MAX; Thermo Electron, U.S.A.), MALDI–Mass (Voyager-DE STR Biospectrometry Workstation; Applied Biosystems Inc., U.S.A.), and <sup>1</sup>H NMR (Varian 400-MR, 500 MHz; Varian Inc., U.S.A.) data [9]. Authentic rhapontin was purchased from Sigma. The identified compounds were deposited in the Department of Chemical Engineering and Biotechnology, Korea Polytechnic University.

#### Determination of Growth Inhibition

Clindamycin-sensitive and -resistant *P. acnes* strains were adjusted to the McFarland 0.5 standard and used to inoculate GAM agar

plates. Disks (6 mm diameter) containing 128, 256, and 384 µg of rhapontigenin or DMSO only were placed on the plates. The GAM agar plates were incubated in an anaerobic gas-generating pouch at 37°C for 48 h. The inhibition zone was determined by measuring its diameter.

#### Determination of Minimum Inhibitory Concentrations (MICs)

MICs of rhapontin and rhapontigenin were determined by the CLSI (formerly NCCLS) microbroth dilution method [17]. Rhapontin and rhapontigenin were dissolved in DMSO. A dilute suspension of bacteria was inoculated into each well of a 96-well microplate, each containing a different concentration of rhapontin or rhapontigenin. We performed doubling dilutions of the antimicrobial agents. The range of dilutions was 2,048 µg/ml to 0.5 µg/ml in the culture broth. A final concentration of  $1 \times 10^5$  CFU/ml of test bacteria was added to each dilution. The plates for *P. acnes* were incubated in an anaerobic gas-generating pouch at 37°C for 48 h, where the incubation atmosphere contained 5% CO<sub>2</sub>. The plates for the other test strains were incubated at 37°C for 18–24 h. MIC was defined as the lowest concentration of antimicrobial agent that inhibited bacterial growth, as indicated by the absence of turbidity. Antimicrobial agent-free broths containing 5% DMSO were incubated as growth controls. Minimum bactericidal concentration (MBC) of rhapontigenin against *P. acnes* was determined by inoculating onto GAM agar plates 10 µl of medium from each of the wells from the MIC test that showed no turbidity. MBCs were defined as the lowest concentration of antimicrobial agent for which there was no bacterial growth on the plates.

#### Checkerboard Titrations

For the checkerboard titration, the concentrations tested for rhapontigenin, retinol, and clindamycin ranged from seven 2-fold serial dilutions below the MIC to the MIC for the test strains. GAM broth was used for the checkerboard tests and a final concentration of  $1 \times 10^5$  CFU/ml was inoculated. MICs of the combinations were determined after incubation at 37°C for 48 h. Fractional inhibitory concentration (FIC) indices, determined by averaging all of the FIC values of wells along the growth–no-growth interface, were calculated at 48 h. An index of less than 0.5 was considered as synergism, and of greater than 2.0 as antagonism [24].

All experiments were independently repeated three times and the data in tables are expressed as the mean value of the results.

#### Confocal Microscopy

To examine cell morphology changes after exposure to rhapontigenin, confocal microscopy was performed. Each strain was grown to mid-exponential phase, in Luria–Bertani (LB) broth for *E. coli* and in GAM broth for *P. acnes*, and then treated with rhapontigenin at MIC (256 and 128 µg/ml for *E. coli* and *P. acnes*, respectively) or with DMSO as a control for 3 h at 37°C. A 1-ml sample was transferred to a microtube, pelleted by centrifugation, washed once in phosphate-buffered saline (PBS), and resuspended in 100 µl of PBS containing 10 µM FM4-64 (558/734 for excitation/emission). The samples were incubated in the dark with rotation for 30 min at 37°C. Each sample was pelleted by centrifugation, washed twice in PBS, repelleted, and resuspended in 100 µl of PBS. Ten µl was placed onto a slide, allowed to air dry in the dark, covered with a cover slip, and mounted. Samples were visualized with a confocal laser scanning microscope (LSM 5 Exciter, Carl-Zeiss).

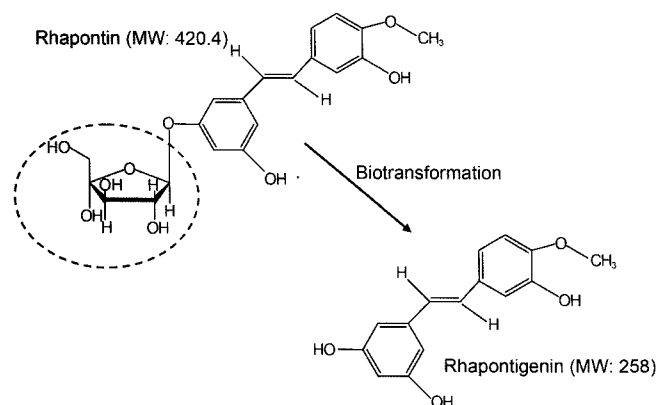


Fig. 1. Biotransformation of rhapontin into rhapontigenin.

**Table 1.** MIC values of rhapontin and rhapontigenin against the test strains.

| Strain                           | Rhapontin ( $\mu\text{g/ml}$ ) | Rhapontigenin ( $\mu\text{g/ml}$ ) |
|----------------------------------|--------------------------------|------------------------------------|
| Gram-positive                    |                                |                                    |
| <i>S. aureus</i>                 | $\leq 1,024$                   | $\leq 128$                         |
| <i>E. faecalis</i>               | $\leq 1,024$                   | $\leq 64$                          |
| <i>P. acnes</i>                  | $\leq 1,024$                   | $\leq 128$                         |
| <i>P. acnes</i> (R) <sup>a</sup> | $\leq 1,024$                   | $\leq 128$                         |
| Gram-negative                    |                                |                                    |
| <i>E. coli</i>                   | $\leq 1,024$                   | $\leq 256$                         |
| <i>P. aeruginosa</i>             | $\leq 1,024$                   | $\leq 256$                         |

<sup>a</sup>Clindamycin-resistant strain

## RESULTS

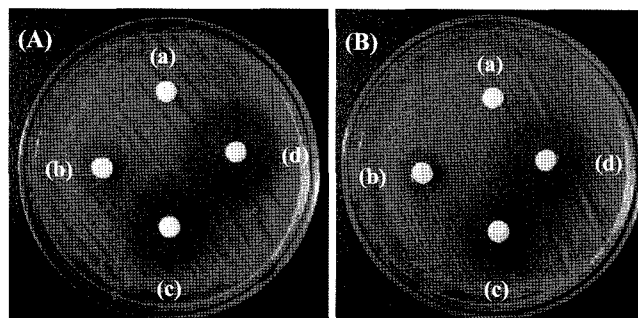
### Antimicrobial Activity of Rhapontigenin

Rhapontigenin was converted from rhapontin (Fig. 1). To investigate the antimicrobial activity of rhapontigenin, we examined its growth inhibition effect against Gram-positive and Gram-negative test strains. The MICs of rhapontin and rhapontigenin against the test strains are shown in Table 1. Rhapontigenin showed 4–16 times higher antimicrobial activity than rhapontin. Rhapontin showed no difference in MIC values between Gram-positive and Gram-negative strains. In contrast, rhapontigenin showed lower MIC values against Gram-positive strains than Gram-negative strains. Thus, the inhibition activity of rhapontigenin was higher in Gram-positive strains than in Gram-negative strains. The MIC values against clindamycin-sensitive and -resistant *P. acnes* strains were not different, although the clindamycin-resistant strain showed slightly more resistance to rhapontigenin in the inhibition zone test (Table 2, Fig. 2). Rhapontigenin acted in a concentration-dependent manner.

The MICs and MBCs of rhapontigenin and retinol against *P. acnes*, along with ampicillin, penicillin G, chloramphenicol, erythromycin, and clindamycin, are shown in Table 3. Retinol is the most commonly used antioxidant showing anti-*P. acnes*. Under the conditions used, there was no difference between clindamycin-sensitive and -resistant strains in their degree of sensitivity to rhapontigenin, which were  $\leq 128 \mu\text{g/ml}$  and  $\leq 512 \mu\text{g/ml}$  for MIC and

**Table 2.** Growth inhibition of rhapontigenin against the *P. acnes* strains<sup>a</sup>.

|                                  | Rhapontin ( $\mu\text{g}$ ) |     |     | Rhapontigenin ( $\mu\text{g}$ ) |                |                |
|----------------------------------|-----------------------------|-----|-----|---------------------------------|----------------|----------------|
|                                  | 128                         | 256 | 384 | 128                             | 256            | 384            |
| <i>P. acnes</i>                  | - <sup>c</sup>              | -   | -   | 15.7 $\pm$ 0.6                  | 24.3 $\pm$ 1.2 | 29.0 $\pm$ 1.7 |
| <i>P. acnes</i> (R) <sup>b</sup> | -                           | -   | -   | 14.3 $\pm$ 1.2                  | 23.7 $\pm$ 1.2 | 28.0 $\pm$ 2.0 |

<sup>a</sup>The unit of the inhibition zone is in millimeters.<sup>b</sup>Clindamycin-resistant strain.<sup>c</sup>No inhibition zone.**Fig. 2.** Inhibition zone of *P. acnes* ATCC 6919 (A) and *P. acnes* CCARM 9010 (B) by rhapontigenin 128  $\mu\text{g/ml}$  (b), 256  $\mu\text{g/ml}$  (c), 384  $\mu\text{g/ml}$  (d), and DMSO only (a).

MBC, respectively. The MICs of retinol were  $\leq 8 \mu\text{g/ml}$  and  $\leq 16 \mu\text{g/ml}$  against clindamycin-sensitive and -resistant strains, respectively. The MICs of clindamycin were  $\leq 0.0625 \mu\text{g/ml}$  and  $\leq 32 \mu\text{g/ml}$  against clindamycin-sensitive and -resistant *P. acnes*, respectively.

### Synergistic Effect of Rhapontigenin and Antibiotic

The efficacy of combination of rhapontigenin was evaluated against only clindamycin-resistant *P. acnes*, a major etiologic agent of acne, and compared with that of retinol, because the growth of sensitive strains is easily inhibited with a very low concentration of clindamycin alone. For the test, checkerboard tests were performed with clindamycin and rhapontigenin or retinol. The results of the checkerboard tests are summarized in Table 4. All the combinations were synergistic according to the FIC index. From the most synergistic concentrations of antibiotic in the checkerboard results, the concentrations of clindamycin combined with rhapontigenin or retinol required 4  $\mu\text{g/ml}$  and 2  $\mu\text{g/ml}$  to inhibit antibiotic-resistant *P. acnes*, respectively, which were less than an eighth and sixteenth of the amount (32  $\mu\text{g/ml}$ ) required using clindamycin alone, respectively. In this case, the amounts of rhapontigenin and retinol

**Table 3.** Antimicrobial activity of rhapontigenin, retinol, and other antibiotics against clindamycin-sensitive and -resistant *P. acnes*.

| Antimicrobial agent | Clindamycin-sensitive strain |                          | Clindamycin-resistant strain |                          |
|---------------------|------------------------------|--------------------------|------------------------------|--------------------------|
|                     | MIC ( $\mu\text{g/ml}$ )     | MBC ( $\mu\text{g/ml}$ ) | MIC ( $\mu\text{g/ml}$ )     | MBC ( $\mu\text{g/ml}$ ) |
| Rhapontigenin       | $\leq 128$                   | $\leq 512$               | $\leq 128$                   | $\leq 512$               |
| Retinol             | $\leq 8$                     | $\leq 256$               | $\leq 16$                    | $\leq 512$               |
| Chloramphenicol     | $\leq 0.25$                  | $\leq 0.5$               | $\leq 0.5$                   | $\leq 0.5$               |
| Erythromycin        | $\leq 0.0625$                | $\leq 0.0625$            | $\leq 512$                   | $\leq 1,024$             |
| Penicillin G        | $\leq 0.0625$                | $\leq 0.0625$            | $\leq 0.0625$                | $\leq 0.0625$            |
| Ampicillin          | $\leq 0.0625$                | $\leq 0.0625$            | $\leq 0.0625$                | $\leq 0.0625$            |
| Clindamycin         | $\leq 0.0625$                | $\leq 0.0625$            | $\leq 32$                    | $\leq 64$                |

**Table 4.** Checkerboard results for clindamycin-resistant *P. acnes*.

| Drug combination | FIC index   | Most synergistic concentration ( $\mu\text{g/ml}$ ) | Maximum concentration tested ( $\mu\text{g/ml}$ ) |
|------------------|-------------|-----------------------------------------------------|---------------------------------------------------|
| RHA/CLI          | 0.133–0.531 | 1/4                                                 | 128/32                                            |
| REL /CLI         | 0.070–0.531 | 0.125/2                                             | 16/32                                             |
| RHA /REL         | 0.375–1.125 | 16/4                                                | 128/16                                            |

Abbreviations: RHA, rhapontigenin; REL, retinol; CLI, clindamycin.

required to inhibit antibiotic-resistant *P. acnes* were also reduced from 128  $\mu\text{g/ml}$  to 1  $\mu\text{g/ml}$  and from 16  $\mu\text{g/ml}$  to 0.125  $\mu\text{g/ml}$ , respectively. In the case of combining rhapontigenin with retinol, the amount of rhapontigenin was reduced to an eighth of the amount required when used alone and the amount of retinol required was 4  $\mu\text{g/ml}$ .

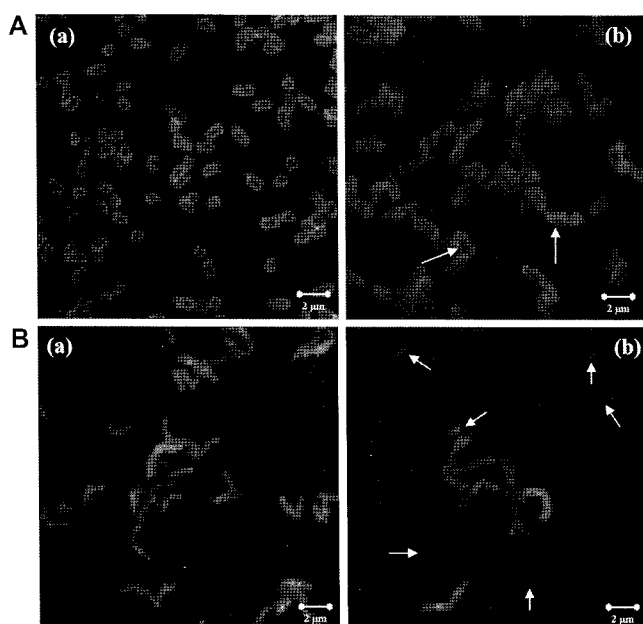
### Confocal Microscopy

Phenolic compounds disrupt cell membrane, and rhapontigenin is a phenolic compound. We used confocal microscopy to obtain images of cells after treatment with rhapontigenin to assess whether the inhibitory activity was associated with cell membrane disruption. We used FM4-64, a widely used fluorescent dye for plasma membrane staining. Microscopic observations revealed the membranes of cells treated with rhapontigenin were disrupted (Fig. 3A and 3B, b) compared with untreated cell membranes (Fig. 3A and 3B, a). Cells treated with rhapontigenin (Fig. 3A and 3B, b) were also elongated compared with untreated cells (Fig. 3A and 3B, a). *P. acnes* is markedly pleomorphic and often stains unevenly, as shown in Fig. 3B. *P. acnes* cells treated with rhapontigenin

produced many unstained cell debris (Fig. 3B, b). *E. coli* treated with rhapontigenin showed a regular cell shape, although the cells became longer. On the other hand, *P. acnes* showed a more irregular cell shape and irregular staining pattern. This might cause the difference in viability between the two strains.

### DISCUSSION

Flavonoids, extracted from plant materials, generally contain glycosides. Flavonoid aglycones exhibit more antimicrobial activity than their corresponding glycosides [31]. Therefore, biological activities, such as antimicrobial action, can be improved by eliminating glycosides through a bioconversion process. Phenolic compounds isolated from *Dalea spinosa* potentiated antibiotic activity against multidrug resistant *S. aureus* by inhibiting the efflux pump mechanism [1]. 6-Oxophenolic triterpenoids showed antimicrobial activity against Gram-positive *Bacillus subtilis* and caused cell membrane alterations [6]. Rhaponticin has inhibitory activity against nitric oxide production and its glucoside moiety reduced the activity, suggesting the aglycone form plays an important role in its inhibitory activity [10]. The glucoside moiety of rhaponticin also reduced its scavenging activity [15]. In the present study, rhapontigenin prepared from rhapontin by bioconversion showed 4–16 times higher antimicrobial activity, suggesting the glucoside structure interferes with antimicrobial activity. Phenolic compounds extracted from honey showed antimicrobial and antioxidant activity; generally, Gram-positive bacteria were more sensitive to the compounds than Gram-negative bacteria [8]. Phenolic antioxidants used as food additives are more effective against Gram-positive than against Gram-negative bacteria [27], which is consistent with our results. In addition, the MICs of rhapontigenin against antibiotic-sensitive and -resistant *P. acnes* were the same, which means that *P. acnes* did not cause resistance against rhapontigenin (Table 1). Although phenolic compounds are produced for protection against microbial infection in plants, there are contradicting reports on the relationship between the structure of such compounds and their antimicrobial activity. Flavonoids lacking hydroxyl groups on their B ring showed higher activity than those with hydroxyl groups [2]. On the other hand, free hydroxyl



**Fig. 3.** Confocal microscopy of *E. coli* (A) and *P. acnes* (B) untreated (a) and after treatment with rhapontigenin (b).

The fluorescence images stained with FM4-64. Arrows indicate cells with membrane damage.

groups of flavonoids were reported to be necessary for antimicrobial activity [16]. Although we could not clearly elucidate its inhibitory mechanism against *P. acnes*, we assume that rhapontigenin has the same mechanism of inhibition as other phenolic compounds have.

Clindamycin is a commonly used topical antibiotic for treatment of acne. Clindamycin-resistant strains of *P. acnes*, however, have been isolated worldwide [4]. The use of combination treatments of topical antibiotics is preferred to enhance the inhibitory activity and to reduce the incidence of antibiotic-resistant *P. acnes*. In the present study, the three combination formulations (clindamycin with either rhapontigenin or retinol; and rhapontigenin and retinol) exhibited a synergistic inhibition of *P. acnes* growth. Although the combination of rhapontigenin and retinol showed synergistic effect, the combination is less effective than those of clindamycin and rhapontigenin or retinol.

Cell shape influences cell growth and survival. Cell morphology changed after exposure to rhapontigenin, which may have affected cell viability, resulting in growth inhibition. Several antibacterial inhibitory mechanisms of phenolic compounds are proposed, such as disruption of the cytoplasmic membrane, change in the permeability of the membrane, and inhibition of the membrane respiratory chain [12, 25, 27]. Lipophilic compounds easily bind to cell membranes, changing membrane properties. Membrane damage blocks its proper function, a proposed mechanism of the antimicrobial action of phenolic compounds [5]. The images from confocal microscopy showed rhapontigenin disrupted cell membranes, possibly owing to the hydroxyl groups and lipophilic groups of rhapontigenin. In addition, the structure of rhapontigenin is simpler than rhapontin, which causes less steric hindrance in penetrating cell membranes. These factors may have contributed to the increased inhibition in bacterial cell growth found in rhapontigenin treatment. We found rhapontigenin disrupted cell membranes to some extent and changed cell morphology, factors which may decrease cell viability in bacterial strains. Clindamycin is a bacteriostatic antibiotic that interacts with 23S rRNA in 50S ribosomal subunits and inhibits protein synthesis. Retinol has been considered as an anti-infective agent, but the mechanism of the antimicrobial activity has not been fully elucidated. The synergistic effect in combination of rhapontigenin with clindamycin, shown in Table 4, might be based on the cooperation action of the membrane disruption of rhapontigenin and the inhibition of the protein synthesis of clindamycin. Rhapontigenin is lipid-soluble and it provides an advantage to be used for the treatment of acne, because lipid-soluble compounds are generally used to treat acne. Further studies are needed to elucidate the clear inhibitory mechanism of rhapontigenin.

In conclusion, rhapontigenin possesses antioxidant activity and antimicrobial activity against *P. acnes*, an

etiologic agent of acne. Moreover, the antimicrobial activity of rhapontigenin was enhanced by appropriate combination with a topical antibiotic. Those activities of rhapontigenin were improved by removing glucosides from rhapontin through biotransformation.

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