Disruption of methicillin-resistant *Staphylococcus aureus* protein synthesis by tannins

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Abstract

Introduction **Methicillin-resistant *Staphylococcus aureus* (MRSA) is a worldwide public health threat, displaying multiple antibiotic resistance that causes morbidity and mortality. Management of multidrug-resistant (MDR) MRSA infections is extremely difficult due to their inherent resistance to currently used antibiotics. New antibiotics are needed to combat the emergence of antimicrobial resistance.**

Methods **The *in vitro* effect of tannins was studied against MRSA reference strain (ATCC 43300) and MRSA clinical strains utilizing antimicrobial assays in conjunction with both scanning and transmission electron microscopy. To reveal the influence of tannins in MRSA protein synthesis disruption, we utilized next-generation sequencing (NGS) to provide further insight into the novel protein synthesis transcriptional response of MRSA exposed to these compounds.**

Results **Tannins possessed both bacteriostatic and bactericidal activity with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 0.78 and 1.56 mg/mL, respectively, against all tested MRSA. Scanning and transmission electron microscopy of MRSA treated with tannins showed decrease in cellular volume, indicating disruption of protein synthesis.**

Conclusion **Analysis of a genome-wide transcriptional profile of the reference strain ATCC 43300 MRSA in response to tannins has led to the finding that tannins induced significant modulation in essential ribosome pathways, which caused a reduction in the translation processes that lead to inhibition of protein synthesis and obviation of bacterial growth. These findings highlight the potential of tannins as new promising anti-MRSA agents in clinical application such as body wash and topical cream or ointments.**

Keywords **tannins; MRSA; antimicrobial assays; ribosomal protein synthesis**

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global public health burden due to emergence of resistance to currently used antibiotic classes and the limited therapeutic options.[1] It also caused the increment of healthcare costs.[2] It causes a diverse range of clinical diseases such as endocarditis, sepsis, skin and soft tissue infections, implanted device infections, and several other relevant human diseases.[3,4] Some MRSA have been discovered resistant to new antibiotics such as linezolid, quinupristin/dalfopristin, daptomycin and telavancin[5-7] which has limited the drug's effectiveness. Thus, there is an intense need for new antibiotics to combat drug-resistant MRSA.

Plants have served as useful source of antimicrobial drugs for over 100 years and offer

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new potential compounds for the development of new antibacterial agents.[8] Promising compounds from plants include tannins. There are studies that prove increased production of tannins in plants contracting a disease. Therefore, tannins may have a biological role in protection against infection.[9] A few studies have been performed to screen anti-MRSA activity of tannins. Tannins from green tea waste, acorn and chestnut hulls have been screened for anti-MRSA properties and have been shown to exhibit antibacterial activity against MRSA.[10] Tannins from *Pimenta dioica* leaves also exhibited anti-MRSA effects with minimum inhibitory concentration (MIC) value of 2500 µg/mL.[11] Thus, this study attempts to explore the anti-MRSA activity of tannins.

This study mainly focused on the effect of tannins against both reference and clinical strains of MRSA by means of antimicrobial assays and a next-generation sequencing (NGS) approach. To the best of our knowledge, this study explored the anti-MRSA effect of tannins most comprehensively to date revealing morphological and ultra-structural alterations of the treated MRSA by electron microscopy and profiling the significant changes in gene expression in response to these compounds. Genes expression profiling enabled the measurement of the expression of thousands of genes in order to render the entire insight of cellular function in affected MRSA.

Methods

Tannins preparation

Tannins were purified from *Phyllanthus columnaris* stem bark methanol extract, which was obtained from previous study.[12] A total of 5 g methanol extract was dissolved with methanol (Merck, Darmstadt, Germany) and filtered into a separating funnel. Diethyl ether (Merck, Darmstadt, Germany) was then added until the formation of white precipitation. Separating funnel stopcock was opened and the solution was collected in a beaker and was discarded. The tannin fraction, which was dark brown precipitate embedded in the separating funnel wall, was collected and weighed.

Bacterial strains and materials

Two clinical isolates primarily collected from patients' blood and wound were obtained from Hospital Serdang, Malaysia and named MRSA 1 and MRSA 7. The isolates were identified by Vitek 2 (bioMérieux, Missouri, USA) and the antibiotic susceptibility was profiled (Table 1). A reference strain MRSA ATCC 43300 was obtained commercially. All strains were maintained and enriched on respective brain-heart infusion (BHIA) agar slopes (Oxoid Ltd., Basingstoke, UK) and brain-heart infusion broth (BHIB) (Oxoid Ltd.).[13] Mueller–Hinton agar (MHA) (Oxoid Ltd.) and Mueller–Hinton broth (MHB) (Oxoid Ltd.) were utilized as growth media for antimicrobial and NGS studies.

Table 1. Methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates and their antimicrobial susceptibility profile.

MRSA isolates	1	7
Oxacillin 1 mg	R	R
Vancomycin 30 mg	S	S
Chloramphenicol 30 mg	S	S
Tetracycline 30 mg	S	R
Ampicillin 10 mg	R	R
Penicillin 10 mg	R	R
Cefoxitin 30 mg	R	R
Erythromycin 15 mg	R	R
Kanamycin 30 mg	S	R
Rifampicin 5 mg	S	S
Trimethoprim-sulfamethoxazole 23.75/ 1.25 mg	S	S

The results were interpreted according to CLSI 2015; S – susceptible; R – resistant.

Antimicrobial assays

MIC test was performed as outlined by Clinical and Laboratory Standard Institute (2015) in 96-well sterile microplates[14] while minimum bactericidal concentration (MBC) test was performed as outlined by Clinical and Laboratory Standards Institute (1999).[15] Tannins were tested on MRSA within the range of 12.50-0.02 mg/mL while vancomycin hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was at 4.00-0.01 mg/mL (as antibiotic positive controls). MRSA with MHB (5 x 10^5 CFU/µL)

and MHB only were included as respective inocula viability and sterility controls. Tests were done in duplicates. The plate was incubated at 35°C overnight before adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent prior to obtaining the MIC value. For MBC test, 5 µL of sample from wells with no growths by MIC was streaked onto MHA and incubated at 35°C for 24 h. The lowest concentrations showing no growth on the agar were recorded as the MBC values.

Scanning electron microscopy

MRSA ATCC 43300 was treated with tannins at 0.78 mg/mL prior to inoculation on BHIA after 24 h incubation at 37°C. Untreated MRSA was included as a control. The cultured agar was then sliced into 1 cm^3 and fixed with 2% glutaraldehyde for 24 h at 4°C. After fixation, samples were washed with 0.1 M phosphate buffer prior to subsequent dehydration in graded ethanol, critical-point dried in CO_2, mounted onto stubs, and coated with gold. The samples were then observed and photographed using a Carl Zeiss LEO 1450VP scanning electron microscope.

Transmission electron microscopy

MRSA ATCC 43300 with or without addition of tannins was reaped by centrifugation and rinsed once with phosphate buffer saline, pH 7.4. Cells pellet were fixed in 2% glutaraldehyde for at least 24 h at 4°C prior to post-fixing in 1% osmium tetroxide for 2 h at room temperature. The samples were then parched in graded ethanol, ingrained in epoxy resin, polymerized, sectioned, and stained with uranyl acetate followed by Reynolds' lead citrate. Finally, the ultrathin sections were scrutinized by means of a Philips CM12 transmission electron microscope.

Total RNA isolation

MRSA ATCC 43300 cultures were treated with tannins at 0.39 mg/mL (1/2 × MIC) for total RNA isolation. Culture without addition of tannins was included as a control. RNA isolation was executed by utilizing an innuPREP RNA Mini Extraction Kit (Analytik Jena AG, Jena, Germany) according to the manufacturer's protocol followed by RNA concentration determination by measuring the absorbance at 260 nm (A260) on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA concentration was assessed to confirm it was contaminants free.

Next-generation sequencing

Purified total RNA was utilized for cDNA synthesizing as outlined by the manufacturer's protocol (Tetro cDNA Synthesis Kit; Bioline, London, UK). NGS was then conducted by RNA sequencing (RNA-Seq) using a MiSeq Sequencing System (Illumina Inc., San Diego, CA).

Next-generation sequencing analysis

The raw data obtained from Illumina RNA-Seq was trimmed prior to further analyze using the Illumina platform system software. Differential expression of a particular gene was studied by comparing control samples (as a reference) with treated samples. A threshold value of ≥2-fold change between treated and control samples and a significance level of <5% were used to discriminate the differentially expressed genes. Statistical analysis was based on count-based statistical analysis, Kal's test, and false discovery rate (FDR)-corrected p-values. Gene ontology (GO) analysis was then carried out using David Bioinformatics Database (DAVID Bioinformatics Resources 6.7; National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) prior to revealing the pathways affected by the differentially expressed genes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html).

Results

Antimicrobial effects of tannins on MRSA

Tannins of *Phyllanthus columnaris* stem bark exerted both inhibitory and bactericidal effects on all MRSA tested with same MIC and MBC values of 0.78 and 1.56 mg/mL, respectively. Bacterial growth was noted in positive control and no growth was marked in negative control.

Electron microscopy study

Morphological and ultra-structural alterations caused by tannins at 0.78 mg/mL after 24 h on MRSA ATCC 43300 were photographed by electron microscopy and compared with control. In scanning electron microscopy, untreated MRSA showed normal morphology with regular, smooth surface and spherical in grape-like clusters – Figure 1(a) – whereas MRSA treated with tannins showed many distorted cells – Figure 1(b).

In transmission electron microscopy, untreated MRSA ATCC 43300 cells were observed as normal by showing normal structure integrity and intact cell walls – Figure 1(c). Treated MRSA had a very notable effect of disrupted cell walls and shortage of cytoplasm and cell volume – Figure 1(d). Apparently, most of the cells were without cell walls. Some of the cells were irregular in shape. Cytoplasmic constituents outside of the cells were also markedly noticed.

Effects of tannins on MRSA protein synthesis

Next-generation sequencing analysis revealed that an enormous number of genes were differentially regulated in MRSA ATCC 43300 treated with tannins compared with the control. All the differentially expressed genes affected by tannins were implicated in varying functional categories, including RNA synthesis, protein synthesis, DNA modification and repair, etc. However, this study mainly focuses on the crucial protein synthesis pathway, i.e., ribosomal proteins synthesis, which gave rise to the

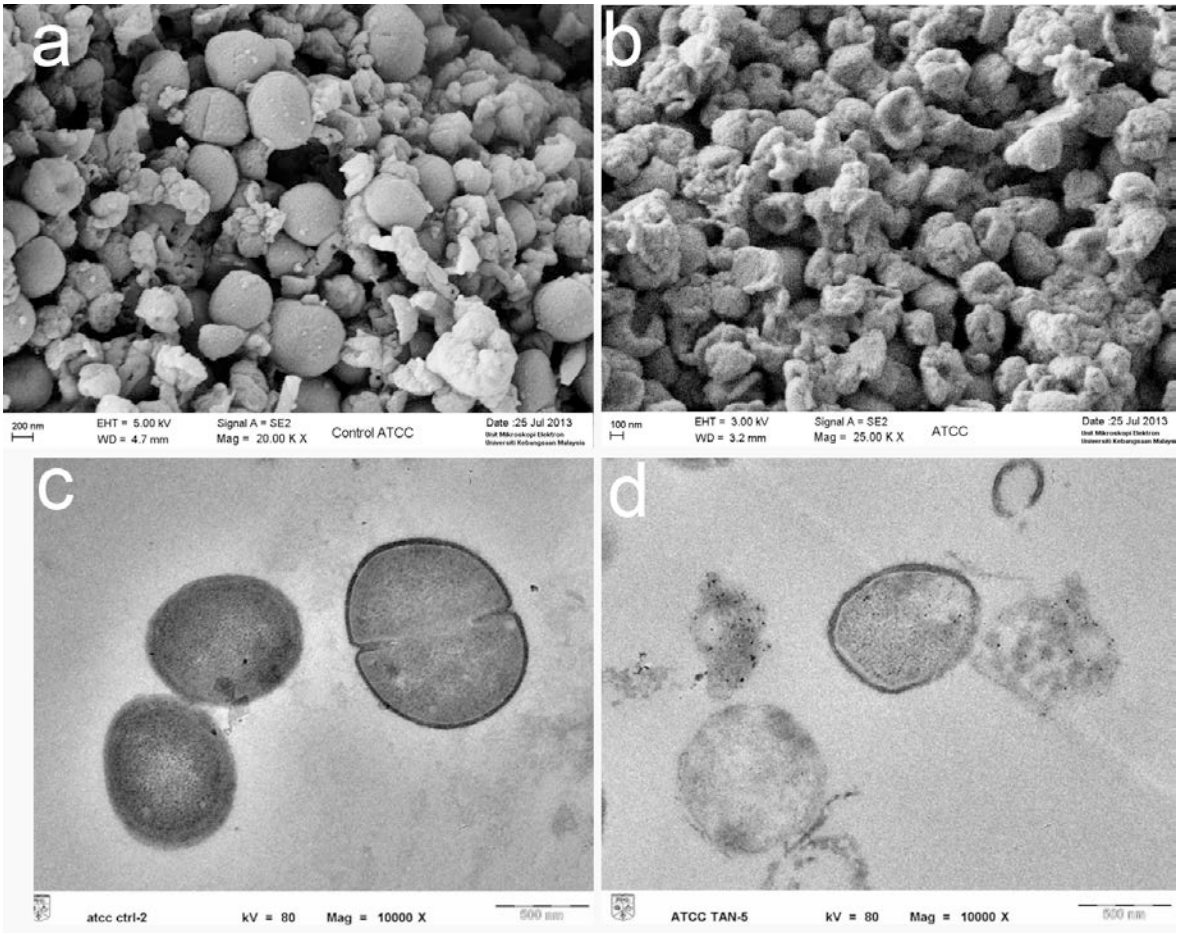


Figure 1. Scanning and transmission electron microscopy of MRSA ATCC 43300 treated with tannins for 24 h at 37°C. (a) & (c) control; (b) & (d) tannins at 0.78 mg/mL.

Bars (a) = 0.2 μm; (b) = 0.1 μm; (c) and (d) = 0.5 μm. n = 2.

morphological and ultra-structural changes of the treated MRSA as revealed in the electron microscopy study.

Tables 2 and 3 show the differences in relative gene expression for genes that were affected by tannins and that are involved in the translational function of ribosomal subunit proteins. Most of this group of genes were strongly downregulated (>2-fold and up to 26.6-fold). Overall, tannins downregulated 44 genes encoding ribosomal proteins, among which 18 were 30S proteins and 26 were 50S proteins.

Table 2. Selected genes involved in translational function of the small ribosomal subunit protein that are differentially regulated in methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 treated with tannins.

Gene	Product	Fold change ± standard deviation
rpsB	30S ribosomal protein S2	5.9 ± 0.8
rpsC	30S ribosomal protein S3	9.7 ± 1.8
rpsD	30S ribosomal protein S4	9.1 ± 0.3
rpsE	30S ribosomal protein S5	12.8 ± 0.4
rpsF	30S ribosomal protein S6	5.7 ± 0.3
rpsG	30S ribosomal protein S7	13.5 ± 1.8
rpsH	30S ribosomal protein S8	15.1 ± 1.2
rpsI	30S ribosomal protein S9	4.9 ± 2.2
rpsJ	30S ribosomal protein S10	6.8 ± 0.4
rpsK	30S ribosomal protein S11	5.3 ± 0.6
rpsL	30S ribosomal protein S12	13.8 ± 1.7
rpsM	30S ribosomal protein S13	9.0 ± 1.0
rpsN	30S ribosomal protein S14	12.6 ± 0.8
rpsO	30S ribosomal protein S15	9.0 ± 0.7
rpsQ	30S ribosomal protein S17	12.5 ± 0.3
rpsS	30S ribosomal protein S19	13.6 ± 1.6

Table 3. Selected genes involved in translational function of the large ribosomal subunit protein that are differentially regulated in methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 treated with tannins.

Gene	Product	Fold change ± standard deviation
rplA	50S ribosomal protein L1	6.8 ± 1.5
rplB	50S ribosomal protein L2	9.1±0.7
rplC	50S ribosomal protein L3	9.7±0.4
rplD	50S ribosomal protein L4	11.3±0.7
rplE	50S ribosomal protein L5	11.4±1.4
rplF	50S ribosomal protein L6	13.3±2.8
rplJ	50S ribosomal protein L10	17.6±1.4
rplK	50S ribosomal protein L11	8.8±1.4
rplL	50S ribosomal protein L7/L12	26.6±0.7
rplM	50S ribosomal protein L13	4.3±0.4
rplN	50S ribosomal protein L14	11.7±0.8
rplO	50S ribosomal protein L15	8.3±0.9
rplP	50S ribosomal protein L16	7.2±1.0
rplQ	50S ribosomal protein L17	7.5±1.1
rplR	50S ribosomal protein L18	15.8±1.7
rplU	50S ribosomal protein L21	6.6±0.5
rplV	50S ribosomal protein L21	13.3±1.6
rplW	50S ribosomal protein L21	14.6±0.7
rplX	50S ribosomal protein L24	12.7±1.3
rplY	50S ribosomal protein L25	5.3±1.5
rpmB	50S ribosomal protein L28	4.4±2.2
rpmC	50S ribosomal protein L29	15.2±1.4
rpmE2	50S ribosomal protein L31 type B	24.1±0.7
rpmG	50S ribosomal protein L33 1,2,3	3.4±1.6
rpmJ	50S ribosomal protein L36	11.2±1.0

Discussion

Antimicrobial activity of tannins on MRSA

Tannins exhibited inhibitory and bactericidal effects on all tested MRSA at 0.78 and 1.56 mg/mL, respectively. With reference to a published work, tannins were also noted to possess the same MIC value of 2500 µg/mL against all their three tested MRSA strains but different MIC values on other genus of bacteria in the same experiment.[11] Large discrepancy in MIC values between bacteria, fungi or yeasts have been noted due to the different mode of action

against the individual microorganisms.[16] We believe that the polar nature of the bioactive compounds in tannins easily penetrates the microbial cell walls to display the antimicrobial effects. Tannins are water-soluble polyphenols and are categorized into four major groups namely gallotannins, ellagitannins, complex tannins and condensed tannins.[17] Other studies have also proved that tannins have antimicrobial activity against *S. aureus*[18] and oral microbiota.[19] The highly probable biochemical compounds in tannins that may be responsible for inhibiting and killing MRSA might have possessed lower MIC and MBC values.

Molecular effects of tannins on inhibition of MRSA ribosomal protein synthesis

Tannins remarkably downregulated 44 ribosomal proteins, which is the highest number of ribosomal genes downregulated compared to other previous studies.[20] The pathway is crucial and essential in protein synthesis. The synthesis of ribosomal proteins begins with the transcription of rRNA followed by assembly in the bacterial ribosome.[21,22] Suppression of these proteins is commensurate to inhibition of translation in obviating cell growth.[23]

Generally, tannins are capable of crossing the MRSA cell wall because they are ligands which can form complexes by fixing to different polysaccharides and proteins in the cell walls. Certain studies claimed that tannins primarily act on and destruct the bacterial cell membrane.[24] There was also a study that reported their direct effect on oxidative phosphorylation, which affects the microbial metabolism.[16]

The remarkable effects of tannins on MRSA have proved their potential use as anti-MRSA agents. Tannins from *P. columnaris* can be further explored in the development of external medications such as body wash and topical cream or ointments. Viljoen et al. (2003) have proposed tannins as body wash or nasal ointments for MRSA treatment.[25]

A study that conducted cytotoxicity assay of tannins demonstrated that tannins have high LC_{50} (4.67 mg/mL) and low IC_{50} (12.26 µg/mL) values, and could be utilized as plausible source for pharmacologically pragmatic products.[26] Tannins are also being profitably utilized as additives in poultry feed as growth-promoting factors (AGP) in order to restrain diseases and to enhance animal performance.[27]

Conclusion

Tannins possessed both bacteriostatic and bactericidal effects against MRSA with MIC and MBC values of 0.78 and 1.56 mg/mL, respectively. Morphological and ultra-structural alterations of MRSA treated with tannins exhibited cell wall disruption, release of cytoplasmic contents and a decrease in cellular volume. Transcriptomic analysis by NGS revealed that tannins suppress the genes involved in RNA synthesis, protein synthesis, DNA modification and repair. These in turn lead to the reduction in protein synthesis and finally suppress the bacterial growth.

Authors' contributions statement: SNA carried out the laboratory work, analyzed the data, conducted the statistical analyses and drafted the manuscript. NI designed and supervised the study and edited the manuscript. WAY supervised the chemistry related study and performed the background literature review for the manuscript. All authors reviewed and approved the final version of the manuscript.

Conflicts of interest: All authors – none to disclose.

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