

A Clinical Trial to Evaluate the Efficacy of α -Viniferin in *Staphylococcus aureus* – Specific Decolonization without Depleting the Normal Microbiota of Nares

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Abstract

Staphylococcus aureus is currently a significant multidrug-resistant bacterium, causing severe healthcare-associated and community-acquired infections worldwide. The current antibiotic regimen against this pathogen is becoming ineffective due to resistance, in addition, they disrupt the normal microbiota. It highlights the urgent need for a pathogen-specific drug with high antibacterial efficacy against *S. aureus*. α -Viniferin, a bioactive phytochemical compound, has been reported to have excellent anti-*Staphylococcus* efficacy as a topical agent. However, so far, there were no clinical trials that have been conducted to elucidate its efficacy. The present study aimed to investigate the antibacterial efficacy of α -viniferin against *S. aureus* in a ten-day clinical trial. Based on the results, α -viniferin showed 50% minimum inhibitory concentrations (MIC₅₀ values) of 7.8 μ g/ml in culture broth medium. α -Viniferin was administered in the nares three times a day for ten days using a sterile cotton swab stick. Nasal swab specimens were collected before (0 days) and after finishing the trial (10th day), and then analyzed. In the culture and RT-PCR-based analysis, *S. aureus* was reduced significantly: 0.01. In addition, 16S ribosomal RNA-based amplicon sequencing analysis showed that *S. aureus* reduced from 51.03% to 23.99% at the genus level. RNA-seq analysis was also done to gain insights into molecular mechanisms of α -viniferin against *S. aureus*, which revealed that some gene groups were reduced in 5-fold FC cutoff at two times MIC conditions. The study results demonstrate α -viniferin as a potential *S. aureus*-specific drug candidate.

Key words: α -viniferin, clinical trial, MRSA, PCR, 16S rRNA amplicon sequencing analysis

Introduction

Staphylococcus aureus is one of the most common opportunistic pathogens carried by approximately 30–50% of humans and continues to be a leading cause of infection-related deaths, ranging from 6–40% around the globe (Frank et al. 2010; Islam et al. 2020). Although the axilla, throat, and perineum are essential reservoirs, the anterior nares are the primary niche for *S. aureus* and serve as a reservoir for the pathogen's spread (Lowy 1998). *S. aureus* nasal colonization causes a range of mild infections to life-threatening conditions, including fatal endocarditis, pneumonia, bacteremia, or chronic osteomyelitis. Additionally, it is a risk factor for life-threatening surgical site infections and infections in

dialysis (Mitchell and Howden 2005). So far, antibiotic therapy has been the best option for treating *S. aureus*. However, *S. aureus* infections have become a serious global challenge because of resistance to a wide range of clinically significant antibiotics and a limited number of new antibiotics. Previous studies showed that the rate of discovery of new antibiotics is slowing, while the occurrence of antibiotic-resistant infections is increasing sharply, therefore, the rate of antibiotic withdrawal from the healthcare system is higher than that of approvals (Kinch et al. 2014). In addition, the current antibiotic regimen reduces and changes the skin's microbial composition and supports pathogen overgrowth (Chambers and Deleo 2009; Song et al. 2018), highlighting the need to develop new drugs that

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not only serve as effective alternatives to the current drug regimen but also preserve skin microbiota.

Natural products and secondary metabolites produced by living systems, mostly plants, have a wide range of pharmacological properties, including antimicrobial and anti-inflammatory ones, and a high potential for treating human diseases, such as coronary heart diseases, cancer, diabetes, and infectious diseases (Chabán et al. 2019). According to the World Health Organization, 65–80% of the world's population depends on traditional medicine to treat many diseases (Chew et al. 2011). α -Viniferin is a phytochemical compound extracted from *Carex humilis*, a medicinal plant that grows in eastern Asian countries, such as Japan, China, and Korea (Seo et al. 2017). It was also identified from *Iris clarkei*, *Caragana Sinica*, and *Caragana chamlagu* (Chung et al. 2003). α -Viniferin isolated and identified as a stilbene oligomer has various biological activities, including antioxidant, anti-tumor, anti-cancer, and anti-arthritis. It has also been reported to inhibit cyclooxygenase, acetylcholinesterase, and prostaglandin H-2 synthase (Sim et al. 2014; Seo et al. 2017). Additionally, α -viniferin shows antibacterial activity against drug-susceptible and drug-resistant strains of *Mycobacterium tuberculosis* and excellent anti-*Staphylococcus* activity against three *Staphylococcus* species, including methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), and methicillin-resistant *Staphylococcus epidermidis* (MRSE) (Seo et al. 2017). Previous studies using animal models have demonstrated that α -viniferin improves general health in mammals and is rapidly absorbed in the blood stream (Baur et al. 2006; Fan et al. 2020). Although α -viniferin is assumed to be a potential *S. aureus*-specific drug, there were no clinical studies on its effects on *S. aureus* to the best of our knowledge. In this study, we investigated α -viniferin clinical efficacy in eradicating *S. aureus* from the nasal carriage. Additionally, we looked over the effectiveness of α -viniferin against nasal MRSA.

Experimental

Materials and Methods

α -Viniferin and its antimicrobial inhibition. We purchased the α -viniferin, pale brown powder (Cat No.: CFN97068) from Chem Faces (Wuhan, Hubei, China) and confirmed its specific toxicity against 20 bacterial strains obtained from the National Culture Collection for Pathogens (NCCP) (Chungbuk, Korea). The minimal inhibitory concentration (MIC) of α -viniferin against each bacterial strain was determined using cation-adjusted Mueller-Hinton broth according to the Clinical and Laboratory Standards Institute (CLSI).

Each well of a 96-well plate was inoculated with 200 μ l of the inoculum suspension (1×10^5 to 1×10^6 CFU/ml) and the plates were incubated for 24 h. The MIC values were determined as the lowest drug concentrations that showed complete inhibition of visible growth. The bacterial strains were as follows: *Escherichia coli* NCCP 14762, *Proteus vulgaris* NCCP 14765, *Shigella boydii* NCCP 14745, *Shigella flexneri* NCCP 14744, *Shigella dysenteriae* NCCP 14746, *Staphylococcus aureus* NCCP 14780, *Staphylococcus epidermidis* NCCP 14768, *Staphylococcus aureus* MRSA NCCP 14769, *Corynebacterium diphtheriae* NCCP 10353, *Salmonella enteritidis* NCCP 14771, *Acinetobacter baumannii* NCCP 14782, *Streptococcus sanguinis* NCCP 14775, *Streptococcus pyogenes* NCCP 14783, *Streptococcus pneumoniae* NCCP 14774, *Serratia marcescens* NCCP 14770, *Citrobacter freundii* NCCP 14766, *Enterobacter aerogenes* NCCP 14761, *Proteus mirabilis* NCCP 14763, *Klebsiella pneumoniae* NCCP 14764, and *Escherichia coli* O157 NCCP 14541.

Study design and subjects. Our study enrolled 20 Korean adult females aged between 20 and 60 years following inclusion and exclusion criteria. The inclusion criteria comprised health and physical fitness, age > 18 years, and the willingness to avoid topical agent applied to the nares during the entire trial. The characteristics of the subjects are shown in Table SI. The Institutional Review Board approved this study of the Korea Dermatology Research Institute (IRB Number: KDRI-IRB-20231, Study Code: KDRI-2020-231). We obtained written informed consent from all participants before conducting the study. The study was carried out according to the 1964 declaration of Helsinki.

Clinical experiment. Following the randomization sequence, we sequentially numbered containers with the study drug, α -viniferin, and provided it to the numerical order participants. Nare's specimens were collected on day 0 and at day ten by a professional. During the trial, each participant's skin moisture content was measured on days 0, 4, and 8 using a corneometer CM 825 (Courage and Khazaka, Germany). Each time, the corneometer measurement was done five times at constant temperature and humidity (20–24°C, 40–60% RH), at the same site, in the same way, and the average value was recorded as an immediate value. The recorded value was then analyzed to observe our study drug's moisturizing power because moisture content plays a key role in maintaining an intact skin barrier (Mojumdar et al. 2017). During and immediately after the test, a dermatologist examined the skin for irritation or allergic symptoms.

Skin irritation test. Before the clinical trials, a skin irritation test for three concentrations of α -viniferin (10 μ g/ml, 100 μ g/ml, 1,000 μ g/ml) was done on 32 Korean adult females in a different place to rule

out the possibility of skin irritation of the test drug. The characteristics of the participants are shown in Table SII. The drug solution was loaded into a clear patch chamber, and then the patches were applied onto the participants' backs. All participants were advised to avoid water, heavy exercise, and scratching. The patch chambers were removed after 48 hours. After thirty minutes of patch removal (total 48 hours test), the reaction site was observed and observed again after 24 hours (total 72 hours of the test). The reactions were graded according to the recommendation of the International Contact Dermatitis Research Group (ICDRG), and Frosch and Kligman (Frosch and Kligman 1979; Lachapelle and Maibach 2020). This study was approved by the Institutional Review Board of the COREDERM Skin Research Center (IRB Number: CDIRB-QR-20-025, Study Code: CDS-2000-005-14). We obtained written informed consent from all participants before conducting this study.

Preparation and application of α -viniferin. We prepared α -viniferin suspension by mixing the powder with sterile distilled water at 100 mg/l. A sterile cotton swab (BD Difco, USA) was used to treat the nares; the swab was moistened with the drug suspension and then gently rubbed the anterior nares while rotating it for 15 seconds. The drug was applied three times daily for ten days in right and left nares. The participants themselves made the application of the drug solution.

Sample collection. An expert did the collection of nasal specimens at day zero (0) and the end of the trial (at day 10), by swabbing the anterior nares with sterile foam-tipped swabs moistened with a mixed solution of 0.15 M NaCl and 0.1% Tween 20 solution (Sigma-Aldrich, France) (You et al. 2019). Each region was swabbed for 15 seconds while rotating the swab and exerting gentle pressure. Each swab was then placed into the swab container without touching any objects and transported to the laboratory in a dry-ice bag container within two hours of collection.

Culture of microorganisms. Upon receiving the lab samples, we added 2 ml 0.15 M NaCl solution into the swab container, vortexed it enough, and transferred it into a fresh microcentrifuge tube. After doing a 10-fold serial dilution four times (10^{-1} to 10^{-4}) in 0.15 M NaCl solution, we placed a 10- μ l sample from each of the dilution-tube in duplicate onto the nutrient agar, a staphylococcus agar medium (BD Difco, USA), and a second staphylococcus agar medium containing methicillin antibiotic (50 μ g/ml) for the culture-based quantification of nasal microbiota, *S. aureus*, and MRSA, respectively, using a spread plate method. After 48 hours of incubation at 37°C, plates were inspected for the growth of colonies with morphology characteristic of *S. aureus*. We distinguished *S. aureus* from other *Staphylococcus* species by its round and golden-yellow colonies. For

each dilution, the average of the two duplicate plates was calculated. When two successive dilutions yielded 30 to 300 colonies, the average count of both dilutions was calculated. We stored the rest of the samples at -80°C for further molecular analysis.

DNA extraction. We extracted DNA for real-time quantitative PCR (qRT-PCR) and next-generation sequencing (NGS)-based 16S rRNA profiling by thawing samples and transferring them to Lysing Matrix B tubes aseptically (MP, Biomedicals, USA), followed by mechanical lysis through bead beading. After centrifuging enough, we collected supernatant without any visible particles. Next, we checked the quality of extracted DNA by agarose gel electrophoresis and visualized it using ethidium bromide (Bio-Rad, ChemiDoc, USA). We also quantified the isolated DNA by a Qubit Fluorometer using a dsDNA HS Assay kit (Invitrogen, USA). We used a 0.15 M NaCl solution as a negative control to ensure no DNA contamination occurred during the whole process (Rainer et al. 2020).

qRT-PCR. We did qRT-PCR (C1000 Thermal Cycler) to test for the presence and quantification of *S. aureus* and MRSA in the nasal swab samples separately, targeting organism-specific genes is a significant correlation between PCR cycle threshold (Ct) value and bacterial load (Dionne et al. 2013; Davies et al. 2018). We used the following primers in this study: SA1 (5'-AATCT-TTGTCGGTACACGATATTCTTCACG-3') and SA2 (5'-CGTAATGAGATTTTCAGTAGATAATACAACA-3') specific for *S. aureus* (Pereira et al. 2010); and MRS1 (5'-TAGAAATGACTGAAC GTCCG-3') and MRS2 (5'-TTGCGATCAATGTTACC GTAG-3') specific for MRSA (Del Vecchio et al. 1995). We also used 16S rRNA universal primers to quantify resident nasal microbiota. We did the assay in a final volume of 20 μ l consisting of a 5- μ l extracted template DNA and 15 μ l reaction mixtures: 4 μ l nuclease-free water, 10 μ l iQ SYBR Green Supermix (Bio-Rad Laboratories, USA), and 0.5 μ l each of the forward and reverse primer (10 pmol/ μ l). We did amplification as follows: an initial DNA denaturation and enzyme activation at 95°C for 3 min, followed by 49 cycles of a 10-s denaturation step at 95°C, a 10-s annealing step at 55°C, and a 30-s extension step at 72°C.

Absolute quantification. To make a qRT-PCR standard curve (threshold cycle versus the number of CFU/ml curve), we cultivated an *S. aureus* stock culture (0.1% inoculation) in Mueller-Hinton broth (BD Difco, USA) for 6–8 hours at 37°C using a shaker incubator (BioFree, Korea). We then checked the optical density (OD at 600 nm) using a spectrophotometer (DR 1900, Hach, USA); it was 0.8 to 1.0. Next, we centrifuged the broth culture to get the bacterial pellet at 1,811.16 \times g for 10 mins employing a Hanil combi R515 (Hanil Scientific Inc., Korea) serially diluted the pellet 10-fold eight times

using sterile distilled water. After that, we extracted DNA through mechanical lysis as described in the DNA Extraction section, amplified it by qRT-PCR, and used the data to create the standard curve in which each target CFU is plotted against the resulting C_t value.

16S rRNA-based amplicon sequencing. Following the DNA extraction, we amplified the V4 hyper-variable region of the 16S ribosomal RNA gene. We used the V4 region-specific primers with a locus-specific overhang sequence: 515F-5' – TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG- GTGCCAGCMGCCGCGGTAA and 806R – 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GGACTACHVGGGTWTCTAAT (overhang sequence-locus specific-sequence). We attached indexes and Illumina sequencing adapters through index PCR using a Nextera XT Index kit v2 (Illumina, USA). We used Agencourt AMPure XP (Beckman Coulter, USA) beads after every PCR step to purify the PCR products and quantified them using the Qubit dsDNA HS Assay kit (Invitrogen, USA). We then normalized the samples, pooled, mixed them with PhiX Control v3 (Illumina), and sequenced them using Illumina iSeq 100 platform (Illumina). Finally, we analyzed the sequence data using Ezcloud software that exhibits the entire microbial profile within the sample tested.

Gene expression profiling. Drug treatment, RNA extraction, and analysis. To understand how α -viniferin downregulate *S. aureus* gene expression, we treated an *S. aureus* broth culture with α -viniferin, extracted RNA, and sent it for the RNA sequencing. We cultivated *S. aureus* culture and measured optical density as described in the absolute quantification section. Then, we distributed the broth culture into three tubes; each tube contained 30 ml of broth. Afterward, we added a specific volume of α -viniferin stock solution to the tubes separately to make the drug concentration $1 \times$ the MIC value (10 $\mu\text{g/ml}$) and $2 \times$ the MIC value (20 $\mu\text{g/ml}$). We also used drug-free 0.15 M NaCl solution in one of the tubes as a negative control. We then incubated the drug-treated and drug-free culture at 37°C for 6–8 hours on a shaker incubator. After the incubation, we extracted total RNA from the broth cultures of *S. aureus* using the RNA Protect Bacterial Reagent and RNeasy Mini kit (QIAGEN, Germany), according to the manufacturer's instructions (RNeasy Mini Handbook). We also used an RNase-free DNase set (QIAGEN) for the degradation of the existing DNA. We assessed the quality of the isolated total RNA through 2% agarose gel electrophoresis since high-quality RNA is a prerequisite for ensuring all expressed genes' representation. We sent RNA samples for RNA sequencing, which has become a standard tool for analyzing gene expression in bacterial infections (Saliba et al. 2017). We did an RNA sequencing assay using a commercial RNA sequencing service (Ebiogen, Seoul, Korea).

Library preparation, sequencing, and data analysis. We used a QuantSeq 3'mRNA-Seq Library Prep Kit (Lexogen, Inc., Austria) to construct an RNA library following the manufacturer's instructions. In brief, we hybridized 500 ng of the total RNA with an oligo-dT primer containing an Illumina-compatible sequence at the 5' end, followed by reverse transcription. We initiated the second-strand synthesis using a random primer containing an Illumina-compatible linker sequence at its 5' end after the degradation of the RNA template. We used magnetic beads to remove all the reaction components and purify the double-stranded library. We then amplified the library to add the entire adapter sequences essential for cluster generation. Finally, we purified the complete library from PCR components, did a high throughput sequencing as single-end 75 sequencing using NextSeq 500 (Illumina). After the sequencing, we used Bowtie2 to align the QuantSeq 3'mRNA-Seq reads. We created Bowtie2 indices from either a representative transcript sequence or a genome assembly sequence for aligning with genome and transcriptome. We used alignment files for assembling transcripts, estimating their abundances, and detecting different expressions of genes. We used Bedtool coverage to find the differentially expressed genes based on counts from unique and multiple alignments. We processed the RT (Read Count) data based on Quantile normalization using EdgeR within R using Bioconductor. We based gene classification on searches done in the DAVID (<http://david.abcc.ncifcrf.gov/>) and Medline databases (<http://www.ncbi.nlm.nih.gov/>).

Statistical analysis. We analyzed the corneometer value, CFU, and qRT-PCR data using Prism 7.0 (Graph-Pad Software, Inc., La Jolla, USA). We used a two-tailed Student's *t*-test to evaluate the statistical significance of the results of three independent experiments done in triplicate. In the graph, data are represented as mean \pm standard deviation (SD). Means were considered to be significant at $p < 0.05$ ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$). We analyzed NGS-based 16S rRNA sequencing data using Ezcloud software. We also used the hypergeometric distribution to analyze the RNA sequencing data.

Results

Antimicrobial inhibition. Antibacterial inhibition of α -viniferin was tested against 20 bacterial isolates. The antibacterial activities of α -viniferin and two references antibiotics VAN and MET, expressed as MICs, are shown in Fig. 1, where MICs values were expressed up to 100 $\mu\text{g/ml}$. However, in this study, α -viniferin showed excellent anti-*staphylococcus* efficacy with the MIC value of 7.8 $\mu\text{g/ml}$ against three *Staphylococcus*

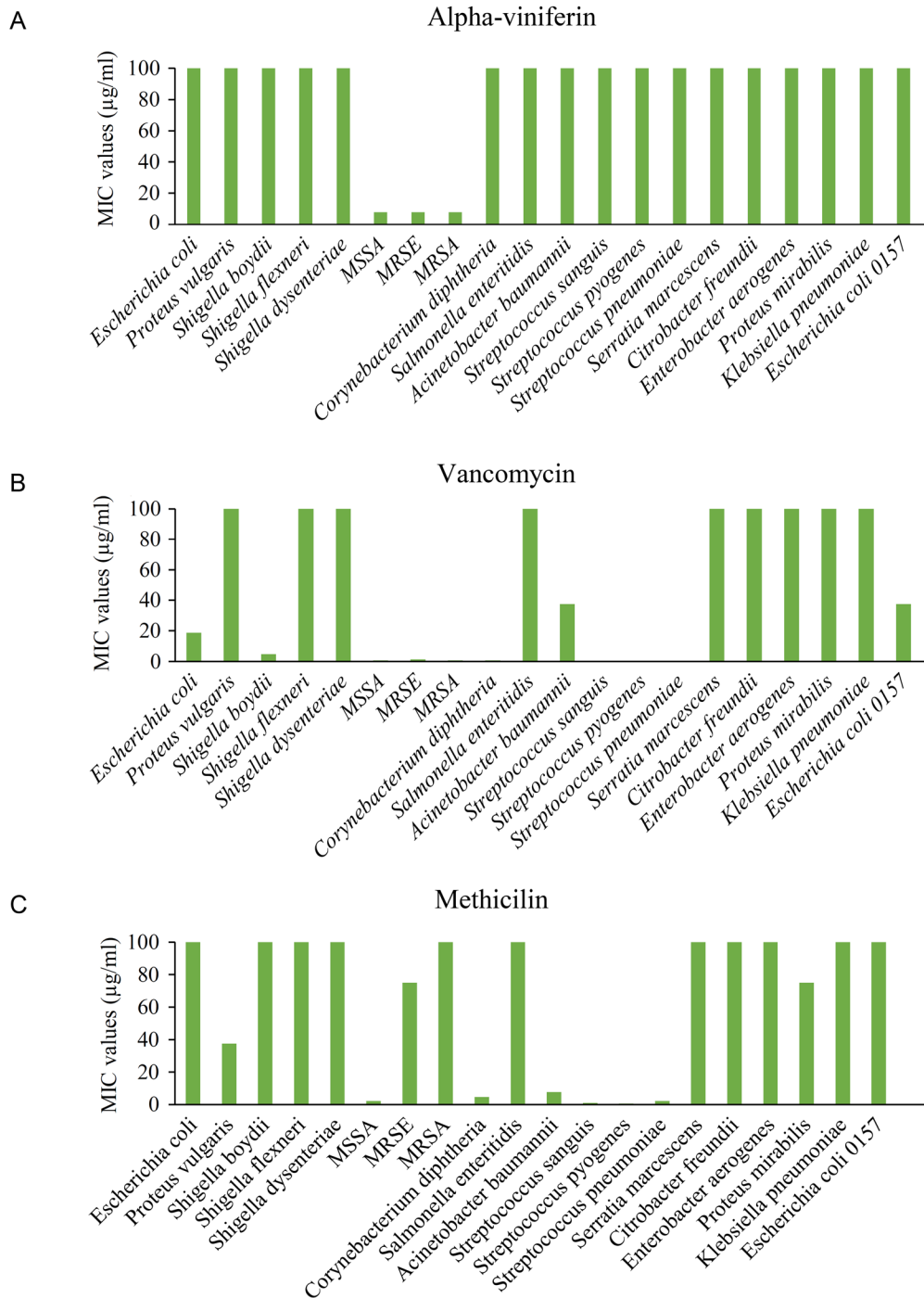


Fig. 1. Antimicrobial inhibition. Antibacterial activities, expressed as MIC₅₀, of α -viniferin (A), Vancomycin (B) and Methicillin (C) against 20 bacterial isolates. Bacterial suspensions (1×10^5 to 1×10^6 CFU/ml) were incubated with varying concentrations of α -viniferin, Vancomycin, Methicillin in Mueller Hinton Broth in a 96 well plate for 24 h at 37°C. *In vitro* MICs were determined by the broth dilution procedures described by the Clinical and Laboratory Standards Institute (CLSI).

species including methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), and methicillin-resistant *S. epidermidis* (MRSE) with no toxicity to other bacterial strains (Fig. 1A). The reference drug VAN was also active against three *Staphylococcus* species, but it also showed toxicity against other bacterial strains (Fig. 1B), and MET was also tested as a second reference drug (Fig. 1C). These results indicate

α -viniferin as a potential antibacterial agent with specific toxicity against *Staphylococcus* group.

Clinical experiment. We performed a 10-day clinical trial to investigate the antimicrobial efficiency of α -viniferin, in which 20 healthy Korean adult females participated. The drug was applied in the right and left anterior nares three times daily. We collected the nasal swab samples before (day 0) and after conducting

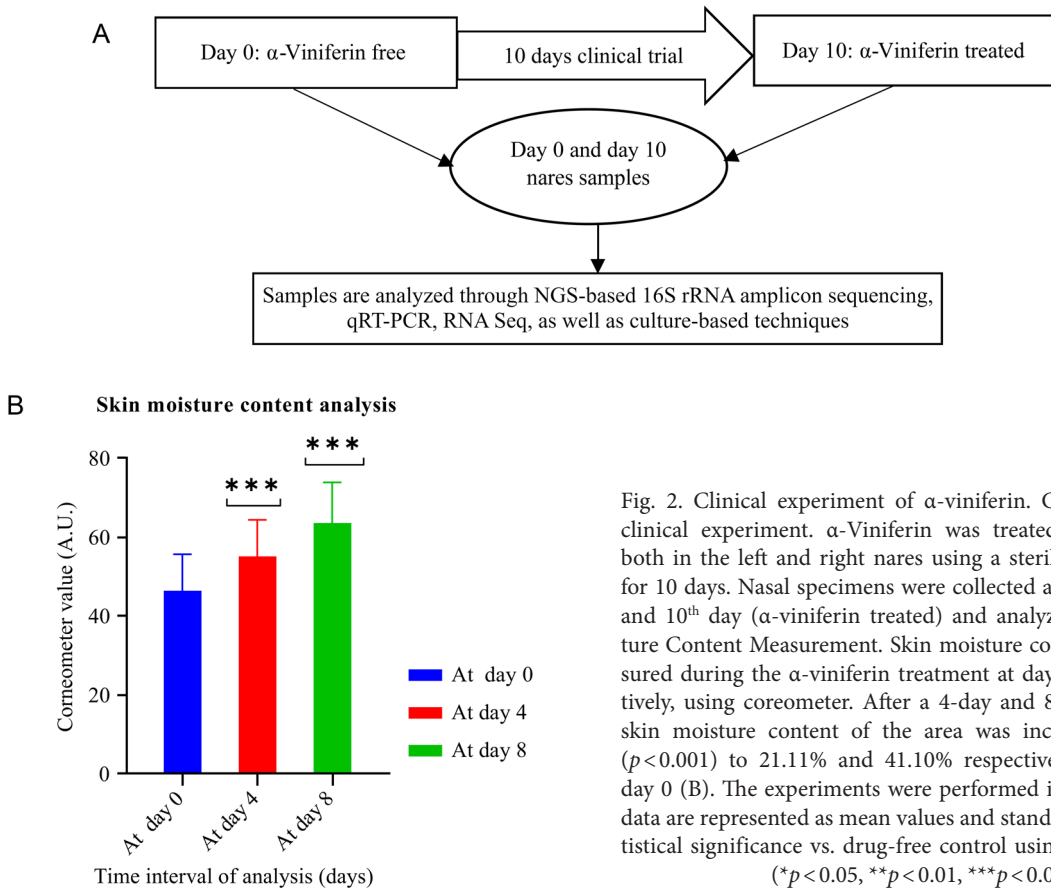


Fig. 2. Clinical experiment of α -viniferin. General overview of clinical experiment. α -Viniferin was treated three times daily both in the left and right nares using a sterile cotton swab stick for 10 days. Nasal specimens were collected at 0th day (drug-free) and 10th day (α -viniferin treated) and analyzed (A). Skin Moisture Content Measurement. Skin moisture content was also measured during the α -viniferin treatment at day 0, 4, and 8 respectively, using coreometer. After a 4-day and 8-day treatment, the skin moisture content of the area was increased dramatically ($p < 0.001$) to 21.11% and 41.10% respectively, compared to at day 0 (B). The experiments were performed in triplicate, and the data are represented as mean values and standard deviations. *Statistical significance vs. drug-free control using one-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

the clinical trial (day 10), and we analyzed them through culture-based and molecular-based techniques (Fig. 2A). We also measured each participant's skin surface's hydration level using a corneometer at day 0, fourth, and eighth, respectively, and then the corneometer value, A.U., was analyzed. We observed that skin moisture content increased to a statistically significant level ($p < 0.001$) at day 4 (21.11%) and at day 8 (41.10%), compared to day 0, demonstrating the moisturizing power of α -viniferin (Fig. 2B).

Skin irritation test. A skin irritation test of α -viniferin was done in 32 healthy females in different loca-

tions before performing the clinical trial; with all participants, after the patch was attached for 48 hours, α -viniferin did not show skin irritation or allergic reactions at 30 mins and 24 hours after patch removal. The mean values with three different α -viniferin concentrations in both cases were 0.00 and were classified as having a low range (0.00–0.87) irritation potential according to the classification criteria (Table I). These results ensure the safety profile of α -viniferin as a topical agent.

Culture-based quantification. To enumerate the culturable bacteria, *S. aureus*, and MRSA in the drug-free and α -viniferin-treated samples, we collected nasal

Table I
Skin irritation test result.

Material's name	No. responder	30 min. after patch removal (48 hrs.)						24 hrs. after patch removal (72 hrs.)						Mean ^a
		0.5+	1+	2+	3+	4+	mean	0.5+	1+	2+	3+	4+	mean	
1. α -viniferin 10	0	- ^b	-	-	-	-	0.00	-	-	-	-	-	0.00	0.00
2. α -viniferin 100	0	-	-	-	-	-	0.00	-	-	-	-	-	0.00	0.00
3. α -viniferin 1,000	0	-	-	-	-	-	0.00	-	-	-	-	-	0.00	0.00
4. Negative control	0	-	-	-	-	-	0.00	-	-	-	-	-	0.00	0.00

^a - Mean: (mean value of skin reaction at 48 hrs. + mean value of skin reaction at 72 hrs.)/2. (Mean score: 0.00–0.87, low; 0.88–2.42, mild; 2.43–3.44, moderate; 3.45–4.00, severe)
^b - "-": No reaction. (Reaction score: 0, -, no reaction; 0.5, ±, Barely perceptible erythema, Doubtful or questionable reaction; 1, +, Slight erythema, either spotty or diffuse; 2, ++, Moderate uniform erythema; 3, +++, Intense redness with edema; 4, +++++, Intense redness with edema and vesicles).

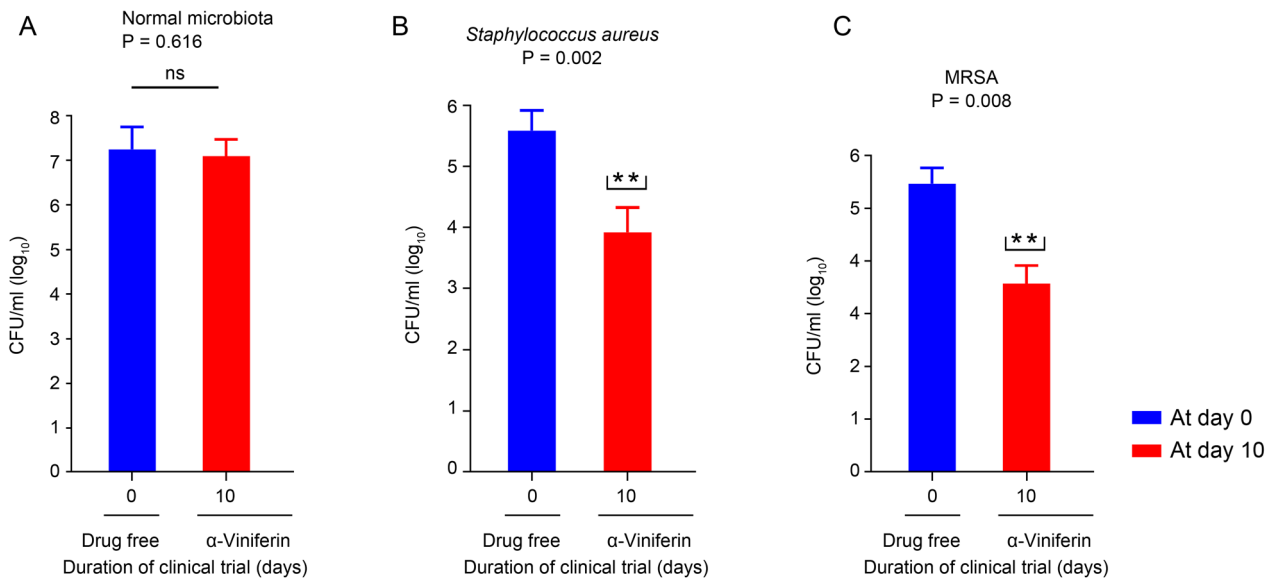


Fig. 3. Culture-based quantification. Nasal swab samples of 0 and 10th days were collected and incubated in nutrient agar, staphylococcus agar media 110, and staphylococcus agar media 110 containing methicillin antibiotic for 48 hours at 37°C by using spread plate technique. After this period, antimicrobial activity was determined by the colony forming unit (CFU) method against normal flora (A), *S. aureus* (B), and MRSA (C), respectively. The experiments were performed in triplicate, and the data are represented as mean values and standard deviations. *Statistical significance vs. drug-free control using unpaired two-tailed Student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

specimens on day 0 and tenth. We assayed them on the nutrient agar, staphylococcus agar medium, and a staphylococcus agar medium containing methicillin (50 µg/ml) through spread-plate techniques. After incubation, we analyzed the resulting CFU data, which revealed that the samples' bacterial content remained almost constant throughout the clinical trial period. The average number of commensal bacteria before and after the treatment was 1.7×10^7 CFU/ml and 1.2×10^7 CFU/ml, respectively, and the p was 0.616 ($p > 0.05$) (Fig. 3A), indicating lack of significant change in bacterial content after α -viniferin treatment. On the other hand, the average number of *S. aureus* before and after the treatment was 3.8×10^5 CFU/ml and 8.3×10^3 CFU/ml, respectively, and the p was 0.002 ($p < 0.01$) (Fig. 3B). The MRSA number was 7.8×10^4 CFU/ml and 2.9×10^3 CFU/ml, respectively, and the p was 0.008 (Fig. 3C), indicating a significant reduction in the number of *S. aureus* and MRSA during the clinical trial. Overall, the culture-based quantification results demonstrate α -viniferin as an antibacterial agent with specific activity against the *staphylococcus* group, including MRSA without affecting the nares' resident normal microbiota.

qRT-PCR-based quantification. We did qRT-PCR using *S. aureus*- and MRSA-specific primers to evaluate and quantify these organisms in the nares samples. We then analyzed the resulting C_t value and found that the C_t values for *S. aureus* and MRSA increased, which indicated the reduction of the organism populations. It is also noteworthy that there was no noticeable change in the C_t value of normal microbiota ($p > 0.05$) (Fig. 43).

In Fig. 4B, the difference of the C_t value between the columns is four, which indicates that the *S. aureus* number was reduced 16-fold while the MRSA number decreased 32-fold (Fig. 4C). Since we know that in each amplification cycle, the target doubles exponentially, and the C_t value is inversely related to the content of starting material. To calculate the reduction of bacterial numbers in the samples, we generated a qRT-PCR standard curve (Fig. 4D). Comparing the analyzed data with this curve, the initial and final average C_t value of *S. aureus* was 31 and 35 ($p < 0.01$), which corresponded to 1.6×10^4 CFU/ml and 1×10^3 CFU/ml, respectively. Furthermore, MRSA's initial and final average C_t value was 32 and 37 ($p < 0.001$), which corresponded to 1×10^4 CFU/ml and 3.2×10^2 CFU/ml, respectively. Based on the C_t values in this study, we can conclude that α -viniferin treatment did not significantly change the microbiota, but the prevalence of *S. aureus*, including MRSA, changed significantly. These findings demonstrated the specific antimicrobial effectiveness of α -viniferin against *S. aureus* and MRSA.

NGS-based 16s rRNA profiling. To determine the whole bacterial composition throughout the α -viniferin-treatment period, we did 16S rRNA amplicon sequencing. Based on the results, the *Staphylococcaceae* family and *Staphylococcus* genus were the most abundant in the nares, covering 51.04% and 51.03% of the whole microbial community. After α -viniferin treatment, we observed that the organisms decreased from 51.04% to 23.99% at the family level and 51.03% to 23.99% at the genus level (Fig. 5).

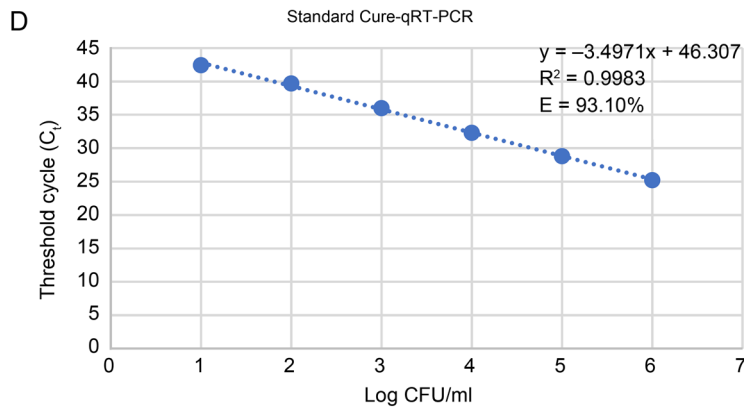
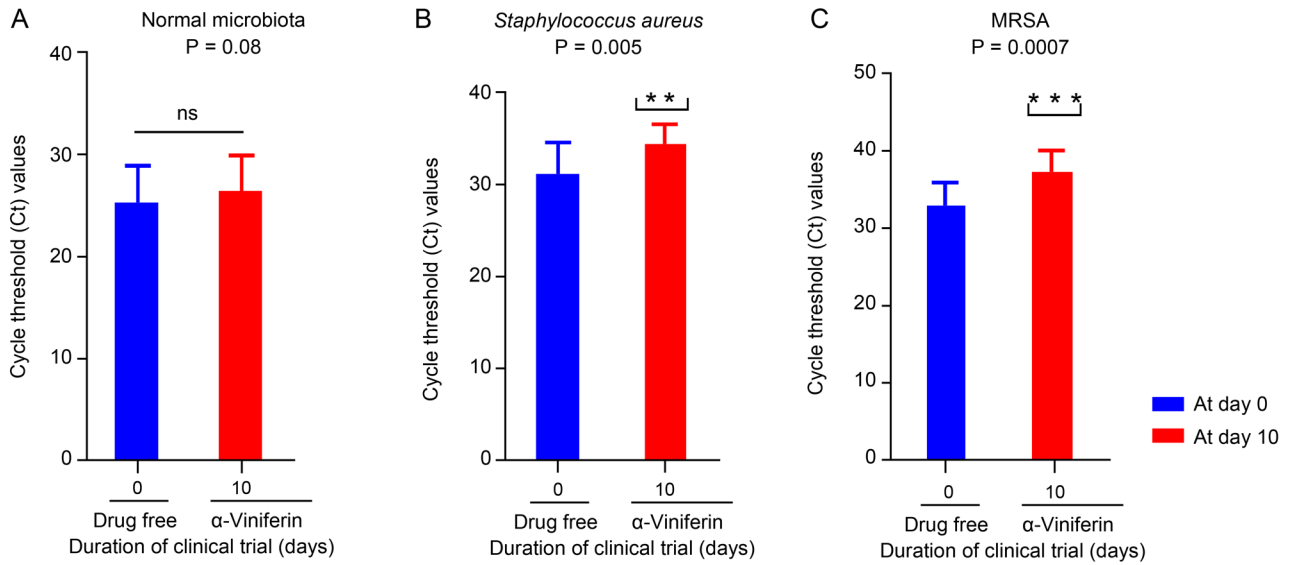


Fig. 4. qRT-PCR-based quantification. Nares swab samples of 0 and 10th days were collected, following by DNA extraction and qRT-PCR was performed. Antimicrobial activity of α-viniferin (A, B, C) was determined by comparing the resulting C_t value with the standard curve (D) against normal flora, *S. aureus*, and MRSA, respectively. The experiments were carried out 3 times, and the data are given as mean values and standard deviations. * Statistical significance vs. drug-free control using unpaired two-tailed Students' *t*-test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

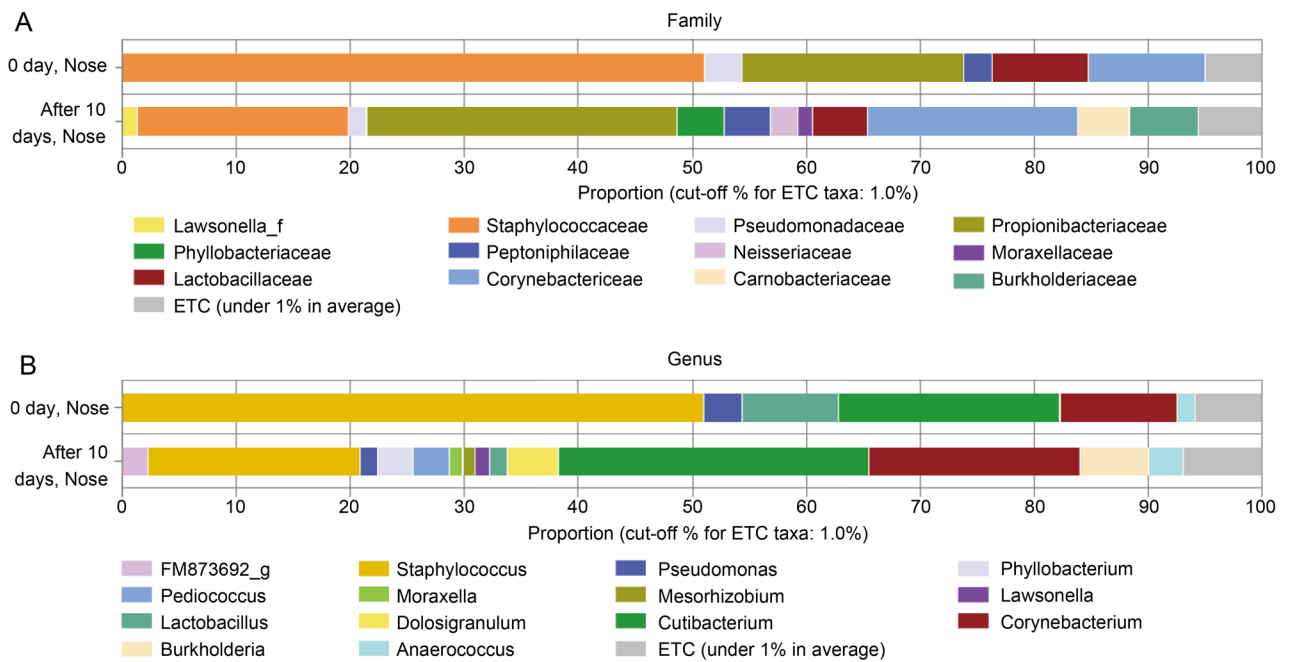


Fig. 5. NGS-based 16S rRNA profiling. To know the whole bacterial composition throughout the α-viniferin treatment, we conducted 16S rRNA amplicon sequencing with 0th (α-viniferin-free) and 10th days (α-viniferin treated) nasal swab samples, and the resulting data was analyzed by Ezcloud software. *Staphylococcaceae* is the most dominant family, which decreased from 51.04% to 23.99% (A), and *Staphylococcus* is the most abundant among the genus level group that reduced from 51.03% to 23.99% (B) after the α-viniferin treatment.

Table II
Number of genes (FC cutoff > 2 and 5) regulated in *S. aureus* by 1× and 2× MIC α-viniferin treatment for 8 hours at 37°C, according to functional class.

Functional classification	Total number of genes	2-fold				5-fold			
		1 × MIC		2 × MIC		1 × MIC		2 × MIC	
		up	down	up	down	up	down	up	down
1 DNA metabolism	92	9	2	20	11	2	0	2	0
2 Energy metabolism	126	10	17	22	31	1	5	5	11
3 Protein synthesis	85	5	4 ^a	12	15	0	0	1	1
4 Transport and binding proteins	195	29 ^b	32 ^b	42 ^a	48 ^b	4	4	16 ^b	19
5 Protein fate	77	5	4 ^a	13	10	2	1	6	1
6 Amino acid biosynthesis	62	13 ^b	5	25	5 ^a	3 ^a	2	11	2
7 Signal transduction	13	0	3	0	3	0	0	0	2
8 Purines, pyrimidines, nucleosides, and nucleotides	37	1	5	2	7	1	0	1	2
9 Regulatory functions; Signal transduction	9	0	2	1	4	0	1	0	1
10 Cellular processes	90	9	11	15	21	0	5	5	8
11 Biosynthesis of cofactors, prosthetic groups, and carriers; Transport and binding proteins	3	0	2	0	2	0	1	0	1
12 Central intermediary metabolism	21	1	4	6	7	0	2	1	3
13 Regulatory functions	55	3	8	6	11	1	1	1	3
14 DNA metabolism; Regulatory functions; Cellular processes	1	0	0	0	0	0	0	0	0
15 Cell envelope	50	4	4	8	11	0	0	1	1
16 Cellular processes; Transport and binding proteins	13	5 ^b	2	5	1	2 ^a	0	3 ^a	0
17 Energy metabolism; Purines, pyrimidines, nucleosides, and nucleotides	2	0	0	0	1	0	0	0	0
18 Transcription	22	1	2	3	3	0	0	0	1
19 Biosynthesis of cofactors, prosthetic groups, and carriers	99	8	7 ^a	25 ^a	13 ^a	1	3	6	2
20 Protein fate; Energy metabolism	4	0	1	1	1	0	0	0	1
21 Fatty acid and phospholipid metabolism	24	0	2	3	3	0	0	0	0
22 Transport and binding proteins; Signal transduction	10	0	2	2	3	0	0	0	1
23 Hypothetical proteins	26	3	3	5	4	1	1	3	3
24 Cell envelope; Central intermediary metabolism	1	0	0	0	0	0	0	0	0
25 DNA metabolism; Mobile and extrachromosomal element functions	3	1	0	1	0	0	0	1	0
26 Cellular processes; DNA metabolism	8	0	2	1	4 ^a	0	0	0	0
27 Protein fate; Transport and binding proteins	1	0	0	0	0	0	0	0	0
28 Regulatory functions; Purines, pyrimidines, nucleosides, and nucleotides	1	0	0	0	0	0	0	0	0
29 Protein fate; Cellular processes	6	1	0	1	0	0	0	0	0
30 Energy metabolism; Central intermediary metabolism	1	0	0	0	0	0	0	0	0
31 Regulatory functions; Cellular processes	2	0	1	0	1	0	0	0	0
32 Signal transduction; Regulatory functions	1	0	0	0	0	0	0	0	0
33 DNA metabolism; Cellular processes	3	0	1	1	1	0	0	0	0
34 Biosynthesis of cofactors, prosthetic groups, and carriers; Central intermediary metabolism	1	0	0	1	0	0	0	0	0
35 Mobile and extrachromosomal element functions	32	1	1	5	1	0	0	0	0
36 Protein synthesis; Cellular processes; Regulatory functions	1	0	0	0	0	0	0	0	0
37 Cellular processes; Cell envelope	2	0	0	0	0	0	0	0	0
38 Transport and binding proteins; Cellular processes	2	0	0	0	0	0	0	0	0
39 Protein fate; Cell envelope	3	0	1	1	0	0	0	0	0

Table II, Continued

Functional classification	Total number of genes	2-fold				5-fold			
		1 × MIC		2 × MIC		1 × MIC		2 × MIC	
		up	down	up	down	up	down	up	down
40 Cell envelope; Cellular processes	4	0	0	1	1	0	0	0	0
41 Cell envelope; Transport and binding proteins	4	1	0	1	0	0	0	0	0
42 Regulatory functions; Transport and binding proteins	2	0	0	0	1	0	0	0	0
43 DNA metabolism; Regulatory functions	3	0	0	0	0	0	0	0	0
44 Cellular processes; Protein fate	4	0	0	0	1	0	0	0	0
45 Mobile and extrachromosomal element functions; Regulatory functions	3	0	1	0	1	0	0	0	1
46 Mobile and extrachromosomal element functions; Hypothetical proteins	2	0	0	0	0	0	0	0	0
47 Protein fate; Signal transduction	3	0	1	1	1	0	0	0	0
48 Regulatory functions; Amino acid biosynthesis	2	0	1	0	0	0	0	0	0
49 Purines, pyrimidines, nucleosides, and nucleotides; Central intermediary metabolism	1	0	0	0	0	0	0	0	0
50 Protein synthesis; Biosynthesis of cofactors, prosthetic groups, and carriers	1	0	0	0	0	0	0	0	0
51 Regulatory functions; Central intermediary metabolism	2	0	1	0	1	0	0	0	0
52 Protein fate; Regulatory functions	3	0	0	2	0	0	0	0	0
53 Regulatory functions; Energy metabolism	1	0	1	0	0	0	0	0	0
54 Mobile and extrachromosomal element functions; Protein fate	1	0	0	1	0	0	0	0	0
55 Regulatory functions; Central intermediary metabolism; Signal transduction	1	0	1	0	1	0	1	0	1
56 Central intermediary metabolism; Cell envelope	1	0	0	0	0	0	0	0	0
57 Cellular processes; Mobile and extrachromosomal element functions	4	0	0	0	0	0	0	0	0
58 Protein fate; Purines, pyrimidines, nucleosides, and nucleotides	1	0	1	0	1	0	0	0	1

^a - $p < 0.05$; ^b - $p < 0.01$; ^c - $p < 0.001$

Exploring α -viniferin-induced gene expression in *S. aureus*. We verified the integrity of extracted total RNA by the sharpness of the ribosomal RNA bands visualized on the 2% agarose gel using ethidium bromide. We observed distinct 23S and 16S rRNA bands without degradation in all samples tested (Fig. S1). Then, we did transcriptional profiling of *S. aureus* using 2,842 whole-genome RNA sequencing to measure the effects of α -viniferin relative to a sample of logarithmically growing *S. aureus*. Compared with the drug-free control group, 583 (1 × MIC, 8 h) and 1,057 (2 × MIC, 8 h) genes had expression levels altered 2-fold. When the FC cutoff was raised to 5-fold, we found that 94 (1 × MIC, 8 h) and 256 (2 × MIC, 8 h) genes had altered expression levels in the α -viniferin-treated group (Table II). To better understand the transcriptional response of *S. aureus* to α -viniferin, we adopted more specific functional classifications. We used a hypergeometric distribution to determine whether the enrichment of genes within a particular functional category in

response to α -viniferin-treatment was significant. We observed that protein synthesis, transport, and binding proteins, protein fate, amino acid biosynthesis, cellular processes; transport and binding proteins, biosynthesis of cofactors, prosthetic group, and carriers, cellular processes, and DNA metabolism functional groups were significantly reduced five-fold when the bacteria were treated with a drug at a concentration equal to 2 × MIC (Table III). These results demonstrated how α -viniferin inhibited the growth of the *Staphylococcus* group.

Discussion

In a 10-day clinical trial, we investigated the decolonization efficiency of α -viniferin as an *S. aureus*-specific drug candidate. Before the clinical trial, we checked the specific toxicity of α -viniferin and two other reference drugs against 20 bacterial isolates. α -Viniferin showed excellent antibacterial effectiveness against the

Table III

Number of (FC cutoff > 2 and 5) specific regulated gene groups in *S. aureus* by 1 × and 2 × MIC α-viniferin treatment for 8 hours at 37°C, according to functional classification.

Functional classification	Total number of genes	2-fold				5-fold			
		1 × MIC		2 × MIC		1 × MIC		2 × MIC	
		up	down	up	down	up	down	up	down
3 Protein synthesis									
a tRNA aminoacylation	12	1	1	1	4	0	0	1	1
b Ribosomal proteins: synthesis and modification	25	2	3	5	4	0	0	0	0
c tRNA and rRNA base modification	37	2	0	6	4	0	0	0	0
d Other	85	9	8	16	18	2	0	6	2
e Translation factors	7	0	0	0	2	0	0	0	0
4 Transport and binding proteins									
a Amino acids, peptides and, amines	32	5	2	7	4	0	1	4a	1
b Cations and iron carrying compounds	72	8	9	12	10	1	0	2	3
c Carbohydrates, organic alcohols, and acids	27	4	6	7	8	1	1	3	5b
d Anions	18	4	2	5	10	0	1	2	5
e Anions; Other	1	1	0	1	0	0	0	1a	0
f Other	85	9	8	16	18	2	0	6	2
g Nucleosides, purines and, pyrimidines	6	0	3	0	5b	0	1	0	3b
h Unknown substrate	20	3	6	6	6	0	0	1	2
i Cations and iron carrying compounds; Anions	3	0	1	0	2	0	0	0	0
5 Protein fate									
a Protein and peptide secretion and trafficking	27	2	2	3	5	0	0	2	0
b Degradation of proteins, peptides, and glycopeptides	18	1	0	5	0	0	0	2	0
c Protein modification and repair	20	0	0	0	3	0	0	0	0
d Protein folding and stabilization	4	2a	1	2	1	2b	1	2a	1
e Degradation of proteins, peptides, and glycopeptides; Protein folding and stabilization	6	0	0	3	0	0	0	0	0
f Degradation of proteins, peptides, and glycopeptides; Protein modification and repair	1	0	0	0	0	0	0	0	0
g Protein and peptide secretion and trafficking; Protein modification and repair	1	0	1	0	1	0	0	0	0
6 Amino acid biosynthesis									
a Aspartate family	15	3	1	8b	0	1	0	3a	0
b Serine family	11	3	0	4	2	0	0	1	0
c Glutamate family	7	0	3a	0	2	0	1	0	1
d Pyruvate family	12	5b	1	8	1	0	1	5	1
e Histidine family	8	0	0	2	0	0	0	0	0
f Aromatic amino acid family	9	2	0	3	0	2b	0	2	0
16 Cellular processes; Transport and binding proteins									
a Toxin production and resistance; Other	13	3	1	4	1	2a	0	3a	0
b Toxin production and resistance; Unknown substrate	3	2a	1	1	0	0	0	0	0
c Detoxification; Other	1	0	0	0	0	0	0	0	0
19 Biosynthesis of cofactors, prosthetic groups, and carriers									
a Glutathione and analogs	4	0	0	1	1	0	0	0	0
b Menaquinone and ubiquinone	17	0	0	3	1	0	0	0	0
c Other	85	9	8	16	18	2	0	6	2
d Heme, porphyrin, and cobalamin	19	3	2	7a	2	0	1	2	1
e Folic acid	6	0	0	0	0	0	0	0	0
f Pyridoxine	2	0	2 ^a	0	2 ^a	0	2	0	0

Table III. Continued

Functional classification	Total number of genes	2-fold				5-fold			
		1 × MIC		2 × MIC		1 × MIC		2 × MIC	
		up	down	up	down	up	down	up	down
g Riboflavin, FMN, and FAD	6	0	0	2	0	0	0	0	0
h Thiamine	7	0	0	0	2	0	0	0	0
i Lipoate	1	0	0	0	0	0	0	0	0
j Other; Thiamine; Pyridoxine	2	0	0	0	0	0	0	0	0
k Pantothenate and coenzyme A	6	0	1	1	1	0	0	0	0
l Biotin	6	2	0	4 ^a	0	1	0	2 ^a	0
m Pyridine nucleotides	2	0	0	0	0	0	0	0	0
n Molybdopterin	4	1	0	2	0	0	0	0	0
26 Cellular processes; DNA metabolism									
a Cell division; Chromosome-associated proteins	8	0	2	1	4 ^a	0	0	0	0

^a - $p < 0.05$; ^b - $p < 0.01$; ^c - $p < 0.001$

Staphylococcus group. On the other hand, several reports showed VAN resulted in rapid depletion of intestinal microflora and significantly promoted the growth of vancomycin-resistant bacteria (Edlund et al. 1997; Isaac et al. 2016) highlighting the drawbacks of VAN even though it was the most effective anti-*Staphylococcus* reference drug in the present MIC study. We have also tested MET as another reference drug.

In this study, we analyzed the nasal swab samples through culture-based techniques to investigate the antimicrobial effectiveness of α -viniferin against the nares normal microflora, *S. aureus*, and MRSA. α -Viniferin was active against *S. aureus* and MRSA but showed no activity against nasal microflora. To further confirm this, we did qRT-PCR using *S. aureus*- and MRSA-specific primers since precise detection and quantification are achievable by using this technique. Expectedly, α -viniferin was active against *S. aureus* and MRSA while preserving the resident nasal microflora. Moreover, α -viniferin improved the skin moisture content, which is essential in maintaining skin plasticity and barrier integrity without toxicity.

In addition, we used NGS-based 16S rRNA profiling to investigate further the clinical efficiency of α -viniferin against the *Staphylococcus* group. We also evaluated the effect of α -viniferin against normal nasal microflora using NGS, where NGS upgrades DNA sequencing methodology to the next level through parallel sequencing processes that enable the simultaneous sequencing of different DNA fragments while providing precise identification results (Abayasekara et al. 2017). The NGS results demonstrated *Staphylococcus* as the most dominant group in the anterior nares, supporting similar published findings (Hogan et al. 2016; Warnke et al. 2016; Lu et al. 2018). α -Viniferin drastically reduced the number of *Staphylococcus* in

the anterior nares, while the decrease in the number of other bacteria groups was statistically insignificant. The important thing is that α -viniferin significantly decreased the *Staphylococcus* group. This molecular approach provides evidence of the antimicrobial specificity of α -viniferin as a pathogen-specific potential drug, and the results were consistent with the culture and qRT-PCR-based findings. The results were also in agreement with a previous study finding that demonstrated the *in vitro* activity of α -viniferin against MRSA (Seo et al. 2017).

RNA sequencing technology is a powerful tool for studying the molecular basis of genetic interactions; it makes it possible to examine the relatively unbiased measurements of expression levels across the entire length of a transcript using high-throughput sequencing platforms (Pickrell et al. 2010). In the present study we did RNA sequencing to understand α -viniferin-induced gene expression in *S. aureus*. Our results showed that α -viniferin inhibits the growth of *S. aureus*, which may be attributed to its effects on some of the gene groups mentioned. Further research is required to elucidate this finding. In conclusion, we demonstrated the clinical efficiency of α -viniferin as a potential *S. aureus*-specific drug candidate for the first time through a clinical trial.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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