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The atopic dermatitis-like lesion and the associated MRSA infection and barrier dysfunction can be alleviated by 2,4-dimethoxy-6-methylbenzene-1,3-diol from *Antrodia camphorata*

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ABSTRACT

Background: Atopic dermatitis (AD) is an inflammatory skin disease with an associated barrier dysfunction and *Staphylococcus aureus* infection. The mainstay steroid and calcineurin inhibitor therapy shows some adverse effects. 2,4-Dimethoxy-6-methylbenzene-1,3-diol (DMD) is a benzenoid isolated from *Antrodia camphorata*.

Objective: We investigated the inhibitory effect of DMD on methicillin-resistant *S. aureus* (MRSA), the chemokine production in stimulated keratinocytes, and the AD-like lesion found in ovalbumin (OVA)-sensitized mice.

Methods: The antimicrobial effect and cutaneous barrier function were evaluated using an in vitro culture model and an in vivo mouse model of AD-like skin.

Results: DMD exhibited a comparative minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against MRSA with nalidixic acid, a conventional antibiotic. The MIC and MBC for DMD was 78.1 and 156.3 µg/ml, respectively. DMD also showed the ability to eliminate the clinical bacteria isolates with resistance to methicillin and vancomycin. The DNA polymerase and gyrase inhibition evoked by DMD for bacterial lethality was proposed. In the activated keratinocytes, DMD stopped the upregulation of chemokines (CCL5 and CCL17) and increased the expression of differentiation proteins (filaggrin, involucrin, and integrin β-1). Topical application of DMD readily penetrated into the skin, with AD-like skin displaying 2.5-fold greater permeation than healthy skin. The in vivo assessment using the mouse model with OVA sensitization and MRSA inoculation revealed a reduction of transepidermal water loss (TEWL) and bacterial burden by DMD by about 2- and 100-fold, respectively. Differentiation proteins were also restored after topical DMD delivery.

Conclusion: Our data demonstrated an advanced concept of AD treatment by combined barrier repair and bacterial eradication with a sole agent for ameliorating the overall complications.

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1. Introduction

Atopic dermatitis (AD) is an inflammatory disease characterized by erythema, edema, vesicles, and lichenification. The prevalence of AD has increased 3-fold over the past 30 years [1]. Two features associated with AD are the compromised skin barrier and increased risk of cutaneous infection. The reduced barrier function leads to *Staphylococcus aureus* invasion and the eczema's exacerbation [2]. More than 90% of AD patients are colonized with *S. aureus* [3]. The occurrence of methicillin-resistant *S. aureus* (MRSA) has emerged as a predominant threat for AD treatment. Efficient AD management requires multiple drug treatments. The anti-AD drugs such as coal tar, doxepin, calcineurin inhibitors, and steroids are reported to cause skin itching and stinging [4]. Several investigations assessing anti-AD therapy based on natural sources reveal a potential activity [5]. *Antrodia camphorata* is a fungal parasite on the inner cavity of *Cinnamomum kaehirae* Hayata. It has long served as the traditional medicine for the treatment of skin itching, abdominal pain, hypertension, influenza, and cancers [6]. *A. camphorata* also displays a potential for inhibiting and eradicating the pathogenic bacteria [7,8].

The present study attempted to purify the chemicals from this mycelium to evaluate the anti-AD potency. MRSA was used as the model pathogen to examine the antibacterial activity of the crude extract and compounds. We found that 2,4-dimethoxy-6-methylbenzene-1,3-diol (DMD) was superior in showing the anti-MRSA effect compared to the other compounds. We detected the chemokines in the stimulated keratinocytes by DMD treatment. To assess the in vivo efficiency of DMD, the MRSA burden, transepidermal water loss (TEWL), and differentiation proteins were estimated using a mouse model of AD plus bacterial implantation.

2. Materials and methods

2.1. Extraction and isolation of *A. camphorata*

The agar-cultured mycelium of *A. camphorata* (500 g) was used to prepare the extract in 95% ethanol (1.5:1) for 3 days at room temperature. The extract supernatant (AC) was further partitioned with ethyl acetate as the fractions. The fractions were separated by silica gel column chromatography using the gradient mixtures (400 ml) of *n*-hexane:acetone (9:1, 7:1, 5:1, 3:1, 1:1, total acetone, and total methanol) as eluents to obtain seven fractions.

2.2. Liquid chromatography-mass spectrometry (LC–MS) analysis

The system included a Dionex UltiMate 3000 equipped with a pump, column compartment, autosampler, and a Thermo Finnigan LXQ linear ion trap mass spectrometer operated in positive electrospray ion mode. The Acquity UPLC BEH Shield RP18 column was employed and maintained at 35 °C with a flow rate of 0.3 ml/min. The mobile phase consisted of water and methanol, both containing 0.1% formic acid. The data analysis was performed using Xcalibur 2.0.7 software.

2.3. Bacterial strains

MRSA (ATCC 33591) and vanomycin-intermediate *S. aureus* (VISA, ATCC 700699) were obtained from American Type Culture Collection. Ten drug-resistant clinical isolates were used, five of which were MRSA (KM-1 to KM-5). The others were VISA (KV-1 to KV-5).

2.4. Disk diffusion test

Disk diffusion test was performed according to EUCAST instruction (<http://www.eucast.org>) with Müller-Hinton agar

plate, a MRSA or VISA suspension in TSB solution at a density equivalent to a 0.5 McFarland barium standard ($1-2 \times 10^8$ CFU/ml) as detected by a McFarland densitometer, and the disk loaded with the compounds or crude extract (500 µg). After incubation for 20 h, the inhibition zone diameter was measured.

2.5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A broth twofold serial dilution method was utilized to measure the MIC [9]. The bacterial population was exposed to several dilutions of the compounds ranging from 1.22 to 2500 µg/ml with TSB and incubated at 37 °C for 20 h. For the MBC assay, the bacterial suspension was diluted in PBS and plated on TSB plates. MBC was defined as the lowest concentration that killed $\geq 99.9\%$ of bacteria.

2.6. The bacterial morphology

The morphology of MRSA after DMD treatment was observed by Jeol JEM-1200 EX transmission electron microscopy (TEM) based on previous study [9].

2.7. Biofilm determined by confocal microscopy

The biofilm was treated by DMD, ciprofloxacin, or CPC at a concentration of 500 µg/ml for 24 h. The three-dimensional structure and thickness of biofilm were detected by Leica TSC SP2 confocal microscopy [10].

2.8. Intracellular MRSA killing

The HaCaT keratinocytes and macrophages differentiated from THP-1 monocytes by phorbol myristate acetate (0.1 µM) were used as the host cells to determine the DMD activity toward intracellular MRSA. The detailed information was shown in the previous report [11].

2.9. Genomic DNA preparation and agarose gel analysis of MRSA

The detailed procedure is shown in Supplementary Materials.

2.10. Anti-Taq DNA polymerase activity

The detailed procedure is shown in Supplementary Materials.

2.11. DNA binding ability of Taq DNA polymerase

The detailed procedure is shown in Supplementary Materials.

2.12. Wrapping assay

The detailed procedure is shown in Supplementary Materials.

2.13. Keratinocyte viability

To estimate the effect of DMD on HaCaT cells, the cell viability was examined using the tetrazolium assay. The detailed process of this assay was described previously [12].

2.14. Determination of chemokines in keratinocytes

HaCaT cells (1×10^5 cells/well) were trypsinized into 24-well plates. After incubation in DMEM without FBS for 24 h, tumor necrosis factor (TNF)-α and interferon (IFN)-γ (20 ng/ml of each) were added with or without AC or DMD for 24 h. The supernatant was harvested after 24 h and subjected to ELISA with a murine

monoclonal antibody against human chemokines CC motif ligand 5 and 17 (CCL5 and CCL17). ELISA was performed according to the manufacturer's directions.

2.15. *In vitro* cutaneous absorption test

The *in vitro* cutaneous absorption of DMD was conducted using Franz diffusion cell. DMD penetration into the receptor was quantified by HPLC. The other procedures were the same as in the previous research [13].

2.16. Ovalbumin (OVA)-induced AD-like mouse and MRSA infection

The AD-like skin was induced by a method modified from the previous study [14]. On Day 14 of OVA treatment, 100 μ l MRSA with an OD₆₀₀ of 1 was pipetted onto the gauze, which was administered to the dorsal skin. On Day 15 and 16, 10 mg/ml DMD was spread onto the 1 $\times$ 1 cm gauze. At the end of the experiment, the skin was excised to count MRSA CFU and analyze the differentiation protein gene expression by real-time PCR. Total RNA was extracted with ZYMO Direct-zol[®] reagent. The cDNA was synthesized by reverse transcription by using an iScript[®] cDNA synthesis kit. The real-time PCR was performed using iQ SYBR Green Supermix according to the manufacturer's instructions. The primers used to analyze mRNA were the same as in the previous report [15].

2.17. *In vivo* cutaneous tolerance

The 10% methanol/PBS containing 1 mg/ml DMD was applied daily (0.2 ml) on the dorsal area of the healthy mouse for 5 consecutive days. The DMD vehicle was replaced with a new one every day. After the vehicle removal, the treated skin region was assessed by TEWL and surface pH using Cutometer[®] MPA 580.

2.18. Statistical analysis

The statistical analysis was carried out using GraphPad Prism 5 software. Dual comparisons were made with unpaired Student's *t*-test. Groups of three or more were analyzed by analysis of variance (ANOVA) with Tukey or Dunnett posttests. The significance was indicated as * for *p* < 0.05, ** for *p* < 0.01, and *** for *p* < 0.001 in the figures.

3. Results

3.1. LC–MS determination of *A. camphorata* extract

An LC profile of the ethanolic extract exhibits many peaks as shown in Fig. 1A. Investigation using chromatography led to the identification of five compounds. Fig. 1B illustrates the structures of these compounds. Suppl. Table 1 summarizes the MS analysis of the compounds derived from ethanolic extract. The five

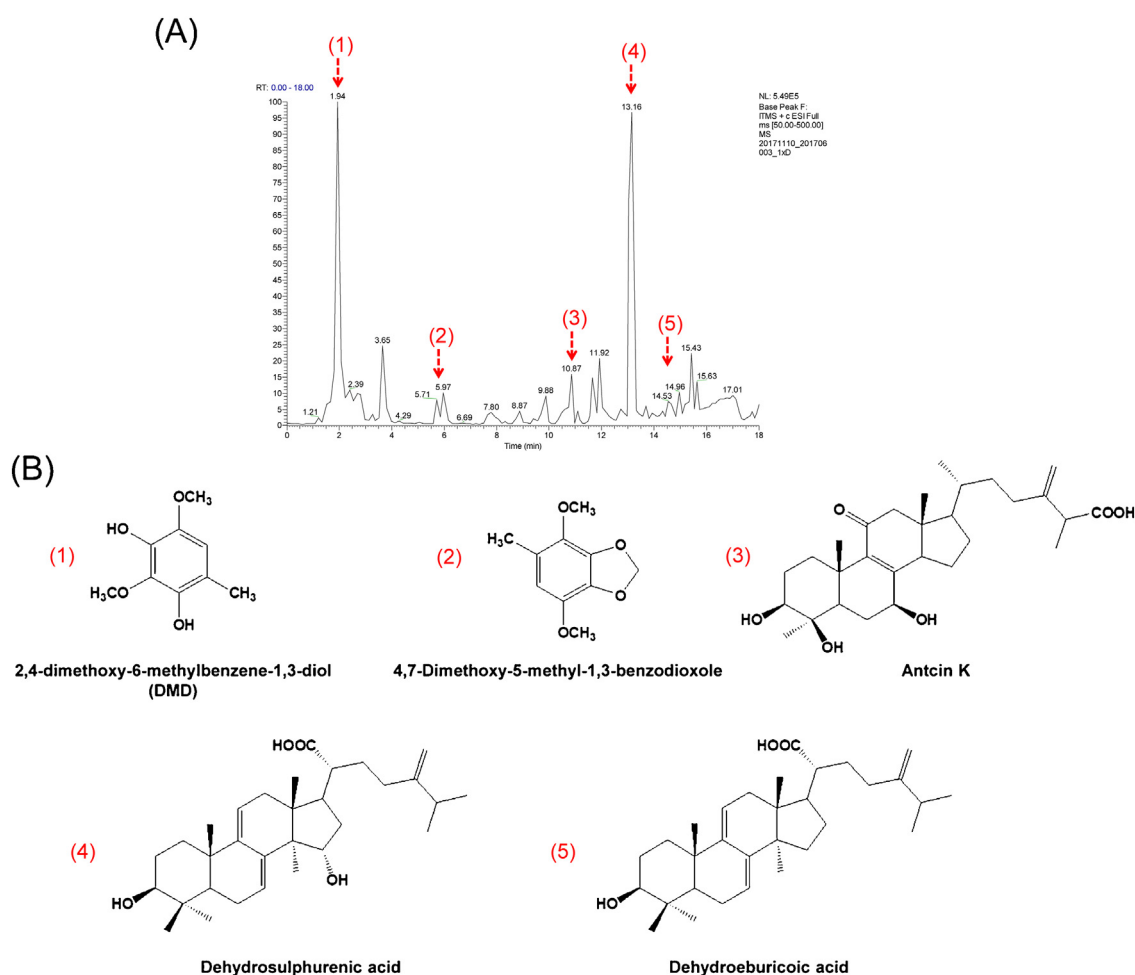


Fig. 1. Structural characterization of *A. camphorata*. (A) LC–MS chromatograms of *A. camphorata* extract. (B) Index compounds of *A. camphorata* mycelium.

compounds were previously found in *A. camphorata* based on the references [16,17].

3.2. DMD inhibits MRSA growth

The ethanolic extract was further separated by column chromatography with different eluents to yield 7 fractions as shown in Suppl. Table 2. The agar diffusion inhibition zone demonstrated the susceptibility of MRSA to the eluents of *n*-hexane:acetone 7:1, 3:1, and 1:1. DMD, antcin B, and 4-hydroxy-benzaldehyde were isolated from these three fractions, respectively. To examine the effect of these compounds on MRSA eradication, the MIC and MBC were measured as compared to the ethanolic extract (AC) as depicted in Suppl. Table 3. DMD demonstrated the strongest MRSA and VISA growth inhibition. The inhibition capability of DMD was comparable to nalidixic acid, a positive control. Nalidixic acid was used because of the similarity of physicochemical properties with DMD. Another positive control was vancomycin, which is the antibiotic used for eliminating MRSA infection in clinics. As expected, vancomycin revealed very low MIC and MBC values to MRSA with efficient growth inhibition. A panel

of drug-resistant clinical isolates was employed to test the MIC and MBC of DMD (Suppl. Table 4). The results showed that DMD was able to inhibit the growth of these isolates to a determined amount.

The MRSA and VISA inhibition was recognized by the agar diffusion assay (Fig. 2A). The trend of inhibition zone for inhibiting MRSA and VISA is DMD > antcin B > 4-hydroxy-benzaldehyde. Flow cytometry was used to determine the MRSA death rate. DMD shows a dose-dependent fashion for MRSA killing (Suppl. Fig. 1). Fig. 2B presents the TEM imaging of MRSA appearance. The intact bacteria exhibited a smooth morphology on the surface. DMD produced no influence on the MRSA shape. As a positive control, CPC disrupted bacterial surface and released cytoplasm. Fig. 2C presents the effect of DMD and CPC on the suppression of MRSA in the biofilm form. DMD significantly reduced CFU inside and outside the biofilm by about 10^3 -fold.

Fig. 2D shows the anti-biofilm activity of DMD, ciprofloxacin, and CPC against MRSA under confocal microscopy. The green signal was faded after treatment of the antibacterial agents, with CPC showing the weakest intensity. CPC is proved to be a strong biofilm inhibitor via the mechanism of disrupting bacterial membrane [18]. DMD, ciprofloxacin, and CPC restricted the biofilm thickness

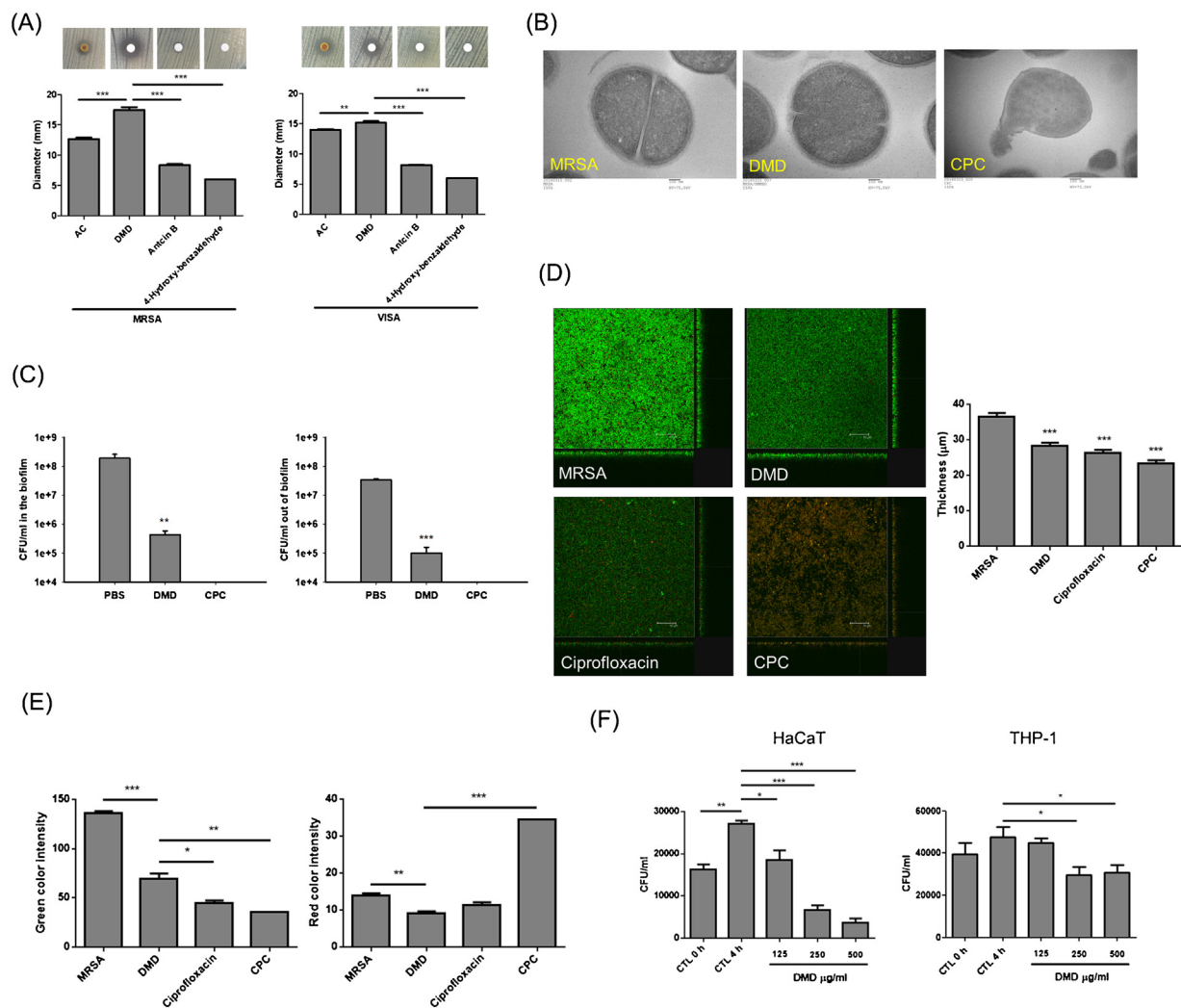


Fig. 2. Determination of the antibacterial activity of *A. camphorata* extracted compound DMD. (A) Zone of inhibition of MRSA and VISA measured from EUCAST guideline. (B) Morphological changes of MRSA viewed under TEM. (C) Measurement of MRSA counts for planktonic and biofilm. (D) The three-dimensional images and corresponding biofilm thickness analyzed by CLSM. (E) Quantification of fluorescence intensity of MRSA biofilm. (F) Intracellular MRSA killing in HaCaT keratinocytes and macrophages (THP-1). Each value represents the mean $\pm$ SEM ($n=3$). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. The statistical methods used for comparing the data of different groups are unpaired *t*-test and ANOVA.

from 36.4 to 28.4, 26.4, and 23.2 μm , respectively. The quantification of biofilm fluorescence intensity shows that CPC markedly reduced the green signal (Fig. 2E). The green fluorescence was significantly decreased by DMD and ciprofloxacin. The MRSA burden in the mammalian cells increased after a 4-h incubation (Fig. 2F). Treatment with DMD dose-dependently reduced the MRSA production. DMD at a dose of 500 $\mu\text{g}/\text{ml}$ could reduce MRSA CFU in keratinocytes by 7.5-fold as compared to the control (4 h).

3.3. Antibacterial mechanism of DMD

We explored the antibacterial mechanism of DMD by genomic DNA detection (Fig. 3A). DMD and ciprofloxacin revealed a similar pattern diminishing DNA concentration. Taq DNA polymerase is important to replicating DNA in MRSA. We further examined the ability of DMD to inhibit DNA polymerase (left panel of Fig. 3B). The PCR products were reduced by DMD in a dose-dependent manner,

confirming the role of DMD as a DNA polymerase inhibitor. Mitomycin C is an antitumor drug with a capability to elicit DNA damage via an interstrand DNA crosslink. This drug may not affect the activity of DNA polymerase. The right panel of Fig. 3B demonstrates a significant presence of PCR products after mitomycin C treatment.

As shown in Fig. 3C, DMD at different concentrations can decrease the polymerase-DNA complex (red arrows) in the ssDNA type but not in dsDNA. We further tested the effect of DMD on the inhibition of the supercoiling activity of DNA gyrase and the relaxing activity of DNA topoisomerase I. The assay demonstrates that DMD exhibited a capacity to deactivate gyrase (left panel of Fig. 3D). However, this effect was not observed in the case of topoisomerase I. Based on the aforementioned experiments, we suggest that during the process of DNA replication, DMD may inhibit the activity of DNA polymerase and gyrase. Fig. 3E illustrates the possible action mechanisms for DMD.

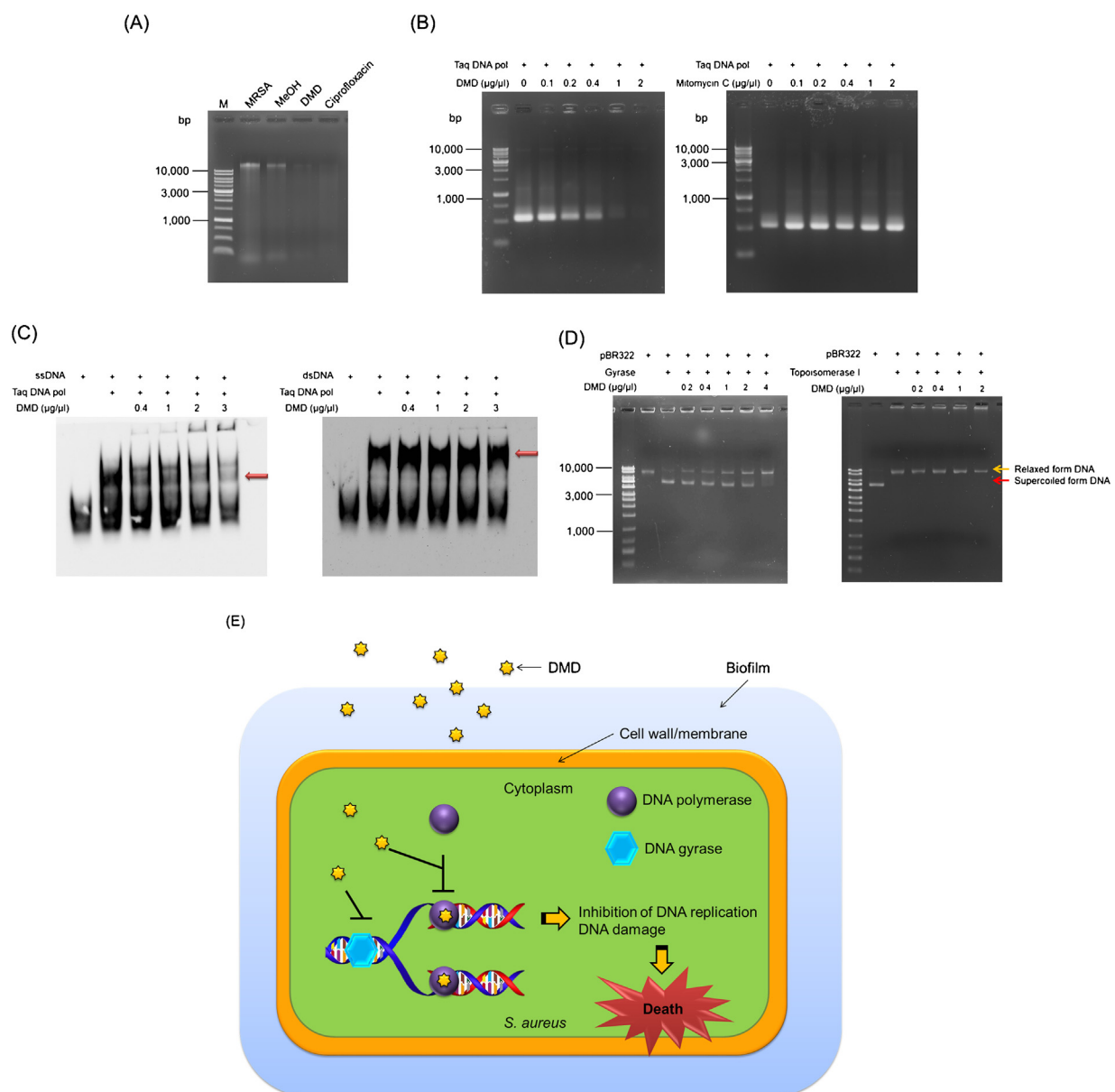


Fig. 3. Antibacterial mechanisms of DMD. (A) Analysis the quality of MRSA genomic DNA after treated with DMD by 0.8% agarose gel electrophoresis. (B) Effects of DMD and mitomycin C on Taq DNA polymerases in PCR. (C) A mobility shift assay for Taq DNA polymerase with ssDNA and dsDNA. (D) Effect of DMD on *E. coli* gyrase and topoisomerase I in wrapping assay. (E) Illustration for antibacterial mechanism of DMD on reduced biofilm production, inhibited DNA polymerase and gyrase activity.

3.4. DMD reduced chemokines in activated keratinocytes

As shown in Fig. 4A, the DMD concentrations of 10 and 50 $\mu\text{g/ml}$ indicate no cytotoxicity. The higher doses of $>100 \mu\text{g/ml}$ further decreased the keratinocyte viability. We next assessed the effect of AC and DMD on the levels of CCL5 and CCL17. The expressions of CCL5 and CCL17 were elevated 4.2- and 30.1-fold with treatment of TNF- α /IFN- γ (Fig. 4B and C). The TNF- α /IFN- γ -induced chemokine increase was significantly inhibited by AC and DMD. DMD at the lower concentration (10 $\mu\text{g/ml}$) was sufficient to return CCL17 expression to the control baseline.

3.5. DMD ameliorates AD-like lesion

In vitro DMD permeation across pig, BALB/c mouse, and AD-like skins was investigated using Franz cell as depicted in Fig. 5A. DMD penetration across mouse skin was greater as compared to that across pig skin because of the thinner and more-permeable features of rodent skin compared to pig skin. We also compared the cutaneous DMD delivery in intact and AD-like skin after a 12-h application. OVA sensitization significantly increased DMD absorption by 2.5-fold compared to healthy skin. An AD-like skin lesion with MRSA infection developed in the mouse. The application of MRSA or OVA on the back skin displayed the symptoms of erythema, edema, scaling, and epidermal excoriation according to the gross and microscopic appearances of the skin surface (Fig. 5B and C). The severity of these lesions was worsened by the combined OVA and MRSA. The signs of AD-like lesion in the DMD-treated groups were significantly lessened compared with MRSA alone or OVA plus MRSA.

In Fig. 5D, we see that MRSA application induced a 1000-fold increase of bacterial CFU in the skin. DMD treatment could decrease the MRSA burden by 10-fold. The combined OVA and MRSA further drove the increase of MRSA inoculation. The antibacterial activity of DMD significantly mitigated the progression of MRSA by about 100-fold. TEWL was measured to evaluate the skin barrier function as shown in Fig. 5E. The water evaporation was increased to 3-fold by combined OVA and MRSA compared to normal skin. The topical administration of DMD significantly reduced TEWL to 8.0 g/cm²/h, approximating the baseline. As illustrated in Fig. 5F, the mRNA level was downregulated by

combined OVA and MRSA. The AD-like skin with MRSA exposure even exhibited a minimized or negligible gene expression of the proteins. DMD markedly enhanced the mRNA of the proteins tested.

3.6. DMD elicits no irritation on intact skin

The repeated treatment of DMD for 5 days generally showed no change in the appearance visualized by a digital camera and handheld microscope (Fig. 6A and B). TEWL was significantly increased after a 4-day treatment of PBS and vehicle (Fig. 6C). This could be due to the methanol and water excessively hydrating the SC and disturbing the barrier property. DMD could reverse the increased TEWL produced by the vehicle effect. This may imply a protective capability of DMD on barrier function. No skin pH change was detected by vehicle and DMD treatments (Fig. 6D).

4. Discussion

AD produces lesions with low antimicrobial peptides [19], leading to the increased bacterial colonization and the following inflammation and barrier disruption exacerbation. Our results demonstrated that DMD had the anti-MRSA activity as evaluated in the forms of planktonic bacteria, biofilm mass, and intracellular infection. According to the TEM imaging and biofilm visualization, the anti-MRSA activity of DMD was not mediated by membrane disturbance. The results of biofilm imaging and genomic DNA assay inferred that DMD might represent a mechanism which traps DNA gyrase and topoisomerase and disarranges DNA replication. We found that DMD could interfere with ssDNA binding capacity to create an unstable polymerase-DNA complex. The DNA replication efficiency was thus lessened. DNA gyrase appears to be an ideal target for bacterial killing [20]. We provided evidence of the gyrase sensitivity toward the inhibition by DMD.

Our skin absorption data showed that topically applied DMD could penetrate the skin, assuring the subsequent anti-AD activity. The cutaneous absorption can be facilitated by the more lipophilic penetrants [21]. DMD revealed a lipophilic characteristic according to a partition coefficient log *P* of 1.8. Another possibility elucidating the absorption of DMD was the low molecular weight (184 g/mol) for preferentially penetrating into the SC. Inflammatory skin

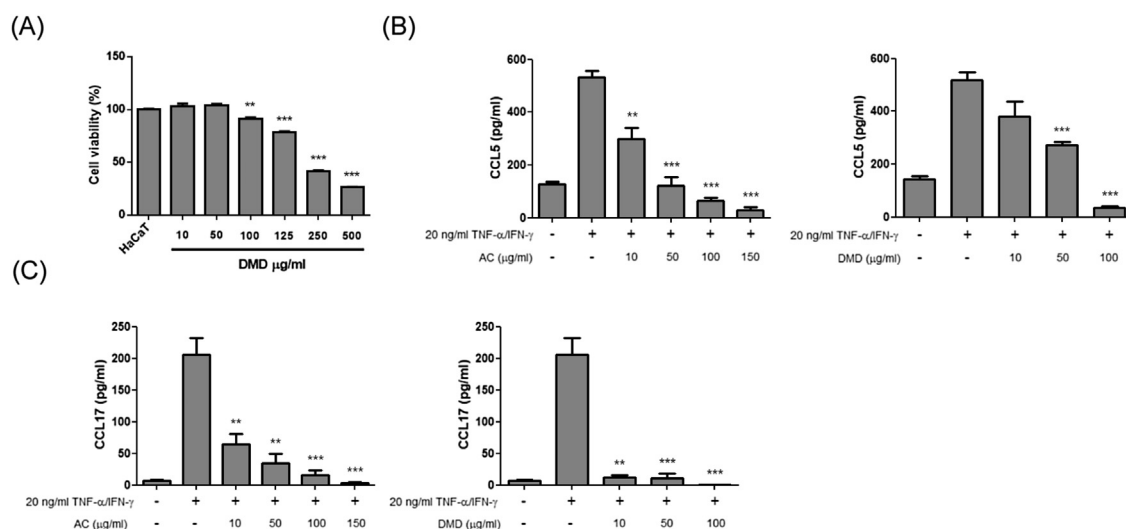


Fig. 4. The effects of crude extract (AC) and DMD on HaCaT keratinocytes. (A) Cell viability of DMD-treated HaCaT cells with respect to untreated control. (B), (C) AC and DMD reduced AD inflammation chemokines marker CCL5 and CCL17. Each value represents the mean $\pm$ SEM ($n = 6$). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. The statistical methods used for comparing the data of different groups are unpaired *t*-test and ANOVA.

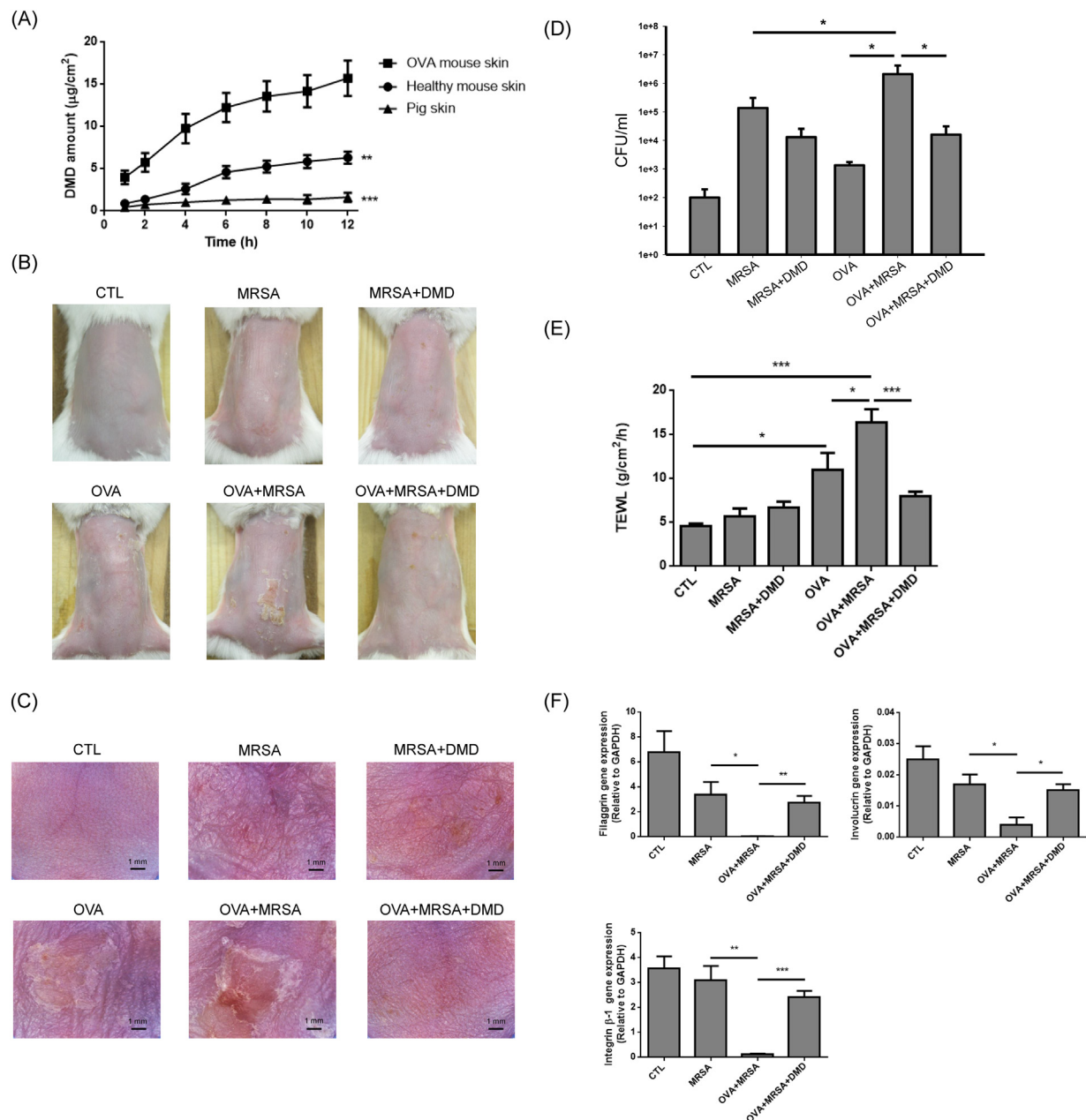


Fig. 5. In vitro absorption-time profiles of DMD and in vivo topical application of DMD against MRSA infection AD-like mouse. (A) In vitro permeation profile of DMD in Franz Cell using OVA mouse, healthy mouse, and pig skins. (B) The skin surface of mouse after treatment of OVA induction or MRSA infection with and without DMD condition at day 17. (C) The magnified images of panel (B). The scale bar is 1 mm. (D) Survival of MRSA in mouse skin treated with DMD. Seventeen days after OVA induction and MRSA infection, skin were cut, homogenized, and bacterial count was determined by CFU assay. (E) Transepidermal water loss (TEWL) of mice skin treated with DMD. (F) qPCR analysis of mouse skin RNA for differentiation markers filaggrin, involucrin and integrin β -1. Each value represents the mean $\pm$ SEM ($n = 6$). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. The statistical methods used for comparing the data of different groups are unpaired t -test and ANOVA.

diseases are always associated with impaired SC integrity and tight junction (TJ) function for the greater drug transport compared to intact skin [22]. Our results confirmed a superior DMD penetration across the lesional skin as compared to healthy skin. The limitation of the present work was the use of murine model but not human skin as the permeation barrier. It is known that human skin exhibits stronger barrier characteristic than murine skin. Whether the topical DMD application is advantageous for skin penetration and AD therapy in humans remains to be elucidated.

S. aureus infiltrates into the skin to trigger keratinocyte stimulation, leading to inflammation. The inflammation produces further barrier defect and hence a vicious cycle [3]. The skin barrier impairment parallels the increased TEWL [23,24]. Not only the skin

inflammation but also the bacteria themselves interact with the SC and TJ to cause barrier damage [25]. Topically applied DMD attenuated the increased TEWL level induced by OVA and MRSA. This compound could even maintain the TEWL at the baseline level to ignore the effect of vehicle disruption on the barrier's nature in the in vivo tolerance study. The upregulation of differentiation proteins in skin tissue suggests that DMD strengthened the SC and TJ barriers. The AD production is partly driven by the terminal keratinocyte differentiation defect [26]. DMD was useful for raising this TJ for barrier function restoration. There is a relationship between the barrier function and microbial infection by estimating the TEWL increase following the decrease of antimicrobial peptides in AD patients [27]. Both the direct bacterial killing

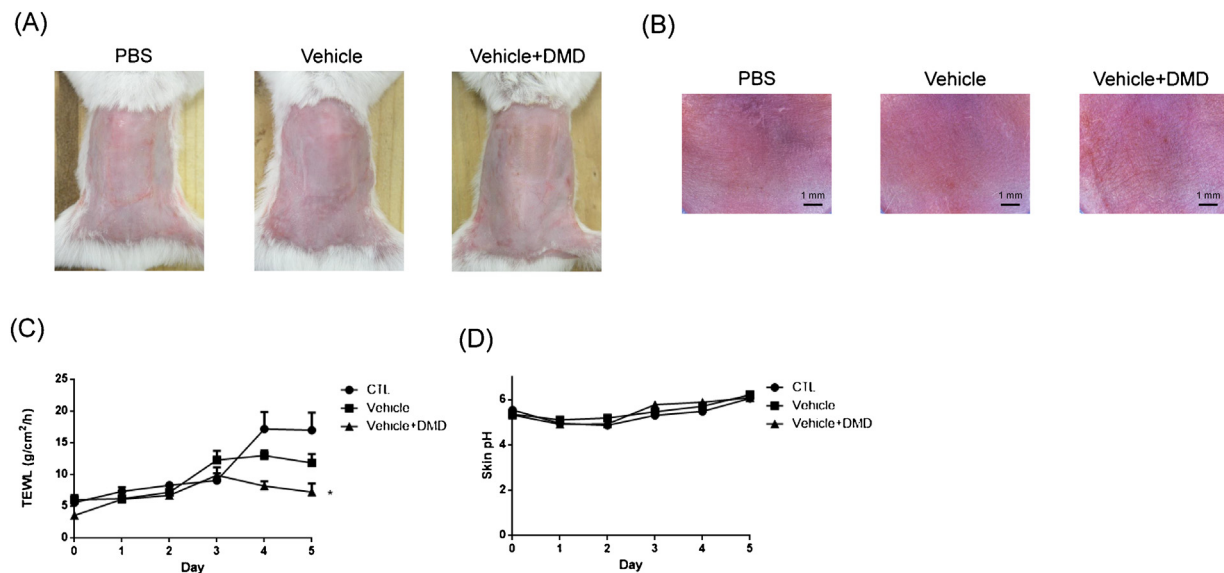


Fig. 6. Skin tolerance examination of mouse skin by a 5-day treatment of topically applied DMD: (A) Macroscopic appearance and (B) magnified images. (C) Transepidermal water loss (TEWL) of mouse skin treated with DMD; and (D) skin surface pH value. Each value represents the mean $\pm$ SEM ($n=6$). * $p < 0.05$. The statistical methods used for comparing the data of different groups are unpaired t -test and ANOVA.

and improvement of epidermis disruption for preventing infection by DMD were helpful for reducing the overall risk of pathogen invasion in AD.

The previous work [28] suggests the anti-inflammatory effect of *A. camphorata* against the production of nitric oxide, IL-1 β , COX-2, and TNF- α in the lipopolysaccharide-activated macrophages. Some benzenoids isolated from *A. camphorata* have attracted attention due to the anti-inflammatory activity in T cells and macrophages [29,30]. The keratinocytes of AD patients indicate a propensity to produce cytokines and chemokines [31], both of which can be employed as the clinical markers. Chemokines are the signaling peptides regulating immune cell trafficking. The inflammatory cell infiltration in AD skin is commonly observed. A previous study [32] demonstrated that *A. camphorata* lowered inflammatory cell infiltration in the epidermis in a mouse skin ischemia model. CCL5 is a chemokine that induces leukocyte infiltration in response to bacterial toxins. The barrier damage elicits the production of CCL17 from keratinocytes, resulting in the attraction of T lymphocytes via CC chemokine receptor 4 (CCR4). Both chemokines are highly associated with the occurrence of AD [23,33]. Our results showed that DMD significantly decreased CCL5 and CCL17 induced by the activated keratinocytes, suggesting that DMD might play a role in the inhibition of T lymphocyte migration into AD lesions.

The in vivo safety rating through topical DMD application manifested a minimal adverse event on normal skin. Although DMD caused in vitro cytotoxicity against HaCaT at the concentrations of $>100 \mu\text{g/ml}$, the in vivo tolerance study demonstrated no irritation of topically applied DMD at 1 mg/ml. The use of topical therapy is beneficial in comparison with systemic therapy as it generally exhibits less adverse risk and increases compliance. Our data proved the feasibility of DMD for topical use because of the capability to penetrate the skin and the improvement of AD symptoms and complications. Topical therapy of bacterial skin infection provides advantages over systemic therapy, including the minimization of systemic toxicity and reduced cost [34]. Chlorhexidine is a topical antiseptic employed in outpatient settings to control MRSA outbreak and prevention of skin infection [35]. The anti-MRSA agent developed in this study achieved similar aims to chlorhexidine. However, the structures and antimicrobial mechanisms of both compounds are quite different. Chlorhexidine is a

cationic disinfectant with broad-spectrum biocide activity against Gram-positive bacteria, Gram-negative bacteria, and fungi [36]. The main mechanism of action is the destabilization of cell wall to interfere osmosis. On the other hand, DMD showed specific antibacterial activity against Gram-positive bacteria but limited activity against Gram-negative bacteria such as *E. coli* and *P. aeruginosa* (data not shown). The mechanism of DMD was the protein synthesis inhibition but not cell membrane damage. The study of AD patient compliance has suggested that use of the anti-inflammatory therapy is too late once the flare-up has started [37]. Currently, there is no primary preventive intervention established for AD [38]. DMD may be developed as a preventative agent because it is safe and even protects the barrier feature in healthy skin.

Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jdermsci.2018.09.002>.

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Supplementary Materials

2.9. Genomic DNA preparation and agarose gel analysis of MRSA

MRSA genomic DNA was extracted using a Presto[®] Mini Bacteria Kit based on the manufacturer's instructions. The aliquot of purified genomic DNA (100 ng) was analyzed by electrophoresis on a 0.8% agarose gel.

2.10. Anti-Taq DNA polymerase activity

The polymerase chain reaction (PCR) was performed with a 50- μ l volume at the following concentrations: 100 ng MRSA genomic DNA as the template, 0.4 μ M primer 1 (isaB-F: 5'-atgaataaaaccagtaaagtttg-3'), 0.4 μ M primer 2 (isaB-R: 5'-ttatttactgtgtttgtatgg-3'), 1x YEA Taq PCR reaction buffer, 2.5 mM dNTP, 2.5 U YEA Taq DNA polymerase, and DMD (0.1 to 2 μ g/ μ l). The PCR reaction was performed in the instrument at 95 °C for 1 min, 30 cycles of 10 sec at 95 °C and 30 sec at 56 °C to generate 528 bp amplicons and analyzed by electrophoresis on a 0.8% agarose gel.

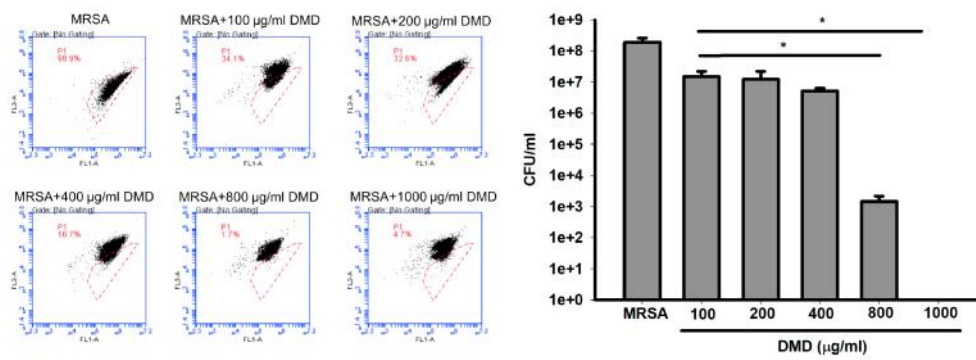
2.11. DNA binding ability of Taq DNA polymerase

To determine whether DMD affected the ability of Taq DNA polymerase to bind ssDNA and dsDNA, we performed the electrophoretic mobility shift assay (EMSA) for the polymerase DNA complexes. The test was carried out using ssDNA or dsDNA biotin-labeled oligonucleotides at the 5' end as the probe. The reaction mixture was incubated at 37°C for 1 h and analyzed using a 6% native PAGE at 100 V for 30 min. The 20,000-fold diluted streptavidin-peroxidase polymer solution was employed to bind biotin-labeled ssDNA or dsDNA.

2.12. Wrapping assay

Relaxed form pBR322 DNA was prepared by incubating supercoiled pBR322 DNA with topoisomerase I according to the manufacturer's instructions. The relaxed DNA was extracted with a Gel/PCR DNA Fragments Extraction Kit and resuspended in

buffer. For the gyrase supercoiling reaction, the reaction mixture (20 μ l) of 200 ng relaxed form DNA, gyrase reaction buffer, 1.25 U gyrase, and DMD (0.2 to 4 μ g/ μ l) was incubated at 37°C for 1 h. The reaction was terminated by raising the temperature to 65°C for 20 min. The DNA products were analyzed by electrophoresis on the agarose gel. The other procedures were the same as for the gyrase supercoiling reaction.



Supplemental Figure 1. Anti-MRSA activity was quantification by (A) flow cytometry and (B) bacteria counting in DMD treated culture broth.

Suppl. Table 1. Characteristic ion of benzenoids and triterpenoids from the mycelium of *A. camphorata*

Peak No.	T _R (min)	Compounds	[M+H] ⁺	MS ⁿ (% base peak)	References
(1)	1.94	2,4-dimethoxy-6-methylbenzene-1,3-diol (DMD)	185	MS ² [185] : 153 (100), 170 (7) MS ³ [185 → 153] : 125 (100), 107 (39), 153 (16), 79 (4) MS ⁴ [185 → 153 → 125] : 97 (100), 107 (65), 93 (48), 85 (27)	(Lien et al., 2014)
(2)	5.97	4,7-Dimethoxy-5-methyl-1,3-benzodioxole	197	MS ² [197] : 167 (100), 139 (13), 169 (2), 137 (1) MS ³ [197 → 167] : 139 (100), 107 (78), 124 (12) MS ⁴ [197 → 167 → 139] : 124 (100), 107 (56), 109 (17), 139 (8)	(Lien et al., 2014)
(3)	10.87	Antcin K	489	MS ² [489] : 435 (100), 453 (84), 471 (39), 407 (30) MS ³ [489 → 435] : 417 (100), 355 (81), 391 (41), 389 (31) MS ⁴ [489 → 435 → 417] : 399 (100), 279 (49), 389 (44), 361 (36)	(Chen et al., 2016)
(4)	13.16	Dehydrosulphurenic acid	485	MS ² [485] : 425 (100), 257 (4), 411 (1) MS ³ [485 → 425] : 257 (100), 191 (8), 395 (5), 410 (5)	(Chen et al., 2016)
(5)	14.53	Dehydroeburicoic acid	469	MS ² [469] : 423 (100), 311 (57), 451 (47), 405 (15) MS ³ [469 → 423] : 405 (100), 443 (68), 285 (51), 325 (26) MS ⁴ [469 → 423 → 405] : 387 (100), 377 (49), 355 (40), 281 (41)	(Lien et al., 2014)

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Suppl. Table 2. Anti-MRSA activity of different elution fractions from *A. camphorata* ethanol extract at a concentration of 25 mg/ml

<i>n</i> -Hexene:acetone	Inhibition zone	Compound
9:1	–	
7:1	++	DMD
5:1	–	
3:1	+	Antcin B
1:1	+	4-Hydroxy-benzaldehyde
Acetone	–	
Methanol	–	

Suppl. Table 3. MIC and MBC of MRSA and VISA after treatment of *Antrodia camphorata* compounds

	MIC (µg/ml)		MBC (µg/ml)	
	MRSA	VISA	MRSA	VISA
AC	1250	625-1250	1250-2500	625-1250
DMD	78.1	156.3	156.3	156.3
Antcin B	625	156.3	1250	625-1250
4-Hydroxy-benzaldehyde	2500	625-1250	2500	625-1250
Nalidixic acid	78.1	156.3	156.3	312.5
Vancomycin	2.4	9.7	2.4	9.8-19.5

All data are presented as the three experiments ($n=3$).

Suppl. Table 4. MIC and MBC of clinical isolates after treatment of DMD

Clinical isolates	DMD (µg/ml)	
	MIC	MBC
KM-1	156.25-312.5	312.5
KM-2	156.25-312.5	312.5
KM-3	156.25-312.5	312.5
KM-4	312.5	312.5
KM-5	156.25-312.5	>312.5
KV-1	156.25-312.5	156.25-312.5
KV-2	156.25-312.5	312.5
KV-3	156.25-312.5	156.25-312.5
KV-4	156.25-312.5	156.25-312.5
KV-5	156.25-312.5	156.25-312.5

KM: MRSA clinical isolates

KV: VISA clinical isolates