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Characterization of *in vitro* antimicrobial activity of gyrophoric acid isolated from *Parmotrema indicum* on methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a group of pathogenic bacteria associated with hard-to-treat infections in humans, which require new, effective antibiotics for treatment. In the search for new candidates for antibiotics against MRSA, we investigated the *in vitro* antimicrobial activity of gyrophoric acid isolated from the lichen *Parmotrema indicum* on MRSA. Gyrophoric acid exerted antimicrobial activity mainly on Gram-positive bacteria and one fungal strain, *Candida albicans*. The minimum inhibitory concentration (MIC) value for MRSA was 32 µg/ml, and at 8 × MIC, gyrophoric acid showed a bactericidal effect on MRSA after 24 hours of culture. This compound showed a low mutation frequency of 2.4×10^{-9} by a single-step resistance test. Gyrophoric acid expressed a synergistic effect with the antibiotic ampicillin that reduced 16-fold the MIC value of ampicillin on MRSA. Gyrophoric acid showed selective toxicity towards MRSA other than human cell lines, with the selective index ranging from 5.43-fold to 8.78-fold. Biofilm formation of MRSA was inhibited by gyrophoric acid starting from the concentration of 2 × MIC, and the biofilm inhibition was 94% at 16 × MIC of gyrophoric acid. The morphology of MRSA under treatment with gyrophoric acid showed a demolition of the cell envelope of the strain revealed by scanning electron microscope. The results of this study proved that gyrophoric acid is a good compound in the "hit validation and declaration" stage of the development procedure of a new antibiotic candidate for MRSA infection treatment.

INTRODUCTION

ESKAPE pathogens are virulent bacteria with multidrug resistance characteristics [1]. They are the leading cause of nosocomial infections in the world, which is associated with a high risk of mortality [2]. These pathogens have recently been identified by the World Health Organization in the list of 12 bacteria that urgently need new antibiotics for treatment. The urgency of new antibiotics for these twelve bacteria is classified into critical, high, and medium-priority [3]. Among ESKAPE pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) is on the list of high-priority groups [3]. In association with different virulent characteristics such as toxins, adhesins, enzymes, and immunomodulators [4], the antimicrobial resistance of MRSA has myriad mechanisms to the major antibiotic classes [5] that confer challenges to the treatment decision of this bacterial infection. Therefore, new antimicrobial candidates are currently sought from several sources (i.e., from plants, fungi, bacteria, and synthetic compounds) [6] to meet the crucial requirements.

Besides the common sources for new antibiotics searching as mentioned above, lichens become highly interesting because these symbionts produce structurally diverse secondary metabolites with various biological activities, including antimicrobial activities [7]. For example, atranorin, salazinic acid, lecanoric acid, and usnic acid isolated from *Bulbothrix setschwanensis* (Zahlbr.) Hale

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lichen exhibited good activities against human fungal and bacterial pathogens [8]; Usnic, barbatic and 4-O-demethylbarbatic acids from *Cladonia borealis* lichen, usnic and perlatolic acids from *Cladina confuse* lichen, atranorin, perlatolic, and anziaic acids from *Stereocaulom ramulosum* lichen, and cryptochlorophaeic and caperatic acids from *Canoparmelia cryptochlorophaea* lichen showed promising minimum inhibitory concentration (MIC) values against pathogenic bacteria including the resistant clinical strains [9]. However, current studies on the antimicrobial activities of lichen compounds have thoroughly focused on the isolation of the active compounds from lichen samples and preliminary investigation by agar diffusion assay and MIC determination technique of their antimicrobial activities. The antimicrobial results from these basic techniques are narrowly used for the hit discovery stage of new antibiotics, which is the earliest step in the pre-clinical development of the drug discovery and development process. Further studies need to be carried out for the hit validation and declaration stage, which include but are not limited to bactericidal or bacteriostatic determination, preliminary assays of spontaneous resistance, initial tests for human safety using animal cell lines for cytotoxicity evaluation, the spectrum of antibacterial activity, and modes of action [10,11]. This stage defines key features of the compound, indicating that the compound has the potential to be a good candidate with no obvious difficulties to be continuously developed in the advanced stages, such as hit-to-lead development, lead-to-candidate development, and pre-candidate profiling before entering the lengthy and costly clinical trials.

Bearing the above ideas in mind, a natural compound, gyrophoric acid, was chemically isolated from *Parmotrema indicum* and tested for its antibacterial activity in this study. Several experiments were subsequently performed to demonstrate the potential to develop this compound in further steps to be an antibiotic candidate for the fight against MRSA.

MATERIALS AND METHODS

Materials

The thalli of the lichen *P. indicum* was collected at Duc Trong District, Lam Dong Province, Vietnam, in May 2020. The scientific name of the lichen was determined by Dr. Thi-Phi-Giao Vo, Faculty of Biology-Biotechnology, VNUHCM-University of Science. A voucher specimen (UEL-001) was deposited in the herbarium of the Department of Organic Chemistry, Ho Chi Minh University of Education.

The microorganisms used for antimicrobial testing include *Bacillus subtilis, Enterococcus faecalis, Klebsiella pneumoniae, S. aureus, Pseudomonas aeruginosa, Escherichia coli, Enterobacter cloacea,* and *Candida albicans* were given by Center for Research and Application in Bioscience (Ho Chi Minh City, Vietnam). Except for *B. subtilis,* the microorganisms were clinically isolated, and they are associated with antibiotic resistance. These microorganisms were preserved in 30% glycerol at −20°C.

Extraction and isolation of gyrophoric acid

The extraction and isolation of gyrophoric acid from *P. indicum* were performed according to the protocol in [12] with modifications. The clean, air-dried, and ground material (3.8 kgs) was macerated with EtOAc at room temperature, and the filtrated solution was concentrated under reduced pressure to afford the crude ethyl acetate extract (719.52 g). While evaporating the filtrated solution to dryness, a precipitate occurred (152.32 g). The crude extract was re-extracted using solvents of n-hexane and n-hexane: EtOAc (1/1, *v*/*v*) to afford n-hexane extract (H, 112 g), n-hexane: ethyl acetate extract (HEA, 56 g), and the remaining (EAR, 70 g).

The remaining EAR (70 g) was applied to silica gel column chromatography and eluted with the solvent system of n-hexane: EtOAc: AcOH (1/1/0.02, *v*/*v*/*v*) to give 10 fractions (EAR1-EAR10). Fraction EAR6 (2.8 g) was subjected to silica gel column chromatography and eluted with the solvent system of n-hexane-chloroform-ethyl acetate-acetone-acetic acid (1/2/2/2/0.01, *v*/*v*/*v*/*v*) to afford six sub-fractions EAR6.1–6. Fraction EA6.6 (4.1 g) was applied to sephadex LH-20 gel chromatography, eluted with methanol to obtain three fractions EA6.6.1–3. Application of fraction EA6.2.2 to silica gel column chromatography using the mobile phase as n-hexane-chloroform-ethyl acetate-acetone-acetic acid (3/2/2/2/0.01, *v*/*v*/*v*/*v*) produced the solid (289 mg). Washing this solid with acetone three times yielded gyrophoric acid (123 mg). Nuclear magnetic resonance (NMR) spectra for this compound were recorded on a Bruker Avance III spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with Tetramethylsilane as the internal standard.

Antimicrobial testing against the indicator microorganisms

The agar diffusion method was employed [13]. The bacteria were cultured in nutrient broth overnight at 37°C with shaking. Then, the bacterial cultures were diluted with sterile 0.9% NaCl to match 0.5 McFarland standards and spread on Mueller-Hinton agar plates. Wells of 8 mm diameter were formed on the surface of the Mueller–Hinton agar (MHA) plates using sterile tips. Gyrophoric acid was dissolved in Dimethyl sulfoxide (DMSO) at the concentration of 1 mg/ml, and 50 µl of the gyrophoric acid solution was placed into wells on the surface of the MHA plates. The plates were incubated at 37°C for 16–18 hours. The antibacterial activity of gyrophoric acid was recorded by measuring the inhibition zones surrounding each well. DMSO was used as the negative control, and ampicillin and apramycin (both in 1 mg/ml) were the positive controls in this experiment.

MIC determination

MIC value of gyrophoric acid on the MRSA strain was determined using the agar dilution method [14]. Gyrophoric acid was dissolved in DMSO and diluted with MHA to the concentration range of 0, 1, 2, 4, 8, 16, 32, 64, and 128 µg/ml. The MRSA strain was cultured in nutrient broth overnight at 37°C with shaking. The bacterial strain was then diluted with sterile 0.9% NaCl to the concentration of 0.5 McFarland. Then, 1 µl of the 1:10 dilution of this bacterial solution (approximately 10^4 CFU) was spotted on the above MHA plates. These MHA plates were incubated at 37°C for 16–18 hours. The MIC value of gyrophoric acid to the MRSA strain was determined at the lowest concentration of gyrophoric acid that inhibited the growth of the MRSA strain.

Time-kill assay

The bactericidal kinetics of gyrophoric acid was investigated using the time-kill assay [15]. Mueller-Hinton broths were supplemented with gyrophoric acid at concentrations of 0×, 1×, 2×, 4 × MIC, and 8 × MIC. The MRSA strain was inoculated in these Mueller-Hinton broths at the final concentration of 1 × 10^6 CFU/ml, and the bacterial cultures were incubated at 37°C with shaking. At each time interval of 0, 3, 6, 12, and 24 hours, a 100 µl aliquot of the bacterial cultures was spread on nutrient agar plates. These plates were incubated at 37°C overnight. The colonies were counted, and the CFU/ml values were calculated for each culture with the corresponding MIC.

Single-step resistance assay

The frequency of single-step resistance was determined using the protocol of Mani *et al*. [16]. Briefly, an MRSA quantity of 10^{10} CFU was spread on nutrient agar plates containing gyrophoric acid at 8 × MIC concentration. The plates were incubated at 37°C for 48 hours. The resistant frequency to gyrophoric acid was calculated by counting the number of resistant colonies per inoculum. Ampicillin was used as the control for this experiment at 8 × MIC concentration.

Synergistic effect test

A standard checkboard assay [17] was used to assess the synergistic effect between gyrophoric acid and ampicillin. 10^4 CFU of the MRSA strain was used for determining the MIC of gyrophoric acid, ampicillin, and the combination between gyrophoric acid and ampicillin. The MIC values were used to calculate the fractional inhibitory concentration index (ΣFICI) using the following equation:

ΣFICI = MIC of antibiotic in combination/MIC of antibiotic alone + MIC of the target compound in combination/ MIC of target compound alone.

FICI ≤0.5 (synergism); 0.5 <FICI <1 (partial synergism); 1 ≤FICI <2 (indifference); and FICI ≥2 (antagonism).

***In vitro* cytotoxicity activity on mammalian cell lines**

The fibroblast and NCI H460 cell lines were used to evaluate the cytotoxicity of gyrophoric acid on mammalian cell lines using the colorimetric SulphoRhodamine-B (SRB) assay [18]. The cells were maintained in E'MEM media supplemented with 2 mM L-glutamine, 20 mM HEPES, 0.025 µg/ml amphotericin, 100 µg/ml streptomycin, 100 units/ml penicillin, and 10% heat-inactivated fetal bovine serum in a humidified 5% (*v*/*v*) CO_2 atmosphere at 37°C. Cells were seeded in 96-well plates with a density of 7.5 × 10^3 cells/well and incubated with complete media for 24 hours. Cells were then treated with gyrophoric acid at different concentrations for 48 hours. After exposure to gyrophoric acid, cells were fixed with 150 µl of 10% trichloroacetic acid (TCA) and incubated at 4°C for 1 hour. The TCA solution was removed, and the cells were washed five times with distilled water. Aliquots of 70 µl SRB solution (0.2% *w*/*v*) were added and incubated in a dark place at room temperature for 10 minutes. Plates were then washed three times with 1% acetic acid and allowed to air-dry overnight. Then, 150 µl of tris base (10 mM) was added to dissolve the protein-bound SRB stain; the absorbance was measured at 492 and 620 nm using a 96-well microtiter plate reader (Synergy HT, Biotek Instruments). IC_{50} was determined using Prism software with multiparameter nonlinear regression and $R^2 > 0.9$. The viability was calculated as (A540 of treated samples/A540 of the untreated sample) × 100, and the inhibitory concentration at 50% (IC_{50}) was determined from the exponential curve of viability versus concentration. Also, the therapeutic index (TI) was determined to estimate the safety and selectivity of the tested compounds by dividing the IC_{50} value by the MIC value of gyrophoric acid.

Determination of MRSA biofilm formation inhibition

The biofilm formation inhibition assay [19] was used to assess the ability of gyrophoric acid to inhibit the biofilm formation of the MRSA strain. The MRSA strain was cultured in 200 µl of Lysogeny broth (LB) broth containing 1×, 2×, 4×, 8×, and 16 × MIC of gyrophoric acid in a 96-well microtiter plate. The plate was incubated at 37°C for 16–18 hours for biofilm formation. The well was washed with Phosphate-buffered saline (PBS) to remove the planktonic MRSA cells, followed by fixation with methanol. Methanol was removed, and the plate was completely air-dried. Next, the plate was stained with 0.1% crystal violet for 5 minutes before the addition of 95% ethanol for decolorization. The biofilm formation and its inhibition were recorded using a microtiter plate reader with an optical density of 595 nm. The biofilm formation inhibition percentage was calculated using the control of no gyrophoric acid treatment.

Scanning electron microscope (SEM) analysis of MRSA treated with gyrophoric acid

The protocol by Weidong *et al*. [20] was applied with minor modifications to examine the morphology of MRSA under the treatment of gyrophoric acid. 1 × 10^8 CFU/ml of the MRSA strain was incubated with 8 × MIC of gyrophoric acid for 4 and 16 hours at 37°C. The MRSA cells were centrifuged at 5,000 g and washed two times with sterile 0.9% NaCl. The cells were fixed with 2.5% glutaraldehyde at 4°C for 2 hours. The cell sample was then dehydrated with increasing concentrations of 30%, 50%, 70%, 90%, and 100% ethanol. The treated cell sample of MRSA was analyzed under the SEM (FE-SEM S-4800, Hitachi). The morphology of MRSA cells treated with gyrophoric acid was compared with those without gyrophoric acid treatment.

Statistical analysis

All experiments were repeated three times independently. The data were expressed as means ± SD. The data were analyzed using Microsoft Excel 2016 and GraphPad Prism 9.

RESULTS AND DISCUSSION

Isolation and identification of gyrophoric acid from *P. indicum*

The dried lichen was sequentially extracted with different solvents to obtain the corresponding extracts, in

which the EAR extract was applied on a silica gel column chromatography and eluted with relevant solvents to obtain fractions. Among the fractions, the fraction EA6.2.2 produced the solid compound, and this compound was washed with acetone to yield gyrophoric acid (Fig. 1). NMR was applied to confirm the structure of gyrophoric acid extracted from *P. indicum* with the following result.

Gyrophoric acid

White amorphous powder. ^{1}H-NMR (500 MHz, acetone-d_6) δ: 11.11 (1H, brs, OH), 6.87 (1H, d, J = 2.5 Hz, H-3′), 6.84 (1H, d, J = 2.5 Hz, H-5′), 6.77 (1H, brs, H-3″), 6.73 (1H, brs, H-5″), 6.39 (1H, d, J = 2.5 Hz, H-3), 6.31 (1H, d, J = 2.5 Hz, H-5), 2.67 (1H, s, 6′-CH_3), 2.67 (1H, s, 6″-CH_3), 2.61 (1H, s, 6-CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 108.5 (C-1), 159.9 (C-2), 100.5 (C-3), 161.0 (C-4), 109.8 (C-5), 140.3 (C-6), 167.0 (C-7), 21.2 (6-CH_3), 116.6 (C-1′), 156.2 (C-2′), 107.2 (C-3′), 152.1 (C-4′), 114.2 (C-5′), 137.9 (C-6′), 165.5 (C-7′), 19.3 (6′-CH_3), 117.3 (C-1′), 158.8 (C-2′), 107.1 (C-3′), 152.2 (C-4′), 114.3 (C-5′), 139.5 (C-6′), 170.3 (C-7′), 20.8 (6′-CH_3). The NMR data were consistent with those reported in the literature [21].

In the literature, gyrophoric acid has several biological activities on cell proliferation, apoptosis, and cell signaling pathways [22]. Gyrophoric acid also showed *in vitro* and *in vivo* antioxidant activities [23,24]. In addition, gyrophoric acid showed antimicrobial activities against bacteria and fungi [25]. Due to its diverse biological activities, gyrophoric acid has been isolated from lichen populations because these composite organisms contain a high concentration of this polyphenolic depside, including lichens of the *Umbilicaria* genus, the lichen *Xanthoparmelia pokornyi* [25], the lichen *Acarospora fuscata* [26]. In this study, gyrophoric acid was isolated from *P. indicum*, a lichen species isolated in Duc Trong District, Lam Dong Province, Vietnam. This is the first time gyrophoric acid has been isolated from *P. indicum,* although species of the *Parmotrema* genus have been reported to contain gyrophoric acid [27,12]. Although showing antimicrobial activities against bacteria and fungi, no study has so far investigated the *in vitro* antimicrobial properties of gyrophoric acid in terms of antimicrobial spectrum, killing kinetics, resistance mutation frequency, synergistic effects with conventional antibiotics, selective antimicrobial activity, and biofilm inhibition. These *in vitro* antimicrobial characteristics are helpful for the initial development (i.e., validating and declaring a hit) of gyrophoric acid as a new antibiotic candidate [10].

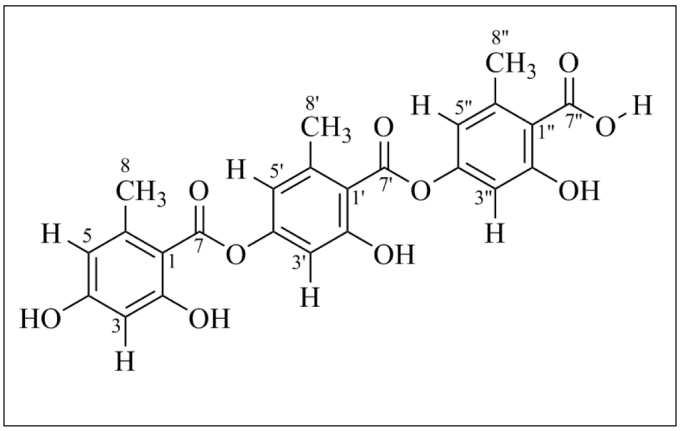

Figure 1. Chemical structure of gyrophoric acid extracted from *P. indicum.*

Antimicrobial activity of gyrophoric acid

Several bacterial strains were used in the evaluation of *in vitro* antimicrobial activity of gyrophoric acid. They included Gram-negative, Gram-positive bacteria. One strain of *C. albicans* was also included in this experiment. The agar diffusion results showed that gyrophoric acid exerted activities against MRSA*, B. subtilis, E. faecalis, K. pneumoniae,* and *C. albicans* with inhibition zones ranging from 13 to 25 mm. The negative control, DMSO, showed no inhibition zone to any testing microorganism. The positive control, apramycin, showed inhibition zones for all microorganisms, while the other positive control, ampicillin, showed inhibition zones for microorganisms except *P. aeruginosa, E. coli,* and *E. cloacea* (Table 1). Gyrophoric acid showed antimicrobial activity mostly on Gram-positive bacteria including *B. subtilis, Enterococcus faecium,* MRSA. It exerted a weak activity on a Gram-negative bacterium as *K. pneumoniae*. It also showed antifungal activity against *C. albicans*, an eukaryotic pathogen. The results on the antimicrobial activity of gyrophoric acid in this study were comparable to those of Candan *et al*. [25], except that the *K. pneumoniae* strain here showed a weak sensitivity to gyrophoric acid with an inhibition zone of 13 mm. Most of the microorganisms in this study were clinically isolated, and more importantly, they are antibiotic resistant. However, the antimicrobial activity of gyrophoric acid was mostly consistent for microorganisms irrespective of nonclinical or clinical strains, sensitive or resistant to antibiotics. The initial antimicrobial results of gyrophoric acid paved the way for the following *in vitro* antimicrobial activity characterization of the compound on MRSA.

MIC of gyrophoric acid toward MRSA

MIC is the value that has been used to determine the effectiveness of antibiotics on bacteria [28]. Antibiotics with lower MIC values are more effective against pathogenic bacteria. The MIC value of gyrophoric acid was determined by the agar dilution technique, and it was 32 µg/ml against the MRSA strain. In comparison, ampicillin had the MIC value of 16 µg/ml (Fig. 2). Ampicillin was chosen to be the control in this experiment and the following experiments because it was effective on the microorganisms in this study in a similar manner as gyrophoric acid (Table 1). In the previous study, gyrophoric acid showed antimicrobial activities against bacteria and fungi, with MIC values varying from 0.47 to 7.5 µg/µl. Of the susceptible bacteria to gyrophoric acid, the *S. aureus* strain showed a MIC value of 3.75 µg/µl [25], which was lower than the MIC value of 32 µg/ml of the MRSA strain in this study. MRSA has been found to be associated with increased levels of antimicrobial resistance to different antibiotics of several classes other than beta-lactam antibiotics [29]. The underlying mechanisms by which MRSA strains had antibiotic resistance

Table 1. Antimicrobial activity of gyrophoric acid.

No	Microorganisms	Inhibition zones (mm)			
		Ampicillin (1 mg/ml)	Apramycin (1 mg/ml)	Gyrophoric acid (1 mg/ml)	DMSO
1	*Bacillus subtilis*	32 ± 0	30 ± 0	20 ± 0	-
2	*Enterobacter cloaceae*	-	23 ± 0	-	-
3	*Enterococcus faecalis*	26 ± 0	24 ± 0	17 ± 0.5	-
4	*Escherichia coli*	-	21 ± 0	-	-
5	*Klebsiella pneumoniae*	13 ± 0	25 ± 0	13 ± 0.5	-
6	*Pseudomonas aeruginosa*	-	21 ± 0	-	-
7	*MRSA*	19 ± 0	25 ± 0	20 ± 0.5	-
8	*Candida albicans*	29 ± 0	30 ± 0	25 ± 0.5	-

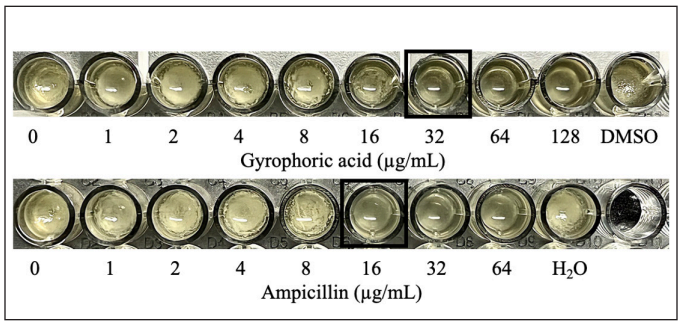

Figure 2. MICs of gyrophoric acid and ampicillin to MRSA.

were very diverse, including the production of beta-lactamase, Single nucleotide polymorphisms (SNPs) in *mprF* Open reading frame (ORF), newly acquired resistance, the transmission of *mecA*, and pangenome contexts [30]. These observations could be used to explain the elevated MIC value of gyrophoric acid on MRSA strains in this study versus the MIC value of *S. aureus* strains in [25,4]. The MIC value of 32 µg/ml of gyrophoric acid for MRSA strain, however, was promising when compared to the MIC value of some current antibiotics for the treatment of MRSA infection in humans. Fosfomycin had a MIC breakpoint to MRSA at 32 µg/ml [31]. Sulfonamide and nitrofurantoin had MIC breakpoints of 32 and 256 µg/ml, respectively, to MRSA [32]. The antimicrobial spectrum on several microorganisms and the interesting MIC value toward MRSA of gyrophoric acid make it be worthwhile for the following *in vitro* antimicrobial activity evaluations such as mutation frequency of antibiotic resistance, synergistic effect with ampicillin, inhibition of biofilm formation, and selective toxicity towards bacterial cells.

Morphology of MRSA under the gyrophoric acid treatment

The cellular morphology of MRSA under the treatment with gyrophoric acid could provide visual information on the effects of gyrophoric acid on the MRSA strain. To this end, SEM was used to show the morphological alteration of *S. aureus* when exposed to gyrophoric acid at the concentration of 8 × MIC. As shown in Figure 3, the surface of the untreated MRSA cells had spherical, full, and smooth shapes. The gyrophoric acid-treated MRSA cells, however, showed a substantial change in cell morphology at 4 hours of treatment. The treated cell had a concave shape inward surface showing indentation and collapsed morphology. This destruction was more visible at 16 hours of treatment with more collapse, lysis, indentation, and destructive morphology. The results showed that gyrophoric acid caused the demolition of the cell envelope of the MRSA strain leading to the death of this pathogen. Antimicrobial agents targeting cell walls and cell membranes, such as daptomycin, also expressed a broad spectrum against several bacteria and fungi. The morphologies of the treated bacteria with this antibiotic had abnormal surfaces, such as the creasing of the *B. subtilis* cell wall surface [33] or antler-like protrusion of the *S. aureus* cell envelope [34]. The results of the antimicrobial spectrum and morphology analysis of gyrophoric acid on *S. aureus* may suggest cell walls and/or cell membranes as the targets of this compound in microbes. As seen in Table 1, the antimicrobial activity of gyrophoric acid was comparable to ampicillin. Ampicillin is an antibiotic acting on the bacterial cell wall synthesis [33] that constitutes a crucial component of cellular structure in Gram-positive bacteria [34], including *S. aureus* strains.

Time-kill analysis of gyrophoric acid on MRSA

Determination of the time-kill kinetics is essential to evaluate the efficacy and killing rate of gyrophoric acid to MRSA. Gyrophoric acid at 8 × MIC concentration showed the bacteriostatic effect on MRSA from 0 to 12 hours of culture as the log CFU/ml over time remained roughly the same as the starting log CFU/ml concentration. After 12 hours of culture, the MRSA viable cell count began to decline, and gyrophoric acid showed the bactericidal effect on MRSA at 24 hours of culture with ≥3 $\log_{10}$ reduction in the MRSA viable cell count relative to the initial inoculum. In comparison, the MRSA cell count reduced early after 3 hours of culture at 8 × MIC of ampicillin, and this reduction was more than 3 $\log_{10}$ after 12 hours of culture, indicating the bactericidal effect of ampicillin on the MRSA strain. The other gyrophoric acid concentrations, i.e., 1×, 2×, 4 × MICs showed no effect on the growth of the MRSA strain over time (Fig. 4). In time-kill analysis, gyrophoric acid at 8 × MIC showed two different effects, the bacteriostatic effect from 0 to 12 hours of culture and bactericidal effect at 24

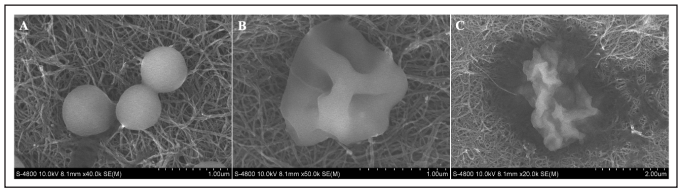

Figure 3. Morphology of MRSA under gyrophoric acid treatment. (A) Control. (B) The effect after 4 hours of treatment. (C) The effect after 16 hours of treatment.

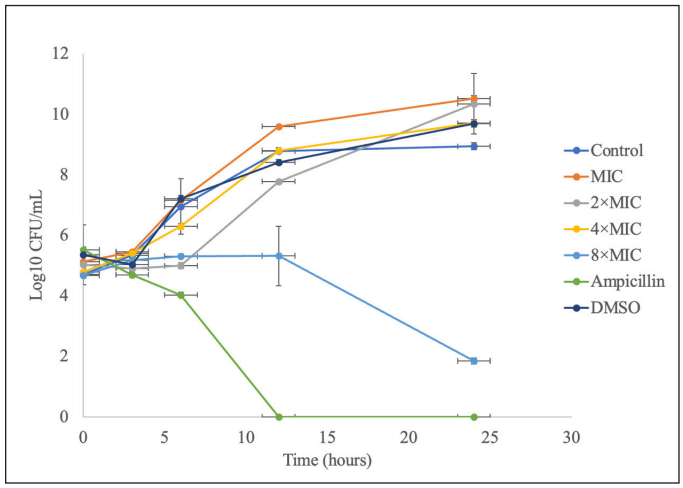

Figure 4. Time-kill kinetics of gyrophoric acid against MRSA.

hours of culture. In fact, the MRSA cell count began to decline after 12 hours of culture, and this reduction continued to 24 hours of culture. In comparison, ampicillin at 8 × MIC showed a bactericidal effect on MRSA only after 9 hours of culture. The time-kill assay provided the time course of the effect of gyrophoric acid on MRSA. These results could be used to find pharmacokinetic-pharmacodynamic models that aid in optimizing the future dosage regimens of gyrophoric acid based on a rational, scientific approach that is more accurate than the simple use of MIC values. Also, the time-kill result may predict the mechanism of action of antimicrobial agents with fast-killing compounds tending to act on the cell membrane, which leads to the leakage of cytoplasmic materials [35] while agents targeting cell walls have a slower killing kinetic [36].

Mutation frequency conferring resistance to gyrophoric acid

Single-step resistant assay is essential to assess the probability of bacteria developing resistance to antimicrobial agents. A lower mutation frequency associated with antimicrobial agents was more desirable. In this study, a single-step resistant assay was performed to study the mutation frequency that confers the MRSA strain the ability to be resistant to gyrophoric acid. An inoculum of 10^{10} CFU of the MRSA strain was spread on the MHA plates containing 8 × MIC of gyrophoric acid. After 24 hours of incubation at 37°C, there was a total of 24 MRSA colonies on these MHA plates showing a mutation frequency of 2.4×10^{-9} for gyrophoric acid. In comparison, ampicillin showed a mutation frequency of 5.55×10^{-8} at the 8 × MIC concentration to the MRSA strain in this study. Gyrophoric acid had a lower rate of spontaneous resistance than ampicillin. In addition, the resistance frequency of MRSA to gyrophoric acid at 8 × MIC was comparable to those of other antibiotics. Rifampin and ciprofloxacin at 8 × MICs had the frequency of single-step mutation resistance of the MRSA strain 1,094 were 8×10^{-7} and 2.2×10^{-10}, respectively [37]. Several MRSA strains showed resistant frequencies to ciprofloxacin, sitafloxacin, moxifloxacin, and gatifloxacin at their 8 × MICs ranging from 2.18×10^{-9} to 4.29×10^{-10} [38].

Synergistic effect between gyrophoric acid and ampicillin on the MRSA strain

The synergy effect between antibiotics was desirable to increase the effect of treating bacterial infection. Gyrophoric acid in this study was evaluated as the adjunct to reduce the MIC of ampicillin against the MRSA strain using the checkboard method to calculate the FICI value. Synergism between gyrophoric acid and ampicillin is found if the ΣFICI value is ≤0.5, and a partial synergism is found if the ΣFICI value is greater than 0.5 and less than 1. Table 2 shows the synergistic and partial synergistic effects between gyrophoric acid and ampicillin. Gyrophoric acid substantially reduced the MIC of ampicillin against the MRSA strain as high as 16-fold at the combination of 8 µg/ml of gyrophoric acid and 1 µg/ml of ampicillin, which showed the ΣFICI value of 0.3125, indicating the synergy between these two antimicrobial agents. In contrast, the MIC of gyrophoric was also greatly reduced when combined with ampicillin. It was the eight-fold reduction in the MIC value with the following combinations of 4 µg/ml of gyrophoric acid plus 2 µg/ml of ampicillin (synergy with the ΣFICI value of 0.25), 4 µg/ml of gyrophoric acid plus 4 µg/ml of ampicillin (synergy with the ΣFICI value of 0.375). The partial synergy between gyrophoric acid and ampicillin was found with the combination of 4 µg/ml of gyrophoric acid and 8 µg/ml of ampicillin with the ΣFICI value was 0.625. The antagonistic effect between gyrophoric acid and ampicillin on MRSA in this study was not found. The synergy effect between gyrophoric acid and ampicillin was equivalent to those of other natural compounds with ampicillin. Sophoraflavanone B, a prenylated flavonoid compound isolated from the roots of *Desmodium caudatum* reduced the MIC of ampicillin to MRSA from 2 to 16-fold [39]. Two isoquinoline alkaloids, 6-acetonyl-dihydrofagaridine, and 6-acetonyl-dihydrochelerythrine, isolated from the roots of *Zanthoxylum nitidum* showed synergistic effects with ampicillin when they reduced four-fold the MIC values of ampicillin to MRSA strains [40]. The synergy between gyrophoric acid and ampicillin could be used to overcome antibiotic resistance to ampicillin in the treatment of bacterial infection with this antibiotic. Indeed, the antibiotic combination has been the concept showing better clinical outcomes for patients infected with multidrug resistance bacterial pathogens [41]. In the case of MRSA, the combination of vancomycin and beta-lactam antibiotics enhanced the clearance rate of MRSA compared with the vancomycin monotherapy [42]. Furthermore, the synergy between gyrophoric acid and ampicillin could reduce

Table 2. Synergistic effects between gyrophoric acid and ampicillin on MRSA.

	Combination	MIC (µg/ml)	FIC	MIC reduction (times)	Interpretation
1	Gyrophoric acid	8	0.3125	4	Synergism
	Ampicillin	1		16	
2	Gyrophoric acid	4	0.25	8	Synergism
	Ampicillin	2		8	
3	Gyrophoric acid	4	0.375	8	Synergism
	Ampicillin	4		4	
4	Gyrophoric acid	4	0.625	8	Partial synergism
	Ampicillin	8		2	

the dose of ampicillin required for the treatment to avoid the dose-related toxicity of this antibiotic, as seen in the case of aminoglycoside antibiotics [43].

Selective toxicity evaluation of gyrophoric acid

The cytotoxicity evaluation of gyrophoric acid was performed with one noncancerous cell line (human fibroblast) and one cancerous cell line (NCI-H460) using the SRB method. For the human fibroblast cells, the number of cells treated with gyrophoric acid at the concentration of 600 µM was reduced by 14% compared to the untreated cells. Therefore, it was assumed that the IC_{50} of gyrophoric acid in the human fibroblast cells was over 600 µM which was equivalent to the concentration of ~281.04 µg/ml of gyrophoric acid. Similarly, the IC_{50} of gyrophoric acid to the lung cancerous cell line NCI-H460 was 373.17 ±15.95 µM which was equivalent to the concentration of ~174 ±7.5 µg/ml of gyrophoric acid. In the previous study, gyrophoric acid showed ineffective to mild cytotoxic effects on nine human cancer cell lines assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with the IC_{50} value over 200 µM for seven cell lines and under 200 µM for HL-60 (146.7 µM) and A2780 (198.3 µM) cell lines [44]. The cytotoxicity results of gyrophoric acid in this study showed consistency in the cytotoxicity of gyrophoric acid toward human cell lines. In particular, gyrophoric acid exhibited an IC_{50} value over 600 µM in the human primary fibroblast cell line, which is a normal cell line isolated from human skin [45]. The IC_{50} values of gyrophoric acid on two mammalian cell lines were used to calculate the TI between IC_{50} and MIC of gyrophoric acid. A higher ratio of IC_{50}/MIC would show a greater bacterial selectivity for the compound. According to the calculation, the ratio of IC_{50}/MIC of gyrophoric acid was greater than 8.78-fold for the fibroblast cell line. For the NCI-H460 cell line, the ratio of IC_{50}/MIC of gyrophoric acid was 5.43-fold, which was lower than that of the fibroblast cell line. The analysis showed that gyrophoric acid had a selective activity towards the MRSA strain versus the mammalian cell lines, with the bacterial selective activity being higher in the noncancerous cell line (fibroblast) than the cancerous cell line (NCI-H460). Equivalent TI values were found in some antibiotics on MRSA, e.g., linezolid (TI = 8) and tigecycline (TI = 9) [46], two of the new antibiotics for the treatment of severe infections caused by MRSA [47].

Inhibition of biofilm formation

Biofilm formation is one of the virulent factors of *S. aureus* bacteria involved in the pathogenesis and antibiotic resistance in this pathogen [48]. Some antibiotics are not effective enough to disrupt biofilm formation [49]. Therefore, it is essential to develop new antimicrobial agents that are able to inhibit the biofilm formation in *S. aureus*. The potential of gyrophoric acid to inhibit the biofilm formation in the MRSA strain in this study was investigated at the concentrations of 1×, 2×, 4×, 8×, and 16 × MICs. Ampicillin was also investigated for its ability to inhibit biofilm formation in the MRSA strain with the same concentration range. The results in Figure 5 showed that the biofilm formation of the MRSA strain began to be inhibited at 2 × MIC of gyrophoric acid with a 15% reduction. Higher MICs of gyrophoric acid had greater reductions in biofilm formation, e.g., 84% reduction at 4 × MIC, 86% reduction at 8 × MIC, and 94% reduction at 16 × MIC. Ampicillin also had an impact on biofilm reduction of the MRSA strain but at weaker levels than those of gyrophoric acid. The biofilm of the MRSA strain began to be inhibited at 4 × MIC of ampicillin, showing 4% reduction. The reduction in biofilm formation was 20% at 8 × MIC and 28% at 16 × MIC of ampicillin. Inhibition of microbial biofilm formation has also been found in other depside compounds originating from lichens. Atranorin, a depside compound produced by some lichens species, showed diverse biological activities [50], including the ability to decrease the biofilm formation and eliminate the established biofilm with regard to *S. aureus* [51]. Another antimicrobial depside, evernic acid, showed antimicrobial and antibiofilm activities against *C. albicans* [52]. Bacterial biofilm inhibition is the result of different actions such as the inhibition of bacterial surface adhesion, interference with the quorum-sensing system, modulation of signaling molecules, chemical inhibition of biofilm maturation, and disruption of mature biofilms [53]. The altered appearance of MRSA under gyrophoric acid treatment revealed by SEM could be the inhibiting mechanism of biofilm formation in MRSA (Fig. 6). Compounds interfering with cell walls specifically severely impair biofilm formation in bacteria [54]. After 24 hours of exposure to carvacrol, cinnamaldehyde, and thymol, *S. aureus* showed distorted morphology with damage to the cell peptidoglycan structure. Carvacrol, cinnamaldehyde, and thymol also expressed the ability to eliminate preformed biofilm and inhibit biofilm formation in *S. aureus* [55].

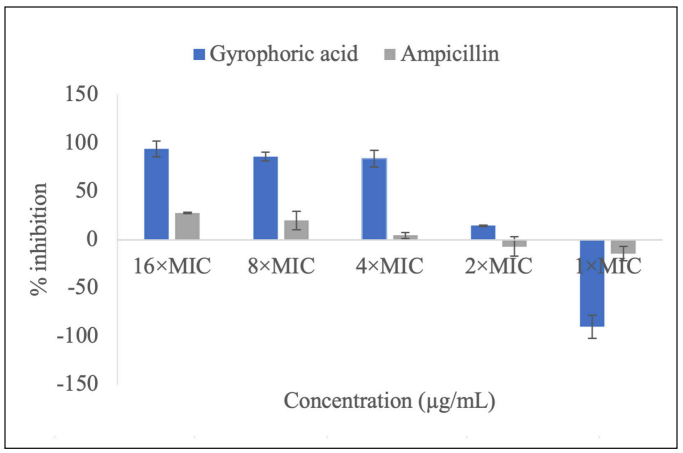

Figure 5. Inhibition of MRSA biofilm formation by gyrophoric acid and ampicillin.

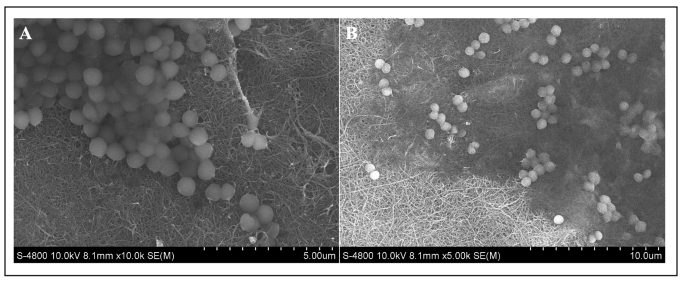

Figure 6. The effect of gyrophoric acid on MRSA biofilm formation. (A) Control. (B) Effect of gyrophoric acid treatment after 16 hours.

CONCLUSION

The lichen *P. indicum* was found to contain gyrophoric acid. This depside showed promising activity against MRSA in terms of MIC, bacterial killing kinetics, frequency of resistance, synergy with antibiotics, selective antimicrobial effect, and inhibition of bacterial biofilm formation. The morphology of MRSA under gyrophoric acid treatment proposed cell wall/cell membrane could be the target of gyrophoric acid. The results of this study demonstrated the possibility of gyrophoric acid being advanced in further steps of the development of new antimicrobial agents, at least for MRSA.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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REFERENCES

1. Mulani MS, Kamble EE, Kumkar SN, Tawre MS, Pardesi KR. Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: a review. Front Microbiol. 2019 Apr 1;10:539.
2. Marturano JE, Lowery TJ. ESKAPE pathogens in bloodstream infections are associated with higher cost and mortality but can be predicted using diagnoses upon admission. Open Forum Infect Dis. 2019 Nov 22;6(12):ofz503.
3. Tacconelli E. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Geneva, Switzerland: WHO; 2017.
4. Watkins RR, David MZ, Salata RA. Current concepts on the virulence mechanisms of meticillin-resistant *Staphylococcus aureus*. J Med Microbiol. 2012 Sep;61(Pt 9):1179–93.
5. Mlynarczyk-Bonikowska B, Kowalewski C, Krolak-Ulinska A, Marusza W. Molecular mechanisms of drug resistance in *Staphylococcus aureus*. Int J Mol Sci. 2022 Jan;23(15):8088.
6. Qadri H, Haseeb Shah A, Mudasir Ahmad S, Alshehri B, Almilaibary A, Ahmad Mir M. Natural products and their semi-synthetic derivatives against antimicrobial-resistant human pathogenic bacteria and fungi. Saudi J Biol Sci. 2022 Sep 1;29(9):103376.
7. Molnár K, Farkas E. Current results on biological activities of lichen secondary metabolites: a review. Z Naturforschung C J Biosci. 2010;65(3–4):157–73.
8. Maurya IK, Singh S, Tewari R, Tripathi M, Upadhyay S, Joshi Y. Antimicrobial activity of *Bulbothrix setschwanensis* (Zahlbr.) Hale lichen by cell wall disruption of *Staphylococcus aureus* and *Cryptococcus neoformans*. Microb Pathog. 2018 Feb;115:12–8.
9. Micheletti AC, Honda NK, Ravaglia LM, Matayoshi T, Spielmann AA. Antibacterial potencial of 12 lichen species. An Acad Bras Ciênc [Internet]. 2021 [cited 2023 Jul 28];93(4):e20191194. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0001-37652021000700904&tlng=en
10. Hughes D, Karlén A. Discovery and preclinical development of new antibiotics. Ups J Med Sci. 2014 May;119(2):162–9.
11. McDowell LL, Quinn CL, Leeds JA, Silverman JA, Silver LL. Perspective on antibacterial lead identification challenges and the role of hypothesis-driven strategies. SLAS Discov. 2019 Apr 1;24(4):440–56.
12. Chi HBL, Bui VM, Phan TQN, Nguyen KPP. Phenolic compounds from the lichen *Parmotrema tinctorum*. VNUHCM J Sci Technol Dev. 2021 Feb 25;24(1):847–51.
13. Balouiri M, Sadiki M, Ibnsouda SK. Methods for *in vitro* evaluating antimicrobial activity: a review. J Pharm Anal. 2016 Apr 1;6(2):71–9.
14. Golus J, Sawicki R, Widelski J, Ginalska G. The agar microdilution method—a new method for antimicrobial susceptibility testing

for essential oils and plant extracts. J Appl Microbiol. 2016 Nov;121(5):1291–9.
15. Clinical & Laboratory Standards Institute. M26-A methods for determining bactericidal activity of antimicrobial agents; approved guideline. Wayne, PA: Clinical & Laboratory Standards Institute; 1999.
16. Mani N, Gross CH, Parsons JD, Hanzelka B, Müh U, Mullin S, *et al*. *In vitro* characterization of the antibacterial spectrum of novel bacterial type II topoisomerase inhibitors of the aminobenzimidazole class. Antimicrob Agents Chemother. 2006 Apr;50(4):1228–37.
17. Wormser GP, Tang YW. Antibiotics in laboratory medicine. Clin Infect Dis. 2005;41(4):577.
18. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc. 2006 Aug;1(3):1112–6.
19. Manandhar S, Singh A, Varma A, Pandey S, Shrivastava N. Evaluation of methods to detect *in vitro* biofilm formation by staphylococcal clinical isolates. BMC Res Notes. 2018 Oct 10;11(1):714.
20. Weidong Q, Zhaohuan S, Wang T, Min Y, Miao L, Jianing Z, *et al*. Antimicrobial activity of eugenol against carbapenem-resistant *Klebsiella pneumoniae* and its effect on biofilms. Microb Pathog [Internet]. 2020 Feb 1;139:103924 [cited 2022 Apr 11]. Available from: https://www.scinapse.io
21. Narui T, Sawada K, Takatsuki S, Okuyama T, Culberson CF, Culberson WL, *et al*. NMR assignments of depsides and tridepsides of the lichen family umbilicariaceae. Phytochemistry. 1998 Jul 1;48(5):815–22.
22. Mohammadi M, Bagheri L, Badreldin A, Fatehi P, Pakzad L, Suntres Z, *et al*. Biological effects of gyrophoric acid and other lichen derived metabolites, on cell proliferation, apoptosis and cell signaling pathways. Chem Biol Interact. 2022 Jan 5;351:109768.
23. Buçukoglu TZ, Albayrak S, Halici MG, Tay T. Antimicrobial and antioxidant activities of extracts and lichen acids obtained from some *Umbilicaria* species from Central Anatolia, Turkey. J Food Process Preserv. 2013;37(6):1103–10.
24. Shim JH. Anti-aging effects of gyrophoric acid on UVA-irradiated normal human dermal fibroblasts. Nat Prod Commun. 2020 Apr;15(4):1934578X2091954.
25. Candan M, Yılmaz M, Tay T, Kıvança M, Türk H. Antimicrobial activity of extracts of the lichen *Xanthoparmelia pokornyi* and its gyrophoric and stenosporic acid constituents. Z Für Naturforschung C. 2006 Jun 1;61(5–6):319–23.
26. Kosanic M, Rankovic B, Stanojkovic T, Vasiljevic P, Manojlovic N. Biological activities and chemical composition of lichens from Serbia. EXCLI J. 2014;13:1226–38.
27. Jayalal U, Divakar PK, Joshi S, Oh SO, Koh YJ, Hur JS. The lichen genus *Parmotrema* in South Korea. Mycobiology. 2013 Mar;41(1):25–36.
28. Kowalska-Krochmal B, Dudek-Wicher R. The minimum inhibitory concentration of antibiotics: methods, interpretation, clinical relevance. Pathogens. 2021 Feb 4;10(2):165.
29. Kaur D, Chate S. Study of antibiotic resistance pattern in methicillin resistant *Staphylococcus aureus* with special reference to newer antibiotic. J Glob Infect Dis. 2015;7(2):78.
30. Ali Alghamdi B, Al-Johani I, Al-Shamrani JM, Musamed Alshamrani H, Al-Otaibi BG, Almazmomi K, *et al*. Antimicrobial resistance in methicillin-resistant *Staphylococcus aureus.* Saudi J Biol Sci. 2023 Apr;30(4):103604.
31. Williams PC. Potential of fosfomycin in treating multidrug-resistant infections in children. J Paediatr Child Health. 2020;56(6):864–72.
32. Clinical & Laboratory Standards Institute [Internet]. M100Ed33 performance standards for antimicrobial susceptibility testing. 33rd ed. Wayne, PA: Clinical & Laboratory Standards Institute; 2023. Available from: https://clsi.org/standards/products/microbiology/documents/m100/
33. Bereda G. Clinical pharmacology of ampicillin. J Pharm Res Rep. 2022 Sep 30;1–3.
34. Jordan S, Hutchings MI, Mascher T. Cell envelope stress response in Gram-positive bacteria. FEMS Microbiol Rev. 2008 Jan 1;32(1):107–46.
35. Jariyarattanarach P, Klubthawee N, Wongchai M, Roytrakul S, Aunpad R. Novel D-form of hybrid peptide (D-AP19) rapidly kills *Acinetobacter baumannii* while tolerating proteolytic enzymes. Sci Rep. 2022 Sep 23;12(1):15852.
36. Hamad M, Al-Marzooq F, Srinivasulu V, Omar HA, Sulaiman A, Zaher DM, *et al*. Antibacterial activity of small molecules which eradicate methicillin-resistant *Staphylococcus aureus* persisters. Front Microbiol [Internet]. 2022 [cited 2023 Jul 28];13. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2022.823394
37. Butler MM, Skow DJ, Stephenson RO, Lyden PT, LaMarr WA, Foster KA. Low frequencies of resistance among *Staphylococcus* and *Enterococcus* species to the bactericidal DNA polymerase inhibitor N3-hydroxybutyl 6-(3′-Ethyl-4′-Methylanilino) uracil. Antimicrob Agents Chemother. 2002 Dec;46(12):3770–5.
38. Bogdanovich T, Esel D, Kelly LM, Bozdogan B, Credito K, Lin G, *et al*. Antistaphylococcal activity of DX-619, a new des-F(6)-quinolone, compared to those of other agents. Antimicrob Agents Chemother. 2005 Aug;49(8):3325–33.
39. Mun SH, Kang OH, Joung DK, Kim SB, Seo YS, Choi JG, *et al*. Combination therapy of sophoraflavanone B against MRSA: *in vitro* synergy testing. Evid Based Complement Alternat Med. 2013 Nov 10;2013:e823794.
40. Zeng Q, Wang ZJ, Chen S, Wang H, Xie TZ, Xu XJ, *et al*. Phytochemical and anti-MRSA constituents of *Zanthoxylum nitidum*. Biomed Pharmacother. 2022 Apr 1;148:112758.
41. Ahmed A, Azim A, Gurjar M, Baronia AK. Current concepts in combination antibiotic therapy for critically ill patients. Indian J Crit Care Med Peer-Rev Off Publ Indian Soc Crit Care Med. 2014 May;18(5):310–4.
42. Dilworth TJ, Ibrahim O, Hall P, Sliwinski J, Walraven C, Mercier RC. β-Lactams enhance vancomycin activity against methicillin-resistant *Staphylococcus aureus* bacteremia compared to vancomycin alone. Antimicrob Agents Chemother. 2014 Jan;58(1):102–9.
43. Wang N, Luo J, Deng F, Huang Y, Zhou H. Antibiotic combination therapy: a strategy to overcome bacterial resistance to aminoglycoside antibiotics. Front Pharmacol [Internet]. 2022 [cited 2023 Jul 28];13. Available from: https://www.frontiersin.org/articles/10.3389/fphar.2022.839808
44. Bačkorová M, Bačkor M, Mikeš J, Jendželovský R, Fedoročko P. Variable responses of different human cancer cells to the lichen compounds parietin, atranorin, usnic acid and gyrophoric acid. Toxicol Vitro Int J Publ Assoc BIBRA. 2011 Feb;25(1):37–44.
45. Kisiel MA, Klar AS. Isolation and culture of human dermal fibroblasts. Methods Mol Biol Clifton NJ. 2019;1993:71–8.
46. Mishra NM, Stolarzewicz I, Cannaerts D, Schuermans J, Lavigne R, Looz Y, *et al*. Iterative chemical engineering of vancomycin leads to novel vancomycin analogs with a high *in vitro* therapeutic index. Front Microbiol. 2018 Jun 7;9:1175.
47. Shariati A, Dadashi M, Chegini Z, van Belkum A, Mirzaii M, Khoramrooz SS, *et al*. The global prevalence of daptomycin, tigecycline, quinupristin/dalfopristin, and linezolid-resistant *Staphylococcus aureus* and coagulase-negative staphylococci strains: a systematic review and meta-analysis. Antimicrob Resist Infect Control. 2020 Apr 22;9(1):56.
48. Idrees M, Sawant S, Karodia N, Rahman A. *Staphylococcus aureus* biofilm: morphology, genetics, pathogenesis and treatment strategies. Int J Environ Res Public Health. 2021 Jul 16;18(14):7602.
49. Roy R, Tiwari M, Donelli G, Tiwari V. Strategies for combating bacterial biofilms: a focus on anti-biofilm agents and their mechanisms of action. Virulence. 2017 Mar 31;9(1):522–54.
50. Harikrishnan A, Veena V, Lakshmi B, Shanmugavalli R, Theres S, Prashantha CN, *et al.* Atranorin, an antimicrobial metabolite from lichen *Parmotrema rampoddense* exhibited *in vitro* anti-breast

cancer activity through interaction with Akt activity. J Biomol Struct Dyn. 2021 Mar;39(4):1248–58.
51. Pompilio A, Pomponio S, Di Vincenzo V, Crocetta V, Nicoletti M, Piovano M, *et al*. Antimicrobial and antibiofilm activity of secondary metabolites of lichens against methicillin-resistant *Staphylococcus aureus* strains from cystic fibrosis patients. Future Microbiol. 2013 Feb;8(2):281–92.
52. Girardot M, Millot M, Hamion G, Billard JL, Juin C, Ntoutoume GMAN, *et al.* Lichen polyphenolic compounds for the eradication of *Candida albicans* biofilms. Front Cell Infect Microbiol. 2021;11:698883.
53. Ghosh A, Jayaraman N, Chatterji D. Small-molecule inhibition of bacterial biofilm. ACS Omega. 2020 Feb 25;5(7):3108–15.
54. Bucher T, Oppenheimer-Shaanan Y, Savidor A, Bloom-Ackermann Z, Kolodkin-Gal I. Disturbance of the bacterial cell wall specifically interferes with biofilm formation. Environ Microbiol Rep. 2015 Dec;7(6):990–1004.
55. García-Salinas S, Elizondo-Castillo H, Arruebo M, Mendoza G, Irusta S. Evaluation of the antimicrobial activity and cytotoxicity of different components of natural origin present in essential oils. Molecules. 2018 Jun 8;23(6):1399.

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