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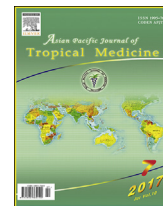
Mechanism of antagonistic effects of *Andrographis paniculata* methanolic extract against *Staphylococcus aureus*

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ABSTRACT

Objective: To investigate the effects of *Andrographis paniculata* (Burm.f.) Wall. Ex Nees (*A. paniculata*) on expressions and activities of catalase, superoxide dismutase and alkylhydroperoxide reductase C in *Staphylococcus aureus* (*S. aureus*) with respect to its survival *in vitro*.

Methods: Antioxidative property of methanolic leaves extract of *A. paniculata* (0.06 mg/mL). Minimum inhibitory concentration (MIC) was determined by its ability to reduce hydrogen peroxide (H_2O_2) toxicity against *S. aureus* ATCC 25923 [(3.8×10^8) cfu/mL]. Effects of the extract on expressions of *katA* (encoding catalase), *sodA* and *sodM* [encoding superoxide dismutases (SODs)], and *ahpC* [encoding alkylhydroperoxide reductase C (AhpC)] in *S. aureus* were determined by RT-qPCR and corresponding enzyme activity assays were performed. Nitroblue tetrazolium reduction (NBT) assay was performed to determine effects of the extract on intracellular and extracellular levels of O^{2-} in *S. aureus*.

Results: Cells challenged with 7.5 mmol/L H_2O_2 showed 0% survival in 30 min whereas 25% survived after treatment with the extract and H_2O_2. Cells that were treated with the extract alone had 43% survival in the same exposure period. Expressions of *sodA* and *sodM* genes in extract-treated cells were lowered 0.8-fold and 0.7-fold, respectively with decrease in total SOD activity of 26.8 U compared to untreated cells, 32.4 U ($P < 0.05$). In contrast, extract-treated *S. aureus* cells showed 3.3-fold increase in *katA* expression with corresponding increase in catalase activity of 1.828 U compared to untreated cells which was 1.248 U, ($P < 0.05$). More profoundly, *ahpC* expression was increased 61-fold in extract-treated cells, ($P < 0.05$) with corresponding increase in AhpC activity of 0.018 U compared to untreated cells, 0.012 U, ($P < 0.05$). Extract-treated cells had significantly lower intra- and extracellular O_2^- levels with absorbance readings ($A_{575\ nm}$) of 0.340 and 0.524 compared to untreated cells which were 0.516 and 0.928 ($P < 0.05$), respectively.

Conclusions: Taken together these results suggest that the low MIC of *A. paniculata* methanolic leaves extract (0.06 mg/mL) reduce H_2O_2 toxicity and more importantly, was in itself effectively inhibitory against *S. aureus*. Further, our observations suggest that a probable mode of its inhibitory mechanism against *S. aureus* is by reducing total SOD activity through downregulation of *sodA* and *sodM* expressions.

1. Introduction

    Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen that colonizes the nasal passages of 20%–80% of individuals and can be transiently present in the gastrointestinal tract, axillae and groin [1]. It causes mild to life threatening infections such as soft tissue and skin infections, bacteremia, endocarditis and others which are easily acquired from both the community and hospital environments [2–4]. Success of *S. aureus* as a pathogen is attributed to its ability to mitigate oxidative stress which involves protection, detoxification and repair mechanisms that are controlled by a network of regulators [5]. It adheres to host cells and evades the immune system to replicate [6–8], displays remarkable oxidative stress resistance and has evolved mechanisms to counter the damaging effects of oxidation products or reactive oxygen species (ROS) [9]. The state of imbalance caused by

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production of ROS and the inability of the cells to remove them results in oxidative stress which damages critical biomolecules and causes cell death [10,11].

Upon entry into the host, *S. aureus* encounters the first line of defence that exposes it to degradation by potent ROS and proteolytic enzymes within phagosomes of polymorphonuclear leukocytes (PMNs) [12] against which it has evolved multi-fold defence strategies. *S. aureus* is well equipped with virulence enzymes that circumvent the host innate human response, to spread and cause infections [13]. It possesses overlapping oxidative stress resistance mechanisms notably, catalase, superoxide dismutase (SOD) and alkylhydroperoxide reductase C (AhpC), amongst others, which confer protection against host defence strategies [14]. Aerobic metabolism paradoxically damages cells as it inadvertently generates ROS including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), hypochlorous acid (HOCL) and others that cause oxidizing damage to DNA, lipids and proteins [5]. Dismutation of O_2^- to H_2O_2 in *S. aureus* is facilitated by two of its major SODs, the homodimeric SodA (encoded by *sodA*) and SodM (encoded by *sodM*) and a heterodimeric SodASodM [15]. SodA is mainly involved in endogenous stress whereas SodM is mainly induced in exogenous stress. Endogenous stress results from aerobic respiration while exogenous stress is due to interactions with the host immune system [16]. In *S. aureus*, H_2O_2 is detoxified to water and O_2 by compensatory roles of catalase, encoded by a single gene *katA*, and AhpC which is encoded by *ahpC* [17,18]. Unscavenged H_2O_2 reacts with Fe (II) via the Fenton reaction to form the highly reactive OH· which directly damages cellular molecules in the cells including DNA, proteins, and lipids [19]. Catalase plays a major role in *S. aureus* towards resistance to high concentrations of H_2O_2 while AhpC works more effectively at low H_2O_2 concentrations and is mainly involved in protection against peroxynitrites and cumene hydroperoxides [9,20]. Both KatA and AhpC are regulated by PerR where reduction in catalase expression increased AhpC activity, presumably as a recovery mechanism to cope with increased oxidative stress induced by H_2O_2 in the absence of catalase [21]. Strains lacking both KatA and AhpC have reduced survival rates probably due to high toxicity of •OH that is generated from H_2O_2 [17]. An MRSA mutant strain lacking in both catalase and AhpC show reduced survival rate in a mouse model of infection although not attenuated [22].

Plants are attested medicinal wonders where their use in traditional medicine and healing date to ancient times. *Andrographis paniculata* (*A. paniculata*) (Burm.f.) Wall. Ex Nees is a medicinal plant with documented pharmacological and curative properties against infections and illnesses [23–26]. Phytochemical compositions in *A. paniculata* differ depending on the geographical location, season and time of harvesting [27]. Samples harvested after 110 d of cultivation contain the highest amount of andrographolide [28], which is the most pharmacologically active component in *A. paniculata* [25,29,30]. Leaves of *A. paniculata* reportedly contain the highest phytochemical content compared to stems, roots and the whole plant also contain phytochemicals with pharmacological activities. Antimicrobial activities are much higher in *A. paniculata* leaves compared to the flowers [31] and to maximize extraction of bioactive compounds, leaves must be dried under shady environment to prevent degradation of components [27]. Inhibitory actions of *A. paniculata* have been demonstrated against several pathogens including *S. aureus* [32–36]. Methanolic extract of *A. paniculata* showed highest antimicrobial activity against *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Escherichia coli* due to high andrographolide and neo-andrographolide contents [25,37] and has been shown to kill drug-resistant Gram-positive bacteria [38]. The ever increasing occurrence and problems encountered in treatment of infections caused by multidrug-resistant bacterial strains such as methicillin-resistant *S. aureus* (MRSA) have spurred research into the discovery of alternative therapeutic agents from natural plant resources. This study looked at probable inhibitory mechanism(s) of *A. paniculata* by investigating its effects on several oxidative stress resistance enzymes in *S. aureus*. Our findings highlight that methanolic extract of *A. paniculata* (Burm.f.) Wall. Ex Nees inhibited SOD activity in *S. aureus* by downregulating expressions of the major genes encoding SODs in *S. aureus* namely *sodA* and *sodM*, which may have contributed to the observed inhibitory effect.

2. Materials and methods

2.1. A. paniculata methanolic extract

A. paniculata was authenticated at the Forest Research Institute Malaysia (FRIM, Reference number: FRIM700-1/7/1 (44)]) and confirmed as *A. paniculata* (Burm.f.) Wall. Ex Nees from the Acanthaceae family. Extraction was performed with modifications [25,39]. Fresh leaves were harvested after 110-d cultivation, washed with distilled water and dried completely in shady environment for two weeks. Dried leaves were macerated in a mechanical blender (Panasonic, Japan) and powdered leaves were soaked in methanol (Sigma–Aldrich, USA) for 72 h for complete extraction [20], filtered (Whatman No. 1, Sigma–Aldrich), reduced to dryness by rotary evaporation (60 °C, auto mode) (EYELA NVC-2100, Japan) and the crude extract was weighed (Shimadzu, Japan). Crude methanolic extract of *A. paniculata* (8 g) obtained from after 3 h was semi solid, dark green in color, and sticky but not oily. Stock extract concentrate was prepared by diluting crude extract in dimethylsulfoxide (DMSO, 10%) to a final concentration of 0.6 g/mL and stored in an airtight container at 4 °C until use.

2.2. Bacterial inoculums

S. aureus was maintained in glycerol stock at −80 °C and species confirmation tests were previously performed which included gram stain, catalase and coagulase tests and were all found positive [40]. Preculture was prepared in brain heart infusion (BHI) broth and grown at 37 °C to $OD_{600\ nm}$ = 0.7 (3.8×10^8 cfu/mL) and cells were harvested at exponential phase by centrifugation ($1520 \times g$), washed with sterile phosphate buffered saline (PBS) and resuspended in sterile Tris–HCl (pH 7.4) [41] and stored at −20 °C until use.

2.3. Minimum inhibitory concentration (MIC)

MIC of methanolic extract of *A. paniculata* against *S. aureus* ATCC 25923 was determined using the broth dilution method [42] in a 96-well microplate. Crude extract was diluted to final concentrations of 0.03, 0.06, 0.12, 0.3, 0.6, 1.2, 3.6, 12, 24, 48, 100, 200 and 250 mg/mL, respectively. Then 100 μL of the *S. aureus*

inoculum was inoculated with equal volumes of BHI broth and plant extract and incubated at 37 °C for 18 h [43]. Control tubes included were; extract control (methanolic extract of *A. paniculata*, BHI broth and 100 μL of *S. aureus*), organism control (sterile PBS, BHI broth and 100 μL of *S. aureus*), positive control (gentamicin (1 U), BHI broth and 100 μL of *S. aureus*) and negative control (uninoculated BHI broth). The lowest concentration of the extract that showed no visible turbidity (growth) after 48-h incubation was taken as the final MIC. The minimum bactericidal concentration (MBC) was determined by subculturing wells with no visible growth onto fresh BHI agar and incubating for 18–24 h at 37 °C [44]. The highest concentration of the extract that showed no growth on the BHI agar plates (99.9% killed) was the MBC value [45]. Purity check was performed which showed Gram-positive cocci in grape-like clusters corresponding to morphology of *S. aureus*.

2.4. Time-kill assay

S. aureus inoculum (10^8 cfu/mL) was mixed with *A. paniculata* methanolic extract at 0.125, 0.25, 0.5, MIC and 2 × MIC respectively. Growth control (10% DMSO, *S. aureus* and BHI broth), positive control [gentamicin (1 U), *S. aureus* and BHI broth], extract control (*A. paniculata* extract, PBS and BHI broth) and organism control (PBS, *S. aureus* and BHI broth) tubes were included in the assay. All tests were performed in triplicate. Tubes were incubated at 37 °C and samplings were performed at 0, 1, 2, 3, 4, 5, 6, 8, 18 and 24 h, respectively. Samples from each tube were diluted twofold in sterile distilled water and plated on BHI agar in triplicate and observed after incubation overnight at 37 °C. Colony forming units were counted and the mean used to determine cfu/mL for each time and extract concentration and percent survival was calculated. Number of cells (cfu/mL) at t_0 was used as the initial concentration of cells in the calculation for percent survival of *S. aureus* cells.

2.5. H_2O_2 sensitivity assay

H_2O_2 sensitivity assay was performed to determine the antioxidative potential of *A. paniculata* extract (0.06 mg/mL) in decreasing the toxic effect of 7.5 mM H_2O_2. *S. aureus* was grown in BHI broth overnight at 37 °C, precultured in fresh BHI broth and grown until exponential phase ($OD_{600\ nm}$ = 0.7), corresponding to (3.8×10^8) cfu/mL. Cells were centrifuged (1 789 × *g*, 5 min, 4 °C), diluted to $OD_{600\ nm}$ = 0.1 in sterile BHI which corresponded to (4.1×10^6) cfu/mL [17]. *S. aureus* inoculum was added into all three tubes, 7.5 mmol/L H_2O_2 in tubes 1 and 2 and extract in tubes 2 and 3 and the final reaction volume in all tubes standardized to 3 mL with sterile PBS. Tubes were kept at room temperature and 50 μL aliquots were sampled at 30, 60 and 90 min intervals and each added directly to 450 μL of sterile PBS containing 10 mg/mL catalase to remove residual H_2O_2. Cells were washed twice in sterile PBS, serially diluted in sterile distilled water and plated in triplicate on BHI agar. Cell counts were determined after overnight incubation at 37 °C.

2.6. *S. aureus* cell cytoplasmic extracts for enzymatic assays

S. aureus cells were grown in BHI broth to exponential phase ($OD_{600\ nm}$ = 0.7) and harvested by centrifugation (1 520 × *g*, 5 min, 4 °C). Pellet was washed twice in sterile PBS and resuspended in Tris–HCl (pH 6.8) [41,46]. Absorbance ($OD_{600\ nm}$) was measured and adjusted to 0.7 (10^8 cfu/mL). *S. aureus* inoculum was added to a tube with *A. paniculata* extract (0.06 mg/mL). For untreated cells, *S. aureus* inoculum was mixed with sterile PBS to a standardized reaction volume of 2 mL. Tubes were incubated for 1 h at 37 °C, centrifuged (1 520 × *g*, 5 min, 4 °C) and washed twice with sterile PBS. Pellets were resuspended in equal volumes of Tris–HCl (pH 6.8), absorbance ($OD_{600\ nm}$) measured, centrifuged (1 520 × *g*, 5 min), resuspended in 5 μL per 0.1 $OD_{600\ nm}$ containing 20 μg of lysostaphin (Sigma–Aldrich, USA) and incubated for 1 h at 37 °C [47]. Cell lysates were centrifuged at 1 750 × *g* at 4 °C and filter sterilized (0.45 μm, Fisher Scientific, USA) into sterile tubes to obtain cell-free lysates for enzyme activity assays.

2.7. Enzyme activity assays

2.7.1. Catalase

Catalase activities were determined by measuring the decrease in H_2O_2 concentration as inferred by change in absorbance at 240 nm. Cell-free lysates obtained from extract-treated and untreated cells were each mixed with phosphate buffer (pH 6.8) which contained 10 mmol/L H_2O_2. Initial absorbance ($OD_{420\ nm}$) of each tube was immediately recorded followed by absorbance readings at 1, 2, 3 and 4 min [48]. The assays included tests for treated and untreated samples, control, and standard. One unit (U) of catalase activity is defined as the amount of enzyme that catalyzed 1 μmol of H_2O_2 per minute at 25 °C. Catalase activity was calculated based on the equation below [48];

$$\text{Catalase activity (kU)} = \frac{2.303}{t} \times \log\frac{S°}{S-M} \times \frac{Vt}{Vs}$$

where, t = time; S° = absorbance of standard; S = absorbance of sample; M = absorbance of control test (correction factor); Vt = total volume of reagents; Vs = volume of sample (lysate).

2.7.2. SOD

Total SOD activity assay was performed according to Das & Bisyashi, 2010 [49]. Cell-free lysates were each mixed with Tris–EDTA–HCl buffer (pH 8.5) and 7.2 mmol/L pyrogallol, incubated at 25 °C for 10 min and reaction terminated by the addition of 1 mmol/L HCl and absorbance ($OD_{420\ nm}$) measured. Treated and untreated samples and control tubes were included in the assay. One unit (U/mg) of SOD activity was defined as the amount of enzyme that inhibited 50% autoxidation of pyrogallol. Total SOD activity was calculated based on the following equations;

$$\% \text{ inhibition of pyrogallol autooxidation} = \frac{\Delta\text{Absorbance(test)}}{\Delta\text{Absorbance(control)}} \times 100\%$$

$$\text{Total SOD activity(U/mg)} = \frac{\% \text{ inhibition of pyrogallol autooxidation}}{50}$$

2.7.3. AhpC (DTT dependent)

AhpC catalyzes the oxidation of dithiothreitol (DTT) in the presence of the peroxide substrate and rate of oxidation was measured at $OD_{310\ nm}$ to monitor the change in absorbance readings [50,51]. We mixed 100 mmol/L KP (pH 7.0), 1 mmol/L EDTA and 10 mmol/L DTT with extract-treated and untreated cell-free lysates in cuvettes respectively and incubated in a temperature controlled circulating water bath at 25 °C [52]. The rate of oxidation of DTT was obtained by calculating the slope over the first 11 s after peroxide was added to the test mixtures and corrected for background DTT oxidation by the peroxidase in absence of enzyme (control) [52].

2.8. Nitroblue tetrazolium (NBT) reduction assay

The NBT reduction assay is an enzymatic assay that determines the level of intracellular and extracellular O_2^- [53–56] by measuring formation of blue-formazan spectrophotometrically at $OD_{575\ nm}$. Reduction of NBT was determined by incubating *S. aureus* cells (10^8 cfu/mL) with methanolic leaves extract of *A. paniculata* (0.06 mg/mL) and NBT (1 mg/mL) at 37 °C for 30 min. For untreated cells, cells were mixed with sterile PBS to standardize the final reaction volumes in all tubes. After the incubation period, 0.1 mole/L HCl was added and the mixture centrifuged ($1\,500 \times g$, 10 min). The supernatant was transferred to a fresh tube and used to measure the extracellular O_2^- levels while the saved pellet was used to measure intracellular O_2^- levels. DMSO was added to the pellet to extract reduced NBT, PBS was added and absorbance was measured at 575 nm [57].

2.9. RT-qPCR analyses

2.9.1. Preparation of cell lysate for cDNA synthesis

Cell lysis was performed using RNA Protect Bacteria Reagent (Qiagen, USA) according to manufacturer's instruction. Total RNA from each sample was isolated using RNeasy mini kit (Qiagen, USA) and cDNA synthesis was performed using Quanti Nova Reverse Transcription Kit (Qiagen, USA) according to manufacturer's instructions.

2.9.2. Quantification of RNA

RNA concentration was measured using a biophotometer (Eppendorf, USA) blanked with RNase-free water which was used to elute RNA sample. A 1:50 dilution of sample was made in diluent, vortexed to avoid concentration gradient and RNA concentration ($A_{260/230}$) was measured. For RNA, the acceptable $A_{260/230}$ value should be between 2.0 and 2.2 [58].

2.9.3. Primers, probes and PCR amplifications

Sequences of targeted genes were accessed from NCBI Gen Bank database. For probe and primers, sequences were each aligned using Sci Tools Real-Time PCR software (Integrated DNA Technologies, USA) to identify unique regions suitable for amplification of the genes of interest (Table 1). Specificity of primers and probes were confirmed using the primer blast and Nucleotide blast tool (NCBI).

2.9.4. Gene expression analyses

Real-time PCR amplifications were performed in 20 μL final reaction mixture containing 2X Quanti Nova Probe PCR Mastermix, respective cDNAs, primers and probes in RNase-free water. Real-time PCR was performed (CFX 76 BIORAD Thermal Cycler, USA) in a 96-well microplate plate according to manufacturer's recommendation for 40 cycles as follows: for each cycle; 2 min at 95 °C initial PCR heat activation, 5 s at 95 °C for denaturation and 30 s at 60 °C for combined annealing/extension process. PCR reactions for each gene expression were performed in triplicate. In the RT-qPCR procedure, several controls were included in the assay to ensure validity of the results obtained. No template control (NTC) is a control without the addition of template and no amplification was observed. If amplifications did occur, then contamination was indicated. The no reverse transcriptase control (NRT-C) was included to detect contamination with genomic DNA (gDNA) and it consisted of the original samples that were not reverse-transcribed. When mRNA was reverse-transcribed into cDNA, residual gDNA may remain in the sample despite addition of buffers to remove gDNA that were incorporated in the protocol (Qiagen, 2011, Quick Start Protocol, USA). In this study, cDNA was used as template; therefore the presence of gDNA would contribute towards erroneous results. Lastly, a negative control was included as an important component to assess validity of RT-qPCR results where all other components were added in the assay except DNA polymerase to detect background signal (Qiagen, 2011, Quick Start Protocol, USA).

Table 1

Sequence of primers and probes for target and housekeeping genes.

Primers and probes	Sequence (5–3)	Target gene
katA-f	GCGTTTGCACCAACTAATATTATTCCA	*katA*
katA-r	GCGCATCGCCATATGAGAATAAAC	
katA probe	CCCTTGCAACATTTTG	
SodA-f	GACAGTGTACCAGCTAACATCC	*SodA*
SodA-r	AGTTTGGTGAAAGTAACTCCCAG	
SodA probe	TAATAATGGCGGTGGAC	
SodM-f	GGATCAGGTTGGACTTGGTTAG	*SodM*
SodM-r	GCATGCTCCCAAACATCAAATAG	
SodM probe	TTGGTTTGGCGTTGTCAC	
ahpC-f	GCCGTGACGCTAGTACATTA	*ahpC*
ahpC-r	TAGCGCCTTCTTCCCATTTAG	
ahpC probe	CGAAGTATGCCCA	
16S rRNA-f	GGTCTGTAACTGATGTG	16S rRNA
16S rRNA-r	GTGGACTACCAGGGTATCTAATCCT	
16S rRNA-probe	5′-/56-FAM/TCCCCACGC/ZEN/TTTCG/3IABkFQ/-3′	
gyrA-f	TCCCTGAATCAACATTACGTCC	*gyrA*
gyrA-r	CCCTACAACTTCGTCACCTTC	
gyrA-probe	FAM/CGTACAGCA/ZEN/ACGGGTGTGAAAGGT 5′-/56	

2.10. Statistical analysis

All experiments were performed in triplicate. Statistical significance between the mean values of triplicate measurements for each test and controls were compared for significance using ANOVA. The *t*-test with confidence interval of 0.05 was used to compare the means of gene expressions and enzyme activities between treated and untreated cells.

3. Results

3.1. MIC against S. aureus

The MIC and MBC values of the methanolic extract of *A. paniculata* against *S. aureus* 25923 were 0.06 mg/mL (60 μg/mL). This MIC was used in all preceding enzyme assays and gene expression analyses in this study.

3.2. Timed-kill assay

Timed-kill assays (Figure 1) were performed *in vitro* to determine the kinetics of killing of *S. aureus* cells after exposure to 7.5–120 μg/mL of methanolic extract of *A. paniculata* for 24 h. No inhibitory effect on cells was observed using 7.5 μg/mL (0.125 MIC) *A. paniculata* extract whereas 39.4% reduction in cell number was seen at 15 μg/mL (0.25 MIC) after 24 h. Extract concentration of 30 μg/mL (0.5 MIC) showed 56.9% reduction of cells whereas treatment of cells with MIC (60 μg/mL) completely killed all cells (100% reduction) in 24 h. A 50% reduction in cell number was observed at MIC concentration after approximately 8 h in

comparison to gentamicin (1 U) which totally killed all *S. aureus* cells in 5 h. The extract concentration at 120 μg/mL (2 MIC) killed all cells in 17.5 h.

3.3. H$_2$O$_2$ sensitivity assay

H$_2$O$_2$ sensitivity assay was performed to determine the antioxidative potential of methanolic leaf extract of *A. paniculata* in decreasing the toxicity effect of H$_2$O$_2$ against *S. aureus*. Results were obtained by calculating the number of viable cells after treatment. The initial cell numbers at T = 0 [(4.1 × 10^6) cfu/mL] was used to determine the percentage of cell survival. Figure 2 shows percent cell survival (%) against challenged period (min). In the first 30 min, 7.5 mmol/L H$_2$O$_2$ showed high toxicity towards *S. aureus*, which killed all cells leading to 0% survival. However, when *A. paniculata* extract (0.06 mg/mL) was added with 7.5 mmol/L H$_2$O$_2$, the percentage of cell survival was increased 25% and complete killing occurred only after 60 min. Of significant interest is that *A. paniculata* extract (0.06 mg/mL) by itself significantly killed *S. aureus* cells with only 43% survival after 30 min exposure and it also completely killed all cells in 60 min.

To determine a probable mode of inhibitory action of *A. paniculata* extract against *S. aureus*, we investigated its effects on activities and expressions of several important enzymes that had contributory roles in conferring oxidative stress resistance in *S. aureus* namely catalase (KatA), SODs (SodA and SodM) and AhpC. RT-qPCR gene expression analyses and enzyme activity assays were performed. Concentrations of recovered RNA samples were; A$_{260}$/A$_{230}$ values of 2.13 and 2.11 corresponding to 163.17 and 101.45 ng/mL for untreated
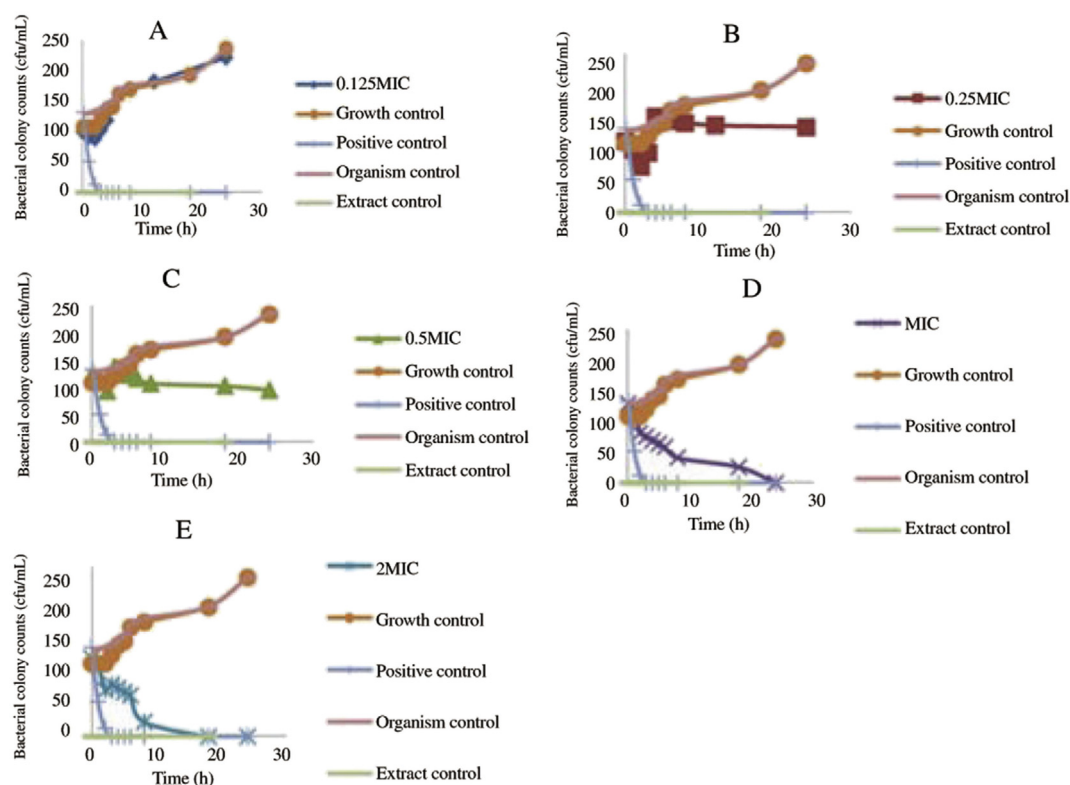

Figure 1. Time-kill curves of *S. aureus* (10^8 cfu/mL) showing colony counts (cfu/mL) after exposure to (A) 7.5 μg/mL (0.125 MIC), (B) 15 μg/mL (0.25 MIC), (C) 30 μg/mL (0.5 MIC), (D) 60 μg/mL (MIC) and (E) 120 μg/mL (2 MIC) methanolic extract of *A. paniculata* for 24 h. Gentamicin (1 U) was included as positive control.

and extract-treated cells, respectively. $A_{260/A280}$ values for both samples were greater than 1.8 at 2.03 and 2.00 respectively.

3.4. Catalase (KatA)

Expressions of *katA* in extract-treated and untreated *S. aureus* cells were analyzed in reference to *gyrA* and 16s rRNA. Based on the results obtained in triplicate, the *katA* gene expression was significantly higher in extract-treated cells at 3.3-fold higher expression compared to untreated cells with 1.0-fold expression ($P < 0.05$) (Table 2). Correspondingly, catalase activity was higher in extract-treated cells which was 9.12 U compared to untreated cells, 6.17 U ($P < 0.05$). These results suggest that methanolic extract of *A. paniculata* leaves increased catalase activity by inducing higher expression of the *katA* gene in *S. aureus*.

3.5. SOD

The effects of methanolic leaves extract of *A. paniculata* (0.06 mg/mL) on the expression of the *sodA* and *sodM* genes in *S. aureus* were investigated using RT-qPCR and are reported as relative gene expression in reference *gyrA* and 16s rRNA. Based on the result obtained in triplicate (Table 2), *sodA* gene expression was significantly higher in untreated cells with 1.0 fold expression but was lowered at 0.8-fold expression in extract-treated cells ($P < 0.05$). Similarly, *sodM* gene expression was lowered at 0.7-fold expression in extract-treated cells showed ($P < 0.05$). Further, *sodM* expression was reduced more than *sodA* ($P < 0.05$) in extract-treated cells. Total SOD (U/mL) activity was correspondingly lowered in extract-treated *S. aureus* cells at 26.8 U compared to untreated cells with 32.4 U activity ($P < 0.05$). These results suggest that methanolic leaves extract of *A. paniculata* reduced total SOD activity presumably resulting accumulation of O_2^- in *S. aureus* cells compared to untreated cells that had higher total SOD activity. Specifically, these results suggest that the extract inhibited expressions of both *sodA* and *sodM* and reduced total SOD activity in *S. aureus*.

Table 2

Relative gene-fold expression of *katA*, *sodA*, *sodM* and *ahpC* transcripts and corresponding enzyme activities (U) of catalase (KatA), SOD and AhpC in *S. aureus* cells treated with *A. paniculata* methanolic extract (0.06 mg/mL).

Gene	Test	Relative normalized expression (gene-fold)	Enzyme	Enzyme activity
katA	Untreated	1.0	Catalase (KatA)	6.17
	Treated	3.3		9.12
sodA	Untreated	1.0	SOD, total	32.4
	Treated	0.8		26.8
sodM	Untreated	1.0	SOD, total	–
	Treated	0.7		–
ahpC	Untreated	1.0	AhpC	0.012
	Treated	61.0		0.018

Significant differences between untreated and extract-treated cells for all genes tested, $P < 0.05$.

3.6. Alkylhydroperoxide reductase C (AhpC)

In contrast, *ahpC* expression was 61-fold higher in extract-treated cells compared to untreated cells with 1-fold expression ($P < 0.05$) (Table 2). Extract-treated cells showed higher AhpC activity at 0.018 U compared to untreated cells at 0.012 U ($P < 0.05$) which suggests that methanolic leaves extract of *A. paniculata* increased both AhpC expression and activity in *S. aureus*. Although *ahpC* expression was drastically increased in extract-treated cells, it was not accompanied by the same level of observed increase in AhpC activity.

3.7. NBT reduction assay

Intracellular and extracellular O_2^- levels in extract-treated *S. aureus* cells were determined using the NBT reduction assay (Figure 3). Extracellular O_2^- levels were determined by measuring the supernatants of cell-free lysates from extract-treated and untreated *S. aureus* cells. Significantly higher
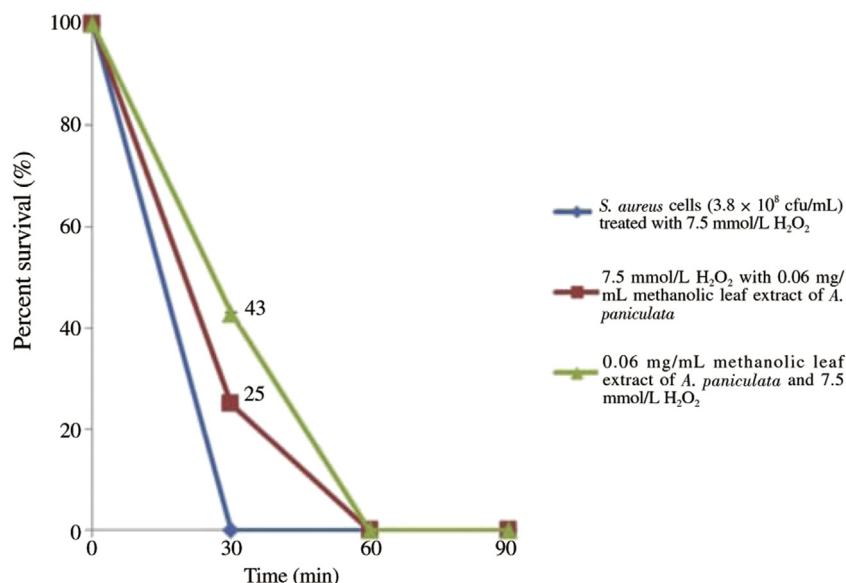

Figure 2. Survival (%) of *S. aureus* cells (3.8×10^8 cfu/mL) treated with 7.5 mmol/L H_2O_2, 7.5 mmol/L H_2O_2 with 0.06 mg/mL methanolic leaf extract of *A. paniculata*, 0.06 mg/mL methanolic leaf extract of *A. paniculata* and 7.5 mmol/L H_2O_2 at 30, 60 and 90 min respectively.
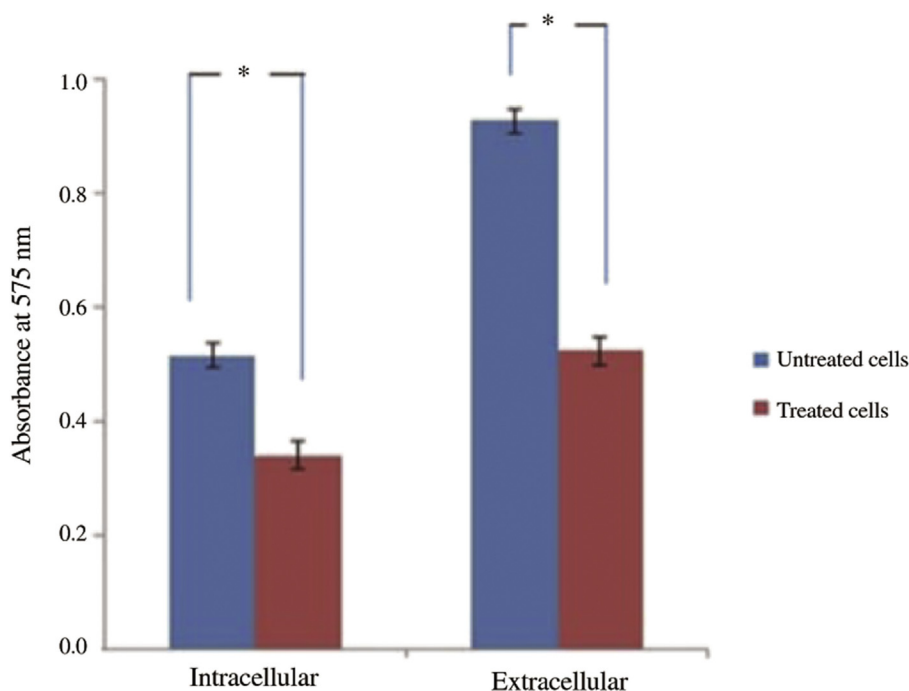
Figure 3. Intracellular and extracellular NBT reductions in *S. aureus* cells treated with methanolic extract of *A. paniculata* (0.06 mg/mL).
[*]$P < 0.05$ compared with untreated cells respectively (ANOVA).

levels of O_2^- were present in untreated cells, $A_{575\ nm} = 0.928$ compared to extract-treated treated cells, $A_{575\ nm} = 0.524$ ($P < 0.05$). Intracellular O_2^- levels were obtained from the pellet after centrifugation, and higher levels of O_2^- was present intracellularly in untreated cells, $A_{575\ nm} = 0.516$ compared to treated cells, $A_{575\ nm} = 0.340$ ($P < 0.05$). These results suggest that *S. aureus* cells treated with methanolic leaves extract of *A. paniculata* (0.06 mg/mL) had significant reduction in extracellular and intracellular O_2^- levels. In untreated cells, higher O_2^- level was seen extracellularly, $A_{575\ nm} = 0.927$ compared to intracellular levels $A_{575\ nm} = 0.516$ ($P < 0.05$). Although O_2^- levels were reduced in extract-treated cells, higher O_2^- levels remained in extracellular, $A_{575\ nm} = 0.524$ compared to intracellular levels, $A_{575\ nm} = 0.340$ ($P < 0.05$). Specifically, the result suggests that methanolic extract of *A. paniculata* leaves reduced O_2^- level in *S. aureus*.

4. Discussion

A. paniculata (Burm.f.) Wall. Ex Nees reportedly affects survival of *S. aureus* [39,59]. Phytochemical analyses that were performed previously on *A. paniculata* extracts that were prepared using various extraction solvents including ethanol, methanol and acetic acid have proven andrographolide as the major bioactive component [34,60–62].

In this study, methanolic leaves extract of *A. paniculata* showed a promising MIC of 0.06 mg/mL (60 µg/mL) against *S. aureus* where total cell killing was attained in 24 h based on the time-kill assay. MIC of an agent in the range between 0.02 and 0.078 mg/mL is considered to have potential for development as an alternative treatment for bacterial infections [63]. *A. paniculata* has been long perceived as safe for use in Traditional Chinese Medicine (TCM), India and Thailand. Previous studies have shown that 60 µg/mL of *A. paniculata* is non-toxic to mammalian cells [64–67] and more recent work on acute and subacute toxicity effects of andrographolide

showed an LD_{50} of greater than 5 g/kg body weight in orally fed mice and no changes in vital signs suggesting its safety for use for medicinal purpose [68]. Further, toxicological assessment of male rats that were treated with standardized extract of *A. paniculata* for 60 d showed no sub chronic testicular toxicity on organ weight, testicular histology, ultrastructural analysis of Leydig cells and testosterone level and with only mild, infrequent and self-limiting adverse effects [62]. Moreover, *A. paniculata* extract offered protection against hepatotoxicity induced by paracetamol [69] and prevented thioacetamide-induced liver cirrhosis in rats [70].

H_2O_2 sensitivity assay was performed to determine the ability of methanolic leaves extract of *A. paniculata* to reduce toxicity of H_2O_2, a potent oxidizing agent capable of damaging cells [5]. We used 7.5 mmol/L H_2O_2 in this study as it was previously shown to be suitable for oxidative stress challenge [71] where it expectedly killed all *S. aureus* cells within 30 min exposure [72,73]. The extract reduced H_2O_2 toxicity against *S. aureus* cells where percentage of cell survival was increased to 25% in the same exposure period, which is in agreement with previous studies that showed its ability to scavenge free radicals of H_2O_2 [36,74] and reaffirms the antioxidant property of *A. paniculata*. More importantly, our observations showed that methanolic leaves extract of *A. paniculata* (0.06 mg/mL) directly killed 57% *S. aureus* cells after 30 min exposure. In attempt to discover the mechanism(s) of this antagonistic property, this study investigated the effects of the extract on major oxidative stress resistance enzymes. Engulfment of *S. aureus* by PMNs is accompanied by increased oxygen consumption (oxidative burst) followed by release of significant amounts of ROS such as O_2^- and degradative enzymes, which damage cells [75]. Increased O_2^- is dismutated to H_2O_2 [76]. Unscavenged H_2O_2 is further reduced to $OH\cdot$ via the Fenton reaction or converted to $HOCl$ [77]. In *S. aureus*, SOD, catalase (KatA) and alkylhydroperoxide reductase C (AhpC) are the major detoxifying enzymes that

convert ROS into less harmful products which enable phagocytosed bacteria to withstand and survive killing by PMNs [16]. Therefore, these enzymes were selected for investigations in this work as they are major enzymes that overcome initial oxidative assault by PMNs on the organism that determines its survival and pathogenicity within the host. SOD is an important oxidative stress resistance enzyme in *S. aureus*, which reduces O_2^- levels in cells by dismutation to H_2O_2 and lessens damaging effects to cells [78]. Absence of SOD in cells increases susceptibility to oxidative damage leading to cell death [79]. In this study, expressions of both *sodA* and *sodM* were lowered in extract-treated cells with reduced total SOD activity. These results suggest that the extract repressed SOD activity resulting in lower dismutation of O_2^- to H_2O_2 presumably resulting in increased O_2^- levels in treated cells. Isoproterenol induced myocardial infarction rats treated with *A. paniculata* methanolic extract showed decreased SOD activity, which corroborates with findings in this study [80]. Further, *sodM* expression was downregulated more in extract-treated *S. aureus* cells compared to *sodA*. Plausible reasons for these observations is that *A. paniculata* methanolic extract, at the concentration tested (60 µg/mL), either directly repressed transcription of *sodA* and *sodM* genes or affected their transcriptional regulatory proteins, SarA and/or SarR [81]. SarA is a protein that negatively regulates *sodA* and *sodM* expression directly as it binds specifically to promoter regions of these genes in modulating stress response, therefore, increased SarA curbs transcriptions and downregulates expressions of both *sodA* and *sodM* in *S. aureus* [82]. SarR is also a regulatory protein, which represses *sarA* expression, therefore it indirectly regulates *sodA* and *sodM* expression via SarA [82]. Increased expression of SarR protein reduces expression of SarA, thereby relieving its repressive effect on expressions of *sodA* and *sodM*, which are expected to increase and vice versa. To prove these assumptions, further investigations on the effect of methanolic extract of *A. paniculata* or its bioactive component on the expressions and activities of *sarA* and *sarR* and consequently, the effects on *sodA* and *sodM* expressions and activities are currently being investigated to enable more definitive conclusions.

Investigations were performed to differentiate the effects of the extract between extracellular and intracellular O_2^- levels using the nitroblue tetrazolium reduction (NBT) assay. Untreated cells had significantly higher O_2^- levels in both the extracellular, 0.928 U and intracellular compartments, 0.516 U compared to treated cells with lowered O_2^- levels of 0.524 U, ($P < 0.05$) and (0.340 U) ($P < 0.05$) respectively. These results are unexpected in reference to the gene expression data, where *S. aureus* cells that were treated with the extract had reduced total SOD activity and therefore, are expected to have higher accumulation of O_2^- compared to untreated cells. Since expression analyses were performed using RT-qPCR, which is a more accurate and sensitive method, therefore we are inclined to accept the data. It may be possible that the NBT assay, in measuring O_2^- levels, reflected the antioxidant activity of the extract that directly scavenged and reduced O_2^- thereby reducing its detectable levels in treated cells [27,83–85]. This suggests that the extract may have affected other regulatory mechanisms in *S. aureus* that affected O^{2-} levels that were not identified in this study and therefore warrants further investigations. The NBT reduction assay data partly corresponded with the *sodA* and *sodM* gene expression data since *sodM* expression was induced higher than *sodA*

presumably due to external induction by the extract. In *S. aureus*, *sodA* is usually responsible for the major SOD activity, however, under oxidative stress, *sodM* becomes the major source of activity [79]. Nevertheless, both *sodA* and *sodM* play important roles because lack of either one reduced virulence of *S. aureus* [82]. Overall, cells treated with methanolic extract of *A. paniculata* showed decreased O_2^- levels which was unexpected as downregulation in *sodA* and *sodM* expressions were expected to cause accumulation of O_2^- resulting in increased levels.

Catalase and alkylhydroperoxide reductase C are involved in detoxification of H_2O_2, an oxidant that damage cells, to H_2O and O_2. In *S. aureus*, catalase is encoded by a single gene, *katA* which converts the potent oxidant H_2O_2 to water and oxygen [86]. In this study, *katA* was expressed three-fold higher in extract-treated *S. aureus* cells with enhanced catalase activity suggesting that methanolic leaves extract of *A. paniculata* increased catalase activity by upregulating *katA* expression in *S. aureus*. These findings are corroborated with a previous study where andrographolide, the major component in *A. paniculata*, significantly increased both catalase and SOD activities in treated mice [87]. Furthermore, oral administration of *A. paniculata* increased catalase activity in erythrocytes [29] and treatment with *A. paniculata* on liver cells of lymphoma bearing mice showed elevated of catalase activities [88]. Alkylhydroperoxide reductase C (AhpC) compensates for the action of catalase but is more effective against low concentrations of H_2O_2, peroxynites and organic hydroperoxides [17]. In *Escherichia coli*, AhpC had higher affinity towards H_2O_2 compared to catalase and is considered as the main scavenger of endogenous H_2O_2 that is generated in the cells [89]. In *S. aureus* however, catalase is the major enzyme that is responsible for catalysis of H_2O_2 and AhpC provides residual and compensatory function of this important role [17]. In this study, the *ahpC* gene was expressed 61-fold higher in cells treated with the extract compared to untreated cells (1.0 fold expression) ($P < 0.05$) with corresponding 0.018 U increase in AhpC activity in treated cells compared to 0.012 U in untreated cells ($P < 0.05$). These results suggest that methanolic extract of *A. paniculata* (0.06 mg/mL) increased both *ahpC* expression and activity in *S. aureus* cells. The *ahpC* gene was expressed significantly higher respective to its enzyme activity in extract-treated cells possibly due to post-translational modifications or protein degradation [90]. Further, the major inducing agents in the extract, which are andrographolide and neo-andrographolide [91] are structurally organic which correlates with the protective nature of AhpC against organic hydroxides and peroxynites [9].

According to Vogel & Marcotte (2013), mRNAs are used only as proxies to determine protein levels as they are not completely translated into proteins [90]. Therefore in performing these kinds of studies, it is crucial to validate results by testing mRNA expression in conjunction with protein activity as was performed in this study [92]. In addition, the profound high increase in *ahpC* expression in response to treatment of cells with the extract may possibly be explained by the propensity of AhpC to confer protection against peroxynitrites and organic hydroperoxides [17]. Since the documented main component in the methanolic *A. paniculata* extract used in this study is a labdene diterpenoid, andrographolide [25], its organic structural composition may likely have contributed towards the high increase in *ahpC* expression. In this study, as

expressions and activities of both catalase and AhpC are increased in *S. aureus* cells that were treated with the extract, a reduction in H_2O_2 concentration is implied [93].

Taken together, our observations suggest that *A. paniculata* extract probably does not exert an antagonistic effect towards *S. aureus* by increasing H_2O_2 as increased catalase and alkyl-hydroperoxide reductase C activities indicate less H_2O_2 accumulation, which is protective to the cells [94–96]. If anything at all our observations point to a more protective effect since higher activities of catalase and AhpC reduce H_2O_2 concentrations [71]. We show in this study that methanolic extract of *A. paniculata* reduced total SOD activity by reducing expressions of the major SODs, which we postulate to be a probable mechanism of its antagonistic effect against *S. aureus*. Observations in this study provide avenues for further investigations especially in mechanisms involving oxidative stress resistance in *S. aureus*, which are essential for its survival and pathogenicity [2].

In conclusion, in this study *A. paniculata* methanolic extract displayed promisingly low MIC of 0.06 mg/mL against *S. aureus* ATCC 25923. The extract significantly inhibited expressions of both *sodA* and *sodM* genes encoding SODs resulting in overall reduction of SOD activity which suggest a possible role in the observed inhibitory effect of *A. paniculata* against *S. aureus*. In contrast, this study showed that *A. paniculata* extract increased activities of catalase and alkyl-hydroperoxide reductase C which are enzymes that are involved in H_2O_2 detoxification and therefore does not imply their roles in the observed killing of *S. aureus* by *A. paniculata*.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgement

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