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# Discovery of Zeylenone from *Piper griffithii* (Piperaceae) as a Potential Anti-Acne Bacterial Agent and Its Mechanism of Action Against Acne-Causing Bacteria

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**Abstract**

An abnormal increase in acne-causing bacteria is the main cause of acne. This study aimed to investigate *Piper griffithii* C.DC. as a new source of compounds for inhibiting acne-causing bacteria and to provide the first elucidation of the mechanism of action against these bacteria. The antibacterial efficacy of 27 *Piper* species was examined against acne-causing clindamycin-resistant bacterial strains. Antibacterial activity of various crude extracts derived from leaves or stems extracted using hexane, ethyl acetate, or ethanol was evaluated. Ethyl acetate leaf extract of *P. griffithii* exhibited the greatest antibacterial effect against all tested bacteria. Zeylenone, an antibacterial substance isolated, purified, and characterized from the ethyl acetate leaf extract of *P. griffithii*, disrupts cell walls and membranes. Minimum bactericidal concentration (MBC) values were 1.25, 2.5, and 7.5 mg/mL for *Cutibacterium acnes*, *Staphylococcus aureus*, and *S. epidermidis*, respectively. Zeylenone derived from *P. griffithii* leaves was nontoxic to human skin keratinocytes (HaCaT). A formulated anti-acne gel with zeylenone was effective in controlling acne-causing bacteria. These results suggest that zeylenone isolated from *P. griffithii* leaves can be further developed as a natural ingredient in anti-acne products. This is the first report of the use of zeylenone from *P. griffithii* for eliminating acne-causing bacteria.

**Keywords:** acne-causing bacteria; antibacterial activity; *Cutibacterium acnes*; *Piper griffithii*; zeylenone

## 1. Introduction

*Acne vulgaris* is a chronic inflammatory disorder of the pilosebaceous unit that affects a large proportion of adolescents and adults worldwide. It represents one of the most prevalent dermatological diseases and a major cause of psychosocial burden [1–3]. Although the pathogenesis of acne is multifactorial, colonization and hyperproliferation of acne-causing bacteria, particularly *Cutibacterium acnes*, *Staphylococcus epidermidis*, and *S. aureus*, play key roles in lesion development and inflammation [3,4]. Among these microorganisms, *C. acnes* is recognized as the predominant contributor to acne due to its distinctive immunomodulatory effect that primarily stimulates inflammatory responses, whereas *S. epidermidis* and *S. aureus* contribute to more severe disease through biofilm formation, opportunistic infection, and synergistic inflammatory responses [3,5,6]. The increasing global prevalence of antibiotic-resistant strains of these bacteria poses a significant

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challenge to acne management and has reduced the long-term effectiveness of conventional antimicrobial therapies [7,8].

Current acne treatments rely predominantly on topical and systemic antibiotics, including clindamycin, tetracycline, erythromycin, benzoyl peroxide, and retinoids. However, their prolonged use has led to adverse effects such as skin irritation, disruption of the cutaneous microbiota, and the emergence of antimicrobial resistance [7–9]. These limitations have stimulated growing interest in alternative therapeutic strategies based on natural products, particularly plant-derived antibacterial compounds that offer broad-spectrum activity with lower toxicity, do not induce resistance, and can potentially be incorporated into topical formulations [10–13]. Several medicinal plants exhibit inhibitory effects against *C. acnes* and Staphylococcal species. Many extracts demonstrate comparable in vitro antimicrobial activity to reference drugs, making them promising candidates for anti-acne development [14–18].

The genus *Piper*, belonging to the family Piperaceae, comprises over 2000 species distributed throughout tropical and subtropical regions. It has long been regarded as a source of medicinal plants and vegetables. Fifty-one species and two varieties of such plants have been identified in Thailand [19]. These plants have a long history of use in traditional medicine for the treatment of infectious, inflammatory, and dermatological conditions [20,21]. Phytochemical research indicates that extracts of *Piper* species are generally rich in bioactive compounds such as amides, alkaloids, flavonoids, tannins, terpenoids, saponins, and phenolic compounds, which possess numerous pharmacological properties, including antimicrobial activity [22]. Amide alkaloids are the most typical constituents produced by the genus *Piper*, with piperine being a predominant and widespread example. They are abundant in *P. longum* and *P. nigrum*, contributing to diverse biological effects, encompassing antimicrobial, antioxidation, and anti-inflammatory properties [23,24]. In recent decades, research has focused on the bioactive compounds present in *P. betle*, *P. longum*, and *P. nigrum* extracts and their biological activities [21,25], with scant information on the anti-acne bacterial activity of *Piper* extracts. Budiman et al. [26] reported on the antibacterial activity of *P. betle* leaf extract against two acne-causing bacteria, *C. acnes* and *S. aureus*. However, there are still many other *Piper* species with significant potential that is currently underexplored in anti-acne research. There is limited information regarding specific active metabolites and their mechanisms of action.

*Piper griffithii* C.DC., a medicinal plant traditionally used in Southeast Asia, remains largely unexplored with respect to its antibacterial properties and bioactive constituents. To date, there have been no reports describing the presence of zeylenone in *P. griffithii* or its antibacterial activity against acne-causing bacteria. Zeylenone, a naturally occurring polyoxygenated cyclohexene biosynthesized via the shikimate pathway, has been found in a few plant families, including Annonaceae and Piperaceae. Zeylenone was originally and is most often isolated from plants in the genus *Uvaria* (family Annonaceae), such as *U. grandiflora* and *U. purpurea*. The biological activity of zeylenone from the *Uvaria* species was reported to trigger apoptosis in both colon cancer cells and cervical carcinoma cells [27–29]. Additionally, zeylenone isolated from the stems of Thai *P. ribesoides* displayed cytotoxicity against human pancreatic cancer cells [30]. Nevertheless, information is lacking on the role of zeylenone in acne-related bacterial inhibition and its underlying mechanism of action.

The present study aimed to evaluate the antibacterial activities of extracts from several native *Piper* species of Thailand against three major acne-causing bacteria, including antibiotic-resistant strains, and identify the most potent source. Additionally, this study sought to isolate and characterize active antibacterial compounds from *P. griffithii*, elucidate

its mechanism of action, assess cytotoxicity toward human keratinocytes, and evaluate its potential for use in a topical anti-acne formulation. This work is the first report of zeylenone from *P. griffithii* and highlights its potential as a natural antibacterial agent for application in anti-acne products.

## 2. Materials and Methods

### 2.1. Plant Materials

Fresh stems and leaves of plants in the genus *Piper* were collected from various fields and plantations in Thailand. All the collected plants were identified by the fourth author (CS). Voucher specimens were prepared and deposited in the Thai herbaria. The scientific names, localities, and voucher specimens of the collected plants are listed in Table 1.

**Table 1.** *Piper* species used in this study.

| Plant Species | Locality (Province) | Voucher Specimens |
|---|---|---|
| *Piper arcuatum* Blume | Nakhon Si Thammarat | *C. Suwanphakdee 223* (BK, BKF, KKU) |
| *P. argyrites* Ridl. ex C. DC. | Nakhon Si Thammarat | *C. Suwanphakdee 394* (BK, BKF, KKU) |
| *P. baccatum* Blume | Nakhon Si Thammarat | *C. Suwanphakdee 488* (BK, BKF, KKU) |
| *P. betle* L. (wild form) | Trang | *C. Suwanphakdee 314* (BK, BKF, KKU) |
| *P. boehmeriifolium* (Miq.) C. DC. var. *boehmeriifolium* | Chiangmai | *C. Suwanphakdee 457* (BK, BKF, KKU) |
| *P. boehmeriifolium* (Miq.) C. DC. var. *glabricaule* (C. DC.) M.G. | Mae Hongson | *C. Suwanphakdee 529* (BK, BKF, KKU) |
| *P. chantaranothaii* Suwanph. & D.A. Simpson | Chiangmai | *C. Suwanphakdee 510* (BK, BKF, KKU) |
| *P. chiangdaoense* Suwanph. & Chantar. | Chiangmai | *C. Suwanphakdee 456* (BK, BKF, KKU) |
| *P. giffithii* C. DC. | Tak | *C. Suwanphakdee 416* (BK, BKF, KKU) |
| *P. kongkandanum* Suwanph. & Chantar. | Kanchanaburi | *A. Laisantad 1* (BK, BKF) |
| *P. kurzii* Ridl. | Narathiwat | *C. Suwanphakdee 453* (BK, BKF, KKU) |
| *P. lanatum* Roxb. | Nakhon Si Thammarat | *C. Suwanphakdee 392* (BK, BKF, KKU) |
| *P. leptostachyum* Wall. ex Miq. | Nakhon Nayok | *C. Suwanphakdee 464* (BK, BKF, KKU) |
| *P. longum* L. | Kanchanaburi | *C. Suwanphakdee 419* (BK, BKF, KKU) |
| *P. macropiper* Pennant | Trang | *C. Suwanphakdee 454* (BK, BKF, KKU) |
| *P. majusculum* Blume | Chumphon | *C. Suwanphakdee 287* (BK, BKF, KKU) |
| *P. obtusissimum* Miq. | Narathiwat | *C. Suwanphakdee 556* (BK, BKF, KKU) |
| *P. polycarpa* Ridl. | Phang Nga | *C. Suwanphakdee 279* (BK, BKF, KKU) |
| *P. porphyrophyllum* N. E. Br. | Narathiwat | *C. Suwanphakdee 449* (BK, BKF, KKU) |
| *P. quinqueangulatum* Miq. | Nakhon Si Thammarat | *C. Suwanphakdee 424* (BK, BKF, KKU) |
| *P. ramipilum* C. DC. | Nakhon Si Thammarat | *C. Suwanphakdee 484* (BK, BKF, KKU) |
| *P. retofractum* Vahl. | Nonthaburi | *C. Suwanphakdee 434* (BK, BKF, KKU) |
| *P. rugocarpum* Y.Banchong and Suwanph. | Trat | *C. Suwanphakdee 405* (BK, BKF, KKU) |
| *P. suipigua* Buch-Ham. ex D. Don | Chiangmai | *C. Suwanphakdee 377* (BK, BKF, KKU) |
| *P. sulcatum* Blume | Ranong | *C. Suwanphakdee 468* (BK, BKF, KKU) |
| *P. umbellatum* L. | Nakhon Si Thammarat | *C. Suwanphakdee 422* (BK, BKF, KKU) |
| *P. wallichii* (Miq.) Hand.-Mazz. | Chiangmai | *C. Suwanphakdee 379* (BK, BKF, KKU) |

### 2.2. Chemicals and Microorganisms Used in the Current Study

All chemicals were of analytical grade and purchased from Sigma Chemical Co., unless otherwise indicated. Mueller Hinton agar (MHA), Mueller Hinton broth (MHB), Tryptic Soy agar (TSA), Tryptic Soy broth (TSB), nutrient broth (NB), nutrient agar (NA), peptone, beef extract, and agar were purchased from Hi-media, India. Fetal bovine serum (FBS), antibiotics-antimycotics, and trypsin-EDTA were purchased from Gibco, USA. Dulbecco's Modified Eagle Medium (DMEM) was obtained from Biowest, USA. *Cutibacterium acnes* (DMST 14916) was purchased from the Department of Medical Science Thailand Culture Collection, while *S. epidermidis* (TISTR 518) and *S. aureus* (TISTR 1466) were purchased from

the Thailand Institute of Scientific and Technological Research Culture Collection. These three inflammatory acne-causing bacterial strains are clindamycin-resistant.

### 2.3. Preparation of Plant Extracts

A sequential extraction was performed using three solvents with varying polarities, hexane, ethyl acetate, and ethanol. Dried powder of the leaves or stems of *Piper* plants was macerated in hexane at room temperature (RT, 30 ± 2 °C) for a week and then filtered. Hexane was subsequently eliminated using a rotary evaporator (BÜCHI Rotavapor R-200) at 45–55 °C to yield a dried hexane extract. The residue was then mixed with a second solvent, ethyl acetate, and the previous steps were repeated to obtain a dried ethyl acetate extract. The residue was then mixed with 95% ethanol at RT for a week, followed by filtration and evaporation to obtain a dried ethanolic extract. The extracts were oven-dried at 70 °C for 6 h to ensure complete solvent evaporation and stored at 4 °C until use. They were resuspended in 10% dimethyl sulfoxide (DMSO) before determining their antibacterial activity.

### 2.4. Extraction and Isolation of Bioactive Zeylenone

*Piper griffithii* leaves were air-dried and then powdered using a grinder. Dried *Piper* leaf powder (400 g) was macerated at RT in hexane for one week and then filtered. This procedure was repeated with the residual *Piper* powder using ethyl acetate. The filtrate was evaporated to yield about 2.60 g of ethyl acetate crude extract, which was redissolved in ethyl acetate (EtOAc) and mixed with silica gel. The mixture was concentrated in a rotary evaporator, applied to the column, and eluted with a gradient of 20–50% EtOAc in hexane. The collected fractions were gathered and analyzed using thin-layer chromatography, yielding eleven fractions. The sixth fraction was further purified by column chromatography with a MeOH:EtOAc:Hexane (1:4:5) solvent system, yielding a light yellow solid (0.60 g), which was recrystallized from a 1:1 mixture of dichloromethane and hexane, m.p. 149–149.5 °C. The light-yellow solid was vacuum-dried for 12 h to completely remove residual solvents. Structural elucidation was carried out using spectroscopic data, $^{1}$H and $^{13}$C NMR spectra (FTNMR 400 MHz, Bruker Corporation, Billerica, MA, USA), and high-resolution mass spectrometry (HRMS, microtof-Q III, Bruker Corporation, Billerica, MA, USA), and by comparison of these data with literature values. The isolated compound is identified as zeylenone, ((1S,5R,6S)-5-(benzoyloxy)-1,6-dihydroxy-2-oxocyclohex-3-en-1-yl)methyl benzoate (Figure 1), and its spectral properties are as follows:

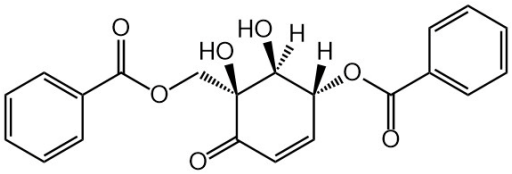



**Figure 1.** The molecular structure of zeylenone isolated from a leaf of *Piper griffithii*.

$^{1}$H NMR (400 MHz, $CDCl_3$): $\delta$ 3.22 (1H, *s*, *br*), 4.11 (1H, *s*, *br*), 4.38 (1H, *d*, $J$ = 4 Hz), 4.60 (1H, *d*, $J$ = 12 Hz), 4.85 (1H, *d*, $J$ = 8 Hz), 5.96 (1H, *d*, $J$ = 4 Hz), 6.34 (1H, *dd*, $J$ = 8, 8 Hz), 6.96 (1H, *dd*, $J$ = 4, 8 Hz), 7.38–7.44 (4H, *m*), 7.56 (2H, *dd*, $J$ = 8, 16 Hz), 7.94 (2H, *dd*, $J$ = 4, 8 Hz), 8.02 (2H, *dd*, $J$ = 4, 8 Hz). $^{13}$C NMR (CDCl3): $\delta$ 65.4, 69.2, 71.6, 77.2, 128.4, 128.5, 128.6, 128.7, 129.1, 129.7, 129.78, 133.4, 133.7, 142.6, 165.3, 166.1, 196.2. Mass spectroscopy *m/z* 383.1125 $(M + 1)^{+}$. IR (KBr, $cm^{-1}$): 712 $cm^{-1}$ (*s*, C—H bending); 1103 $cm^{-1}$ (*s*, C—O stretching); 1277 $cm^{-1}$ (*s*, C—O stretching);

1593 $cm^{-1}$ (*w*, C=C aromatic ring); 1705 $cm^{-1}$ (*s*, C=O); 2933 $cm^{-1}$ (*w*, C=C—H stretching aromatic ring); 3423 $cm^{-1}$ (*s*, O—H stretching).

### *2.5. Antibacterial Activity of the Plant Extracts Against Acne-Causing Bacteria*

#### 2.5.1. Preparation of Bacterial Inocula

Three acne-causing bacteria, *C. acnes* (DMST 14916), *S. epidermidis* (TISTR 518), and *S. aureus* (TISTR 1466), were used. *C. acnes* was inoculated onto MHA and incubated at 37 °C for 72 h under anaerobic conditions in a jar under an anaerobic gas pack (Mitsubishi, Japan). One loop-full of *C. acnes* was inoculated into MHB and incubated at 37 °C for 72 h under static anaerobic conditions until the visible turbidity was equal to the 0.5 McFarland standard, approximately $1.5 \times 10^8$ CFU/mL.

*S. aureus* and *S. epidermidis* were activated on NA and TSA, respectively, and incubated at 37 °C for 24 h. One loop-full of each bacterium was inoculated into 10 mL of NB and TSB, respectively, followed by incubation with shaking at 350 rpm for 3 h at RT until the visible turbidity was equal to the 0.5 McFarland standard.

#### 2.5.2. Determination of Inhibition Zone Diameter

An agar disc diffusion method was carried out to determine the antibacterial activity of the extracts against acne-causing bacteria using the method recommended by the Clinical and Laboratory Standard Institute (CLSI) with some modifications [31]. *S. aureus*, *S. epidermidis*, and *C. acnes* inocula at 0.5 McFarland (approximately $10^8$ CFU/mL) were swabbed onto the surfaces of NA, TSA, and MHA, respectively. Then, 6 mm diameter filter paper discs were impregnated with extracts at 10 mg/disc, followed by oven-drying at 60 °C for 30 min. The paper discs were then placed on the agar surfaces and incubated at 37 °C for 24 h, except for the plates of *C. acnes*, which were incubated at 37 °C for 72 h under anaerobic conditions. After incubation, the inhibition zone diameters were recorded. Tetracycline and clindamycin (1.25 mg/disc) were used as positive controls, while 10% DMSO served as a solvent control. They were oven-dried at 60 °C for 30 min. The experiments were performed in triplicate.

#### 2.5.3. Determination of Minimum Inhibitory and Bactericidal Concentrations

Minimum inhibitory concentration (MIC) of the extracts and isolated compounds was determined using a broth microdilution assay [32]. The extracts and isolated compounds were diluted with 10% DMSO in the culture broth, with final concentrations of 0.08 to 20 mg/mL. The samples (100 µL) and the bacterial inoculum (100 µL), as described in Section 2.5.1, were added into each well of a flat-bottom 96-well microplate. *C. acnes* was incubated at 37 °C under anaerobic conditions for 24 h, while *S. epidermidis* and *S. aureus* were incubated at 37 °C for 24 h. Then, the absorbance at 600 nm was measured with a microplate reader (Thermo Fisher Scientific Inc., Waltham, WA, USA). The lowest concentration resulting in no visible growth of the tested bacteria was the MIC. Tetracycline and clindamycin at 1.25 mg/mL were used as positive controls, whereas 10% DMSO in the culture broth was used as a solvent control.

To determine the minimum bactericidal concentration (MBC), an aliquot from the bacterial suspension and test sample (which exhibited no visible growth) or MIC was streaked onto the appropriate agar medium, as described in Section 2.5.2, and incubated at 37 °C under anaerobic conditions for 72 h (*C. acnes*) or at 37 °C for 24 h (*S. epidermidis* and *S. aureus*). The MBC is defined as the lowest concentration that completely inhibits bacterial growth. All samples were tested in triplicate with separate experimental runs.

### 2.6. Cell Leakage Study

The isolated zeylenone was evaluated for its mechanism of action against acne-causing bacteria. The cytoplasmic material leaked from zeylenone-treated bacteria was studied to preliminarily indicate disruption of bacterial cell membranes, following the modified method of Aiemsaard et al. [33]. Briefly, a double-washed bacterial suspension ($OD_{600}$ = 0.4) was incubated with a zeylenone solution in a 1:1 proportion to achieve final concentrations of MIC and 2MIC. In this study, zeylenone was solubilized in 10% DMSO, and a bacterial suspension containing 10% DMSO served as a solvent control. Samples were gently mixed and incubated at 37 °C for 1, 6, and 12 h, followed by centrifugation at 4000× *g* for 10 min to wash the cells. The supernatant was collected and quantified for leaked cytoplasmic material using a UV/VIS spectrophotometer (UNICO UV-2802, UNICO, Dayton, NJ, USA) at a 260 nm wavelength. All experiments were conducted in triplicate.

### 2.7. Ultrastructural Analysis of Bacterial Cells After Exposure to Zeylenone

Bacterial cells exposed to MIC and 2MIC concentrations of zeylenone were prepared for scanning electron microscopy (SEM). An SEM method, previously described by Vajrobol et al. [31], was employed. Specimens were first fixed overnight in 2% glutaraldehyde in a potassium phosphate buffer (pH 7.2) at 4 °C. The fixed samples were rinsed 3 times before soaking in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 24 h and then dehydrated in an ethanol series. After critical point drying (Polaron Range CPD 7501, Quorum Technologies, Laughton, East Sussex, UK) and sputter coating with gold (Quorum Technology Polaron Range SC7620 Sputter Coater, Laughton, East Sussex, UK), the samples were examined with an FEI Quanta-450 SEM (FEI Company, Hillsboro, OR, USA) at the Scientific Equipment Center, Kasetsart University, Bangkok, Thailand.

### 2.8. Cytotoxicity Determination

Cytotoxicity was evaluated using HaCaT cells received from the CLS Cell Line Service, Germany, and kept in DMEM with 10% FBS and 1% penicillin-streptomycin, in a 5% $CO_2$ humidified atmosphere at 37 °C. Then, the HaCaT cells were washed with Dulbecco's phosphate-buffered saline (PBS) before resuspension in 3 mL of 0.25% trypsin/EDTA. The resuspended cells were centrifuged at 1500 rpm at 4 °C for 5 min and then resuspended in DMEM [34].

Cytotoxicity was determined using the WST-1 assay as described by Asuraphong et al. [35]. Sample solutions in DMEM at different concentrations (0.625–40 mg/mL) were added to separate wells with 200 µL of HaCaT cells ($2 \times 10^4$ cells/mL) and then incubated in a 5% $CO_2$ humidified atmosphere at 37 °C for 24 h. Next, the treated cells were washed with PBS before adding 10 µL of a WST-1 solution and 100 µL of DMEM in each well, followed by further incubation for 30 min. Absorbance was measured at 450 nm using a microplate reader (Thermo Scientific Multiskan Go, Thermo Fisher Scientific). The cell viability (%) was calculated as (absorbance of sample/absorbance of control) × 100.

### 2.9. Development of a Cosmeceutical Formulation with Zeylenone Added

Pure zeylenone, as an active ingredient, was formulated into a gel-form cosmeceutical product. A commercial cosmetic gel base was kindly supplied by the Asoke Skin Hospital (Thailand) for use in this study. The gel base was free of colorants and fragrances with a pH of 6.0. Furthermore, the gel-based formulation is certified as safe by the Thai Food and Drug Administration (FDA). To cover all anti-acne bacterial activities evaluated, the base formulation was supplemented separately with zeylenone at a 1.0% concentration, which corresponds to 10 mg/g of the base gel. The base formulation and zeylenone were thoroughly mixed to ensure sample uniformity

and analyzed immediately after incorporation to assess zeylenone's capability to retain its bioactivities in the gel formulation. A base formulation with 10% DMSO instead of zeylenone and the gel base alone were used as a negative control.

Antibacterial activity of the developed gel formulation containing zeylenone against three acne-causing bacteria was assessed using an agar well diffusion method. *C. acnes*, *S. aureus*, and *S. epidermidis* inocula at 0.5 McFarland were swabbed onto the entire surfaces of MHA, NA, and TSA plates, respectively. Then, a 5 mm diameter hole was aseptically punched into the agar plate with a sterile cork borer. Zeylenone or the gel formulation with zeylenone was introduced into the wells at a 1.0 mg final concentration. The plates were then incubated at 37 °C for 24 h, except for the plates of *C. acnes*, which were incubated at 37 °C for 72 h under anaerobic conditions. The sizes of the inhibition zones were measured after incubation. The experiments were performed in triplicate.

### 2.10. Statistical Analyses

All experiments in the current study were conducted in triplicate, with their results shown as mean ± standard deviation (SD) values. One-way analysis of variance (ANOVA) was used to analyze the experimental data employing the Statistica 10.0 software package (StatSoft Inc., Tulsa, OK, USA). Differences between means for each treatment at the 5% ($p < 0.05$) level were considered statistically significant. Tukey's post hoc test at $p < 0.05$ was applied to detect significant differences between the mean values using SPSS version 26.0 for Windows (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Screening of Crude Extracts Against Acne-Causing Bacteria

The antibacterial activities of 27 *Piper* species from Thailand against three skin bacteria that cause inflammatory acne, *C. acnes*, *S. epidermidis*, and *S. aureus*, were examined in terms of growth inhibition on an agar medium using a disc diffusion technique. Each crude extract was derived from either the leaves or stems of *Piper* plants extracted separately using hexane, ethyl acetate, and ethanol.

A total of 162 samples were screened. Only 13 extracts exhibited bacterial growth inhibition, as shown in Table 2. A comparative analysis of extracts derived from the leaves and stems of *Piper* plants using solvents of varying polarity revealed that leaf extracts consistently exhibited higher bacterial activity than those from the stems. Among the solvents evaluated, ethyl acetate was the most effective for extracting antibacterial constituents, yielding extracts with superior antibacterial activity compared to those obtained using other solvents. In contrast, inhibition against the tested bacterial strains was not detectable in any of the hexane extracts.

Ethyl acetate extracts from the leaves and stems of *P. griffithii* and from the leaves of *P. leptostachyum* were effective in suppressing the growth of all tested bacteria. Additionally, the bacterial strains used in this study were highly sensitive to the antibiotic tetracycline. The effect was different from the antibiotic clindamycin, which showed resistance in all tested bacterial strains. The ethyl acetate extracts from the leaves and stems of *P. griffithii* exhibited a strong inhibitory effect against *C. acnes*, a bacterium triggering inflammatory acne, based on its greater inhibition zone (diameter ≥ 20 mm) [36], which is larger than that of tetracycline and clindamycin. Since ethyl acetate extracts derived from the leaves and stems of *P. griffithii* have a good inhibitory effect against all tested bacteria, especially *C. acnes*, they were selected for further study.

**Table 2.** Antibacterial activities of tested crude extracts derived from *Piper* species against the acne-causing bacteria, *Cutibacterium acnes*, *Staphylococcus epidermidis*, and *S. aureus* using an agar disc diffusion method.

| Plant Species [x] | Part Used | Extraction Solvent | Inhibition Zone (mm ± SD) | | |
|---|---|---|---|---|---|
| | | | *C. acnes* | *S. epidermidis* | *S. aureus* |
| *Piper griffithii* C. DC. | Leaf | EtOAc | 21.5 ± 0.5 | 8.4 ± 0.4 | 15.9 ± 0.4 |
| | | EtOH | 13.5 ± 0.5 | n.d. | 8.6 ± 0.2 |
| | Stem | EtOAc | 20.2 ± 0.6 | 6.7 ± 0.0 | 6.2 ± 0.1 |
| *P. lanatum* Roxb. | Leaf | EtOAc | n.d. | n.d. | 8.5 ± 0.1 |
| | Stem | EtOAc | n.d. | n.d. | 6.8 ± 0.7 |
| *P. leptostachyum* Wall. ex Miq. | Leaf | EtOAc | 19.0 ± 0.5 | 5.5 ± 0.5 | 5.3 ± 0.3 |
| *P. macropiper* Pennant | Leaf | EtOH | n.d. | n.d. | 6.8 ± 0.0 |
| *P. majusculum* Blume | Leaf | EtOH | n.d. | n.d. | 6.8 ± 0.0 |
| | Stem | EtOAc | n.d. | n.d. | 5.2 ± 0.8 |
| *P. retrofractum* Vahl. | Leaf | EtOAc | n.d. | n.d. | 7.8 ± 0.4 |
| *P. umbellatum* L. | Leaf | EtOAc | n.d. | n.d. | 6.8 ± 0.4 |
| | | EtOH | n.d. | n.d. | 6.8 ± 0.6 |
| | Stem | EtOAc | n.d. | n.d. | 5.0 ± 0.9 |
| Tetracycline (1.25 mg/disc) | | | 9.0 ± 0.2 | 27.0 ± 0.3 | 33.0 ± 0.1 |
| Clindamycin (1.25 mg/disc) | | | n.d. | 3.0 ± 0.2 | 3.5 ± 0.6 |

[x] Only plant species with inhibitory effects on the agar medium are included. n.d. = not detected. All data are presented as the mean values ± SD ($n$ = 3). EtOAc = ethyl acetate. EtOH = ethanol. 10% DMSO was the negative control, and no inhibition zones were observed. The amount of extract tested was 10 mg/disc.

The MIC and MBC determinations of ethyl acetate extracts derived from the leaves and stems of *P. griffithii*, the most active species against the acne-causing bacteria using the microdilution method, are shown in Table 3. Based on the MBC/MIC ratio, the effect was classified as bactericidal when the value was ≤4. Conversely, a ratio greater than 4 indicated a bacteriostatic effect [37].

**Table 3.** The MIC and MBC values of ethyl acetate extracts derived from the leaves and stems of *Piper griffithii* against the acne-causing bacteria, *Cutibacterium acnes*, *Staphylococcus epidermidis*, and *S. aureus*.

| Crude Extract | Susceptibility of Bacteria to Crude Extracts (mg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | *Cutibacterium acnes* | | *Staphylococcus epidermidis* | | *Staphylococcus aureus* | |
| | MIC | MBC | MIC | MBC | MIC | MBC |
| *P. griffithii* leaf | 2.5 ± 0.0 | 10.0 ± 0.0 | 10.0 ± 0.0 | 15.0 ± 0.0 | 2.5 ± 0.0 | 10.0 ± 0.0 |
| *P. griffithii* stem | 10.0 ± 0.0 | >20.0 | 10.0 ± 0.0 | 20.0 ± 0.0 | 5.0 ± 0.0 | 15.0 ± 0.0 |

All data are presented as the mean values ± SD ($n$ = 3).

The MIC and MBC values of the *P. griffithii* leaf extract exhibited the strongest antibacterial effect against all tested bacteria, compared to the stem extract, which exhibited similar inhibition zones (Table 3). *C. acnes* and *S. aureus* displayed the highest sensitivity to the ethyl acetate leaf extract of *P. griffithii*, with an MIC value of 2.5 mg/mL. However, the higher concentration of the *P. griffithii* (10 mg/mL) ethyl acetate leaf extract exhibited bactericidal effects against *C. acnes* and *S. aureus*. Additionally, the ethyl acetate leaf extract of *P. griffithii* acted as a bacteriostatic agent against *S. epidermidis* at 10 mg/mL. The greatest antibacterial effect against all tested acne-causing bacteria was from the ethyl acetate extract of *P. griffithii* leaves, which was subjected to more in-depth investigation.

### 3.2. Antibacterial Activity Against Acne-Causing Bacteria by the Isolated Compound

Chromatographic separation of the ethyl acetate leaf extract from *P. griffithii* isolated and identified a pure substance, zeylenone, as the major bioactive compound responsible for suppressing three acne-causing bacteria. The MIC and MBC values of the isolated zeylenone compound required to eliminate the acne-causing bacteria were evaluated. The findings revealed that zeylenone has a bactericidal effect against all tested acne-causing bacterial strains (Table 4). *C. acnes* exhibited the highest sensitivity to zeylenone, followed by *S. aureus* and *S. epidermidis*, with MIC values of 0.63, 1.25, and 5.0 mg/mL, respectively. The bacteriostatic effect of zeylenone against *C. acnes* was greater than that of the standard antibiotics, tetracycline and clindamycin. Additionally, the results indicate that there was no significant difference ($p < 0.05$) in the MIC values for *S. aureus* of zeylenone and tetracycline. However, the MBC values of zeylenone needed to effectively eliminate *C. acnes*, *S. aureus*, and *S. epidermidis* required concentrations above 1.25, 2.5, and 7.5 mg/mL, respectively. This demonstrates that zeylenone was effective in suppressing the growth of the three tested acne-causing bacteria. The MBC/MIC ratio of the extract is displayed in Table 4. Zeylenone can be considered as a bactericidal agent against *C. acnes*, *S. aureus*, and *S. epidermidis*.

**Table 4.** Antibacterial activities of zeylenone purified from a leaf extract of *Piper griffithii* against the acne-causing bacteria, *Cutibacterium acnes*, *Staphylococcus epidermidis*, and *S. aureus*.

| Acne-Causing Bacteria | Susceptibility of Bacteria to Zeylenone (mg/mL) | | |
|---|---|---|---|
| | MIC | MBC | MBC/MIC Ratio |
| *Cutibacterium acnes* | 0.63 ± 0.0 | 1.25 ± 0.0 | 1.98 |
| *Staphylococcus epidermidis* | 5.0 ± 0.0 | 7.5 ± 0.0 | 1.50 |
| *Staphylococcus aureus* | 1.25 ± 0.0 | 2.5 ± 0.0 | 2.00 |

Data are presented as the mean values ± SD ($n = 3$). Tetracycline and clindamycin were used as positive controls. MIC values against *C. acnes*, *S. epidermidis*, and *S. aureus* of tetracycline were 1.25 mg/mL. These three acne-causing bacteria exhibited resistance to clindamycin.

### 3.3. Effect of Zeylenone on Ultrastructural Changes and Cytoplasmic Leakage of Acne-Causing Bacteria

In the present study, zeylenone isolated from *P. griffithii* leaf extract was first investigated for the mechanism of antibacterial action against the acne-causing bacteria, *C. acnes*, *S. aureus* and *S. epidermidis*. The results revealed that zeylenone in MIC and 2MIC concentrations induced cytoplasmic material leakage from the bacterial cells within 1 h (Figure 2).

After exposure to zeylenone in the MIC experiment, changes in acne-causing bacterial cells were analyzed using SEM. Morphological changes in each bacterium caused by zeylenone were compared to the bacterial cells in the untreated control culture. All bacterial cells in the untreated control culture exhibited intact and healthy normal cellular structures with smooth surfaces (Figures 3a, 4a and 5a). Similar morphological characteristics were observed in bacterial cells exposed to the 10% DMSO solvent control (Figures 3b, 4b and 5b), indicating that the solvent alone did not induce detectable structural alterations.

After the bacterial cells were exposed to zeylenone at the MIC value, abnormal cells with holes, dimples, and abrasions on cell surfaces appeared, indicated by white arrows (Figures 3c, 4c and 5c). Furthermore, the zeylenone-treated cells presented critical damage, such as torn cells, as the concentration of zeylenone increased to the 2MIC value (Figures 3d, 4d and 5d). These morphological disruptions were detectable within 6 h of exposure and became more severe with prolonged incubation. The cell walls and membranes of

three acne-causing bacteria were disrupted, leading to consequent cytoplasmic material leakage. These findings are consistent with the observed profiles of cytoplasmic leakage.

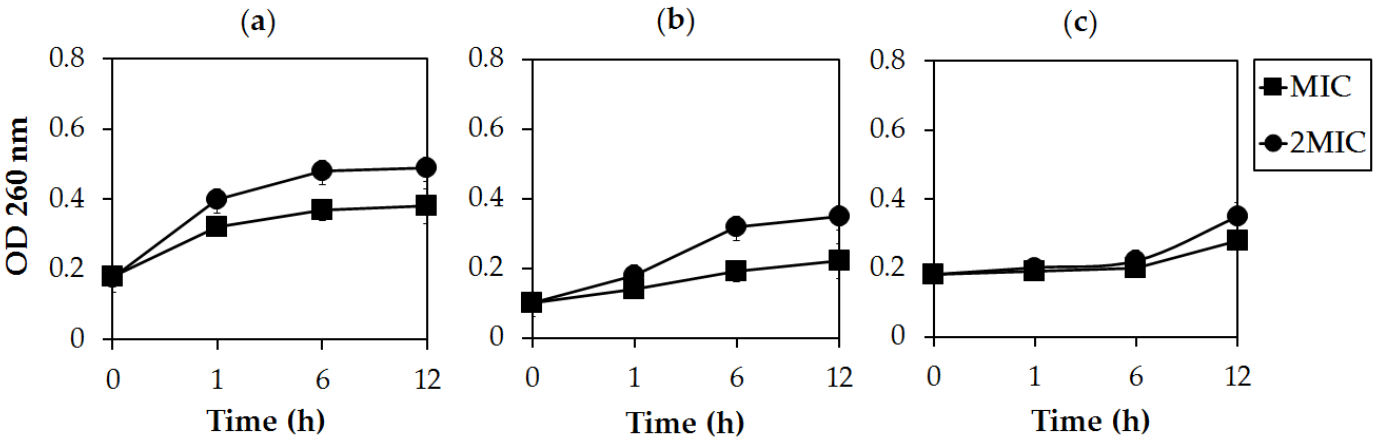



**Figure 2.** Cytoplasmic leakage of *Cutibacterium acnes* (**a**), *Staphylococcus aureus* (**b**), and *S. epidermidis* (**c**) after incubation at 37 °C with MIC (■) and 2MIC (●) concentrations of zeylenone for 6, 12, and 24 h. The data are expressed as the mean values ± SD ($n$ = 3).

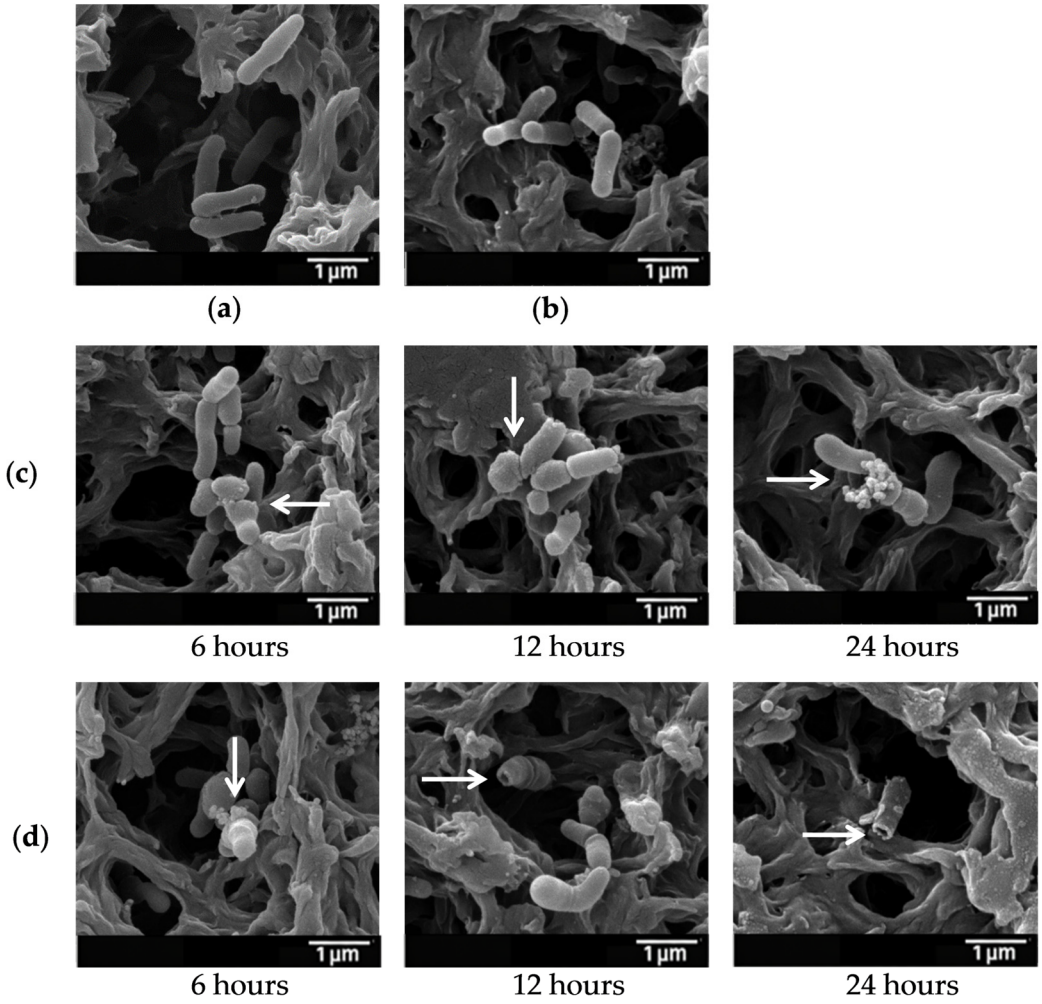



**Figure 3.** SEM micrographs at 30,000× magnification of *Cutibacterium acnes* exposed to the MIC and MBC of zeylenone for 6, 12, and 24 h. (**a**) Control *C. acnes* in Mueller Hinton broth (MHB). (**b**) Control *C. acnes* in MHB with 10% DMSO. (**c**) Treated with zeylenone at MIC = 0.63 mg/mL. (**d**) Treated with zeylenone at 2MIC = 1.25 mg/mL. White arrows illustrate cell wall breaking.

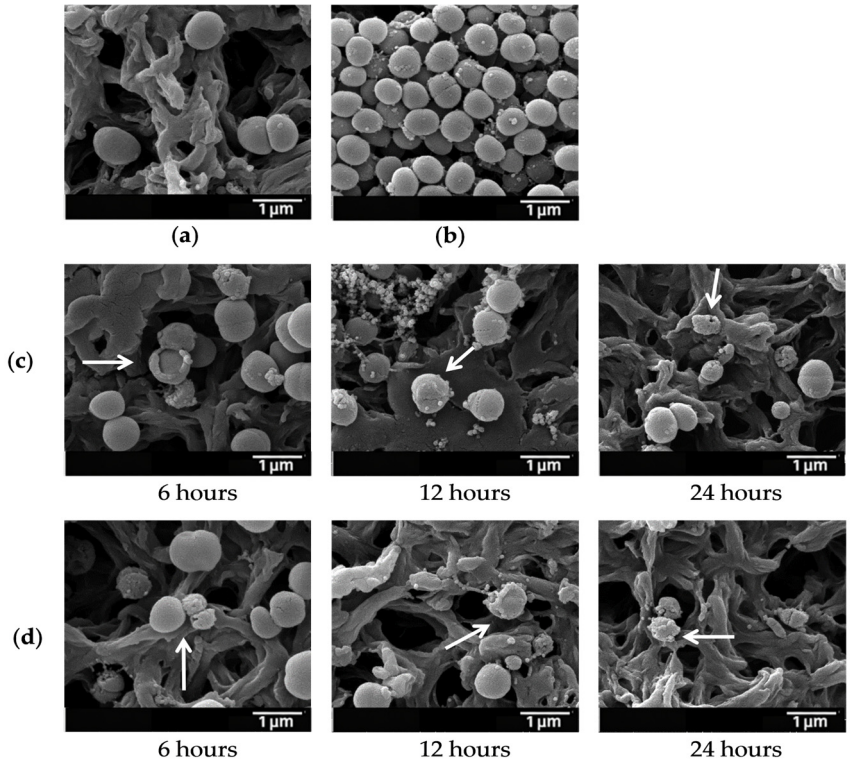



**Figure 4.** SEM micrographs at 30,000× magnification of *Staphylococcus epidermidis* exposed to the MIC and MBC of zeylenone for 6, 12, and 24 h. (**a**) Control *S. epidermidis* in Tryptic Soy broth (TSB). (**b**) Control *S. epidermidis* in TSB with 10% DMSO. (**c**) Treated with zeylenone at MIC = 5.0 mg/mL. (**d**) Treated with zeylenone at 2MIC = 10.0 mg/mL. White arrows illustrate cell wall breaking.

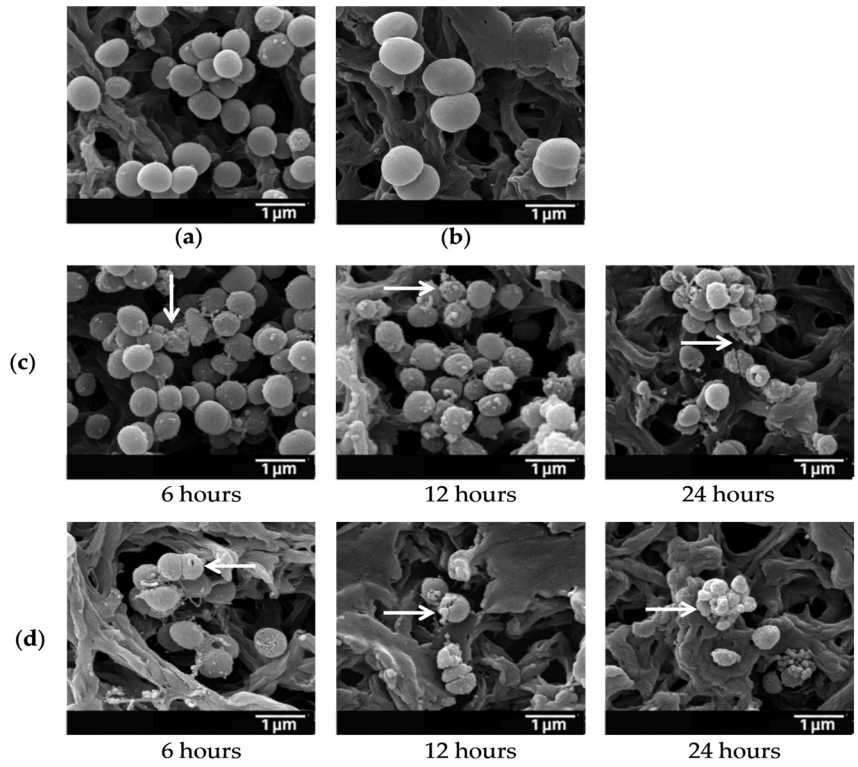



**Figure 5.** SEM micrographs at 30,000× magnification of *Staphylococcus aureus* exposed to the MIC and MBC of zeylenone for 6, 12, and 24 h. (**a**) Control *S. aureus* in nutrient broth (NB). (**b**) Control *S. aureus* in NB with 10% DMSO. (**c**) Treated with zeylenone at 1.25 mg/mL. (**d**) Treated with zeylenone at 2.5 mg/mL. White arrows illustrate cell wall breaking.

### *3.4. Cytotoxicity Analysis*

The effects of zeylenone on the cell viability of the HaCaT cell line were assessed through WST-1 assays to evaluate the safety of zeylenone for further cosmeceutical applications. This cell line has been previously established as a reliable in vitro model. From the results shown in Figure 6, zeylenone exhibited non-toxicity to the HaCaT cell line at concentrations reaching 20 mg/mL. More than 80% cell viability was detected. The maximum concentration tested, 40 mg/mL, inhibited cell viability by up to 30%.

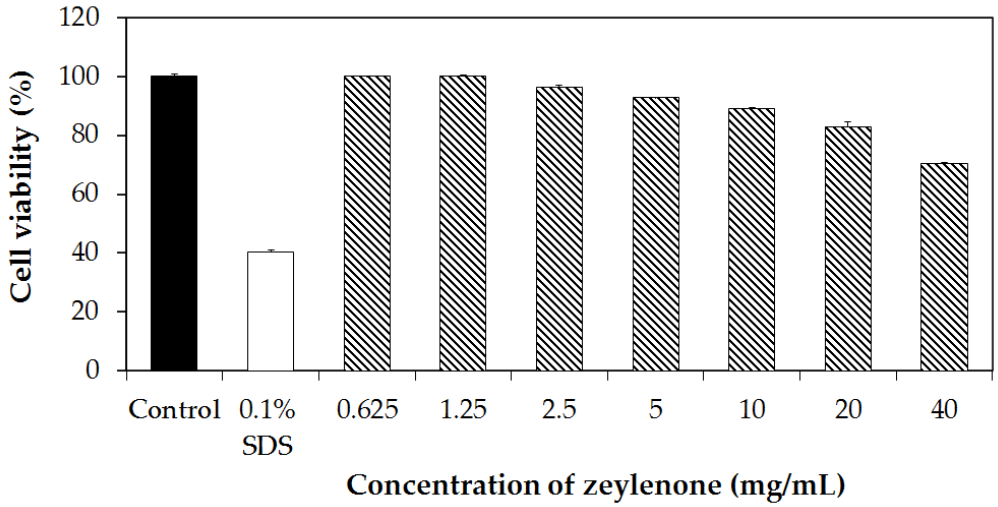



**Figure 6.** Viability of the keratinocyte (HaCaT) cell line after treatment with zeylenone purified from *Piper griffithii* leaves at various concentrations. Data are expressed as the mean values ± SD ($n$ = 3).

### *3.5. Development of a Cosmeceutical Formulation with Zeylenone Added*

As a result of its multi-anti-acne bacterial effects, zeylenone from *P. griffithii* leaves is a promising candidate for use in topical formulations for cosmeceutical applications. The effects of gel formulation on the anti-acne bacterial activities were investigated. A base formulation of gel incorporating 10% DMSO instead of zeylenone and the gel base was used as a negative control and exhibited a lack of antibacterial activity. The antibacterial properties of the produced gel formulation were confirmed. The formulated gel product preserved all antibacterial activities exhibited by zeylenone (Table 5). Visual observation revealed that the formulation was homogeneous. The produced cosmeceutical formulation with zeylenone showed a pale-yellow coloration with a pH of 5.5, which is suitable for application to skin. These results are of interest to the cosmetic industry for minimizing the use of synthetic ingredients. They suggest that zeylenone isolated from *P. griffithii* leaves can be further developed as a natural ingredient for use in anti-acne gel products.

**Table 5.** Anti-acne bacterial properties in a gel formulation based on a zeylenone purified from *Piper griffithii* leaves, 10 mg/mL, using an agar well diffusion method.

| Formulation | Inhibition Zone (mm ± SD) | | |
|---|---|---|---|
| | *C. acnes* | *S. epidermidis* | *S. aureus* |
| Zeylenone | 59.0 ± 0.5 [a] | 18.0 ± 0.1 [a] | 25.3 ± 0.5 [a] |
| Gel + zeylenone | 58.0 ± 0.8 [a] | 17.5 ± 0.9 [a] | 24.5 ± 0.5 [a] |
| Gel + 10% DMSO | 0.0 ± 0.0 [b] | 0.0 ± 0.0 [b] | 0.0 ± 0.0 [b] |
| Gel base | 0.0 ± 0.0 [b] | 0.0 ± 0.0 [b] | 0.0 ± 0.0 [b] |

Data are expressed as the mean values ± SD ($n$ = 3). In the same column, the means followed by different letters indicate significant differences ($\alpha$ = 0.05, ANOVA, Tukey's HSD test).

## 4. Discussion

Plant-derived therapies provide considerable benefits over conventional antibiotics for acne treatment, such as avoiding antibiotic resistance, minimizing adverse effects, and offering multiple therapeutic actions. These advantages highlight their potential as an effective alternative or complementary approach for acne treatment [38,39]. This study provides a systematic comparative evaluation of a broad selection of *Piper* species native to Thailand as potential botanical agents against key skin-associated bacteria implicated in inflammatory acne. It encompasses crude extract screening, bioactive compound isolation, mechanism of action elucidation, cytotoxicity assessment, and formulation feasibility. The results collectively demonstrate that *P. griffithii*, particularly its ethyl acetate leaf extract and the isolated compound zeylenone, exhibit outstanding antibacterial efficacy against *C. acnes*, *S. aureus*, and *S. epidermidis*.

Potential extracts containing high amounts of bioactive compounds depend on the plant species, parts, and extraction solvents used. Therefore, the anti-acne bacterial properties of the 162 crude extracts from 27 species of *Piper* plants were comparatively studied. Each crude extract was obtained from the leaves and stems of *Piper* plants sequentially extracted using hexane, ethyl acetate, and ethanol as solvents. The initial screening revealed that only 13 extracts from seven species of *Piper* plants, including *P. griffithii*, *P. lanatum (syn. P. caninum)*, *P. leptostachyum*, *P. macropiper*, *P. majusculum*, *P. retrofractum*, and *P. umbellatum*, had inhibitory effects against acne-causing bacteria with different sensitivities, highlighting the highly specific nature of antibacterial potential within this genus, which varies significantly across species, plant parts, and solvent types. In contrast, the remaining 149 extracts showed no detectable activity. This lack of efficacy may be attributed to several factors, including diverse phytochemical profiles, suboptimal concentrations of bioactive metabolites, or the selectivity of the extraction solvents. These findings demonstrate the high chemical specificity of bioactive constituents within the *Piper* genus. This observation was consistent with earlier studies reporting that antibacterial activity in *Piper* plants was highly selective and closely linked to specific phytochemical profiles rather than being a universal genus-level trait [40,41].

The present study revealed that ethyl acetate extracts derived from *Piper* leaves consistently exhibited superior antibacterial activity compared to stem extracts and other solvent systems. This finding highlights the importance of both plant-part selection and extraction polarity in maximizing antibacterial efficacy, which aligns with previous findings that leaves accumulate higher levels of phenylpropanoid, flavonoid, and amide alkaloid metabolites involved in plant defense [42]. Similar plant-part-dependent differences in antibacterial efficacy have been documented for *P. betle*, *P. nigrum*, and *P. sarmentosum*, where leaf extracts showed excellent activity against *Staphylococcus* spp. [43–45]. Our study also revealed that solvent polarity played a decisive role in extract bioactivity. Ethyl acetate extracts outperformed hexane and ethanol extracts, while no antibacterial activity was detected in any hexane-derived extract. This pattern suggested that the major antibacterial constituents in the active *Piper* species are moderately polar compounds. Based on the current findings, ethyl acetate is the appropriate solvent to extract bioactive compounds from *P. griffithii* for anti-acne purposes because its extracts had the highest anti-acne bacterial activity. The absence of antibacterial activity detected in hexane extracts from all 27 *Piper* species may occur due to the recovery of primarily lipophilic constituents, which possess limited efficacy against Gram-positive acne bacteria. This solvent-dependent extraction behavior is consistent with previous studies, which highlights the efficacy of ethyl acetate in extracting phenolic and polyketide-derived antibacterial compounds from medicinal plants, whereas non-polar solvents like hexane primarily recovered lipophilic constituents with limited antibacterial relevance against Gram-positive bacteria [46].

Notably, ethyl acetate extracts from both the leaves and stems of *P. griffithii* exhibited strong antibacterial activity against all tested strains, with inhibition zone diameters against *C. acnes* exceeding 20 mm, surpassing those of tetracycline and clindamycin. This finding is particularly significant given the complete resistance of all tested strains to clindamycin, which mirrors current clinical reports of widespread clindamycin resistance among acne-causing bacteria. The inhibition zone against *S. aureus* was significantly different from that of *S. epidermidis*. These bacteria are members of the same genus. They have the same dominant phenotypic properties. The cell wall thickness of *S. aureus* (24 nm) is comparatively thinner than that of *S. epidermidis* (33 nm) [47,48], resulting in a larger diameter extract inhibition zone against *S. aureus*. While tetracycline remained effective, the superior or comparable activity of *P. griffithii* extracts suggests a promising alternative mechanism of action, distinct from conventional antibiotics. In recent years, acne-causing bacteria have developed resistance to numerous synthetic drugs, indicating the necessity for new antibacterial agents with fewer adverse effects for effective treatment. Several studies have explored natural products against multidrug-resistant strains and reported that certain plant species contain promising antimicrobial compounds [49].

MIC and MBC analyses further confirmed that the ethyl acetate leaf extract of *P. griffithii* possesses stronger antibacterial activity than the corresponding stem extract, consistent with our disc diffusion results. This extract exhibited the lowest MIC values against *C. acnes* and *S. aureus*, 2.5 mg/mL. The bactericidal effects against *C. acnes* and *S. aureus* were observed at higher concentrations (10 mg/mL), while exerting bacteriostatic effects against *S. epidermidis*. This differential sensitivity among bacterial species might occur due to differences in cell wall composition, membrane permeability, and stress response mechanisms between anaerobic and facultative aerobic Gram-positive bacteria [50]. This selective activity aligns with emerging acne treatment strategies aimed at suppressing pathogenic bacteria while preserving commensal skin microbiota, a balance rarely achieved by broad-spectrum antibiotics [51]. The pronounced activity against *C. acnes* is particularly significant, as this bacterium plays a central role in acne inflammation by inducing host immune responses [3]. The MIC values of *P. griffithii* leaf extract were lower than those reported for many plant-derived anti-acne extracts, including *P. betle*, *P. hispidum*, *P nigrum*, *Mangifera indica* L. Kernel, mangosteen fruit rind, and *Mesua ferrea* flowers [15,32,52–55]. These findings suggest that the ethyl acetate leaf extract of *P. griffithii* exhibits heightened potential as an anti-acne bacterial agent. Further investigations on the isolated metabolites could offer a deeper understanding of how each compound contributes to the observed antibacterial effects.

Chromatographic isolation has identified zeylenone as the principal active compound responsible for the observed antibacterial activities. Zeylenone exhibited potent bactericidal effects against all tested acne-causing bacteria, with *C. acnes* showing the highest susceptibility (MIC 0.63 mg/mL), followed by *S. aureus* (MIC 1.25 mg/mL) and *S. epidermidis* (MIC 5.0 mg/mL). Notably, the MIC values of zeylenone against *C. acnes* were lower than those of tetracycline and clindamycin, indicating a strong intrinsic antibacterial potency. Although the MIC values against *S. aureus* were comparable to standard antibiotics, the overall bactericidal profile of zeylenone supports its potential as an effective antimicrobial agent against multiple acne-causing pathogens. Previous reports indicated that *S. epidermidis* contributed negatively to acne development by forming biofilms that promote the proliferation of *C. acnes*. These biofilms shield the bacteria from the host's innate immune defenses. Additionally, both *S. aureus* and *S. epidermidis* cause skin infections that trigger inflammatory acne outbreaks [56]. Thus, pure zeylenone and the crude extract of *P. griffithii* leaves, which not only eliminate *C. acnes* growth but also inactivate *S. epidermidis* and *S. aureus*, may be beneficial for managing inflammatory acne lesions, such as papules and

pustules, and may help reduce infections that can progress to abscess formation. Since no previous studies have described the metabolites of this plant, the compounds identified in the present work could not be compared with earlier findings.

The cell wall, a rigid structure that encloses the cell membrane, is vital for maintaining cellular shape and protecting cytoplasmic components from external stress [57]. The cell membrane functions as a selective barrier that regulates bacterial metabolic activities, which preserves material and energy balances, making it a primary target for numerous antimicrobial agents [58]. Leakage of cytoplasmic contents is evidence of irreversible membrane damage [59]. The mechanism of action of zeylenone against three acne-causing bacteria can be clearly seen in SEM images, together with the cytoplasmic leakage results. Zeylenone induced damage to bacterial cell membranes, leading to leakage of intracellular components, severe ultrastructural damage, including surface perforations, and cell lysis within hours of exposure. This indicates loss of membrane integrity and cell wall damage as primary mechanisms leading to bacterial inactivation. Similar membrane-disruptive effects against *S. aureus* have been observed for piperine, the main alkaloid found in *P. nigrum* [60]. Additionally, phenolic compounds and flavonoids of medicinal plants have an antibacterial mechanism against *C. acnes* and *S. epidermidis* that is important in bacterial cell membrane and cell wall disruption [32,61]. Lipophilicity is a crucial factor responsible for the antibacterial activity of phenolic compounds and flavonoids [32,62]. Our study is the first to present the antibacterial properties of zeylenone and its potential mechanism of action against acne-causing bacteria. Previous studies have demonstrated that zeylenone disrupts both the cell wall and plasma membrane of fungi. It increases membrane permeability, induces oxidative stress through reactive oxygen species (ROS), and interferes with fungal energy metabolism of *Phytophthora capsici*. These effects can ultimately result in structural collapse and fungal cell lysis [63]. A similar mechanism may also occur in bacteria.

Topical antibacterial agents that suppress acne-causing bacteria help limit their penetration into the skin and reduce the spread of lesions. Such topical agents must eliminate bacteria while remaining safe for host tissues to be effective. In this study, zeylenone isolated from *P. griffithii* leaves can be safe for topical application, showing negligible toxicity against HaCaT keratinocytes. This safety profile is critical for topical applications and supports the feasibility of incorporating zeylenone into dermatological or cosmeceutical formulations. After incorporation of pure zeylenone into the gel base, a new formulation with pH 5.5 was obtained, which is considered within the suitable pH range for cosmeceutical design. Earlier reports suggest that cosmetic formulations with a pH range of 4.0–6.0 are advantageous because most pathogenic bacteria grow optimally at neutral pH levels. Skin microflora and barrier homeostasis are maintained at these conditions [64]. The successful formulation of zeylenone into a gel-based cosmeceutical product further strengthens its translational potential. The formulated gel retained full antibacterial activity against acne-causing bacteria, whereas the base formulation lacking zeylenone showed no inhibitory effect. These results demonstrate that zeylenone remains chemically stable and biologically active within a topical delivery system, an essential requirement for practical anti-acne applications.

This study is the first report of zeylenone isolated from *P. griffithii* and establishes its potent antibacterial activity against key acne-causing bacteria through a cell-wall- and membrane-disruptive mechanism. The combination of strong antibacterial efficacy, effectiveness against antibiotic-resistant strains, low cytotoxicity, and formulation compatibility highlights zeylenone's prospects as a promising natural candidate for the development of novel anti-acne therapeutics and cosmeceutical products. Additional research is required to determine the formulation stability over various storage periods and permeation in

skin models, as well as in vivo studies and clinical validation to confirm the potential of zeylenone from *P. griffithii* leaves as a natural anti-acne antibacterial agent.

## 5. Conclusions

This study identified *P. griffithii* as a novel botanical source of the antibacterial compound, zeylenone. It is the first report of zeylenone isolated from *P. griffithii*. It demonstrates its strong in vitro activity against key acne-causing bacteria, including antibiotic-resistant *C. acnes*, *S. epidermidis*, and *S. aureus*. The bactericidal mechanism of zeylenone involved disruption of bacterial cell walls and cell membrane integrity, culminating in rapid cytoplasmic leakage and cell rupture. Zeylenone has no toxic effects on human skin keratinocytes and retains its activity when formulated into a gel, indicating potential for topical application. These findings support the further development of zeylenone from *P. griffithii* as a promising natural antibacterial agent for anti-acne therapeutics and cosmeceutical products.

**Author Contributions:** Conceptualization, C.T.; methodology, C.T., T.L., C.S. and A.L.; software, C.T., A.L.; validation, C.T., T.L. and C.S.; formal analysis, C.T., T.L. and C.S.; investigation, C.T. and A.L.; resources, C.T. and C.S.; data curation, C.T. and A.L.; writing—original draft preparation, C.T.; writing—review and editing, C.T., T.L. and C.S.; visualization, C.T. and A.L.; supervision, C.T.; project administration, C.T.; funding acquisition, C.T. and C.S. All authors have read and agreed to the published version of the manuscript.

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