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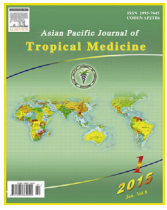

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Mechanism of antagonistic effects of *Andrographis paniculata* methanolic extract against *Staphylococcus aureus*

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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 × *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*

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