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• Research Article

Killing of *Staphylococcus aureus* by allylpyrocatechol is potentiated by induction of intracellular oxidative stress and inhibition of catalase activity

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ABSTRACT

OBJECTIVE: This study investigated the effects of allylpyrocatechol (APC), the major component in ethanolic extract of *Piper betle*, on key oxidative stress resistance enzymes important for the survival of *Staphylococcus aureus*, a major pathogen in the human host.

METHODS: Effects of APC on expressions of genes encoding catalase (*katA*), superoxide dismutases (SODs), including *sodA* and *sodM*, and alkyl hydroperoxide reductase (*ahpC*) in *S. aureus* were quantitated by RT-qPCR in reference to *gyrA* and *16S rRNA*. Corresponding activities of the enzymes were also investigated. The Livak analysis was performed for verification of gene-fold expression data. Effects of APC on intracellular and extracellular reactive oxygen species (ROS) levels were determined using the nitroblue tetrazolium (NBT) reduction assay.

RESULTS: APC-treated *S. aureus* cells had higher *sodA* and *sodM* transcripts at 1.5-fold and 0.7-fold expressions respectively with corresponding increase in total SOD activity of 12.24 U/mL compared to untreated cells, 10.85 U/mL (P<0.05). Expression of *ahpC* was highest in APC-treated cells with 5.5-fold increased expression compared to untreated cells (P<0.05). Correspondingly, *ahpC* activity was higher in APC-treated cells at 0.672 (A_{310nm}) compared to untreated cells which was 0.394 (A_{310nm}). In contrast, *katA* expression was 1.48-fold and 0.33-fold lower respectively relative to *gyrA* and *16S rRNA*. Further, APC-treated cells showed decreased catalase activity of 1.8×10^{-4} (U/L or μmol/(min·L)) compared to untreated cells, which was 4.8×10^{-4} U/L (P<0.05). Absorbance readings (A_{575nm}) for the NBT reduction assay were 0.709 and 0.695 respectively for untreated and treated cells, which indicated the presence of ROS. APC-treated *S. aureus* cells had lower ROS levels both extracellularly and intracellularly, but larger amounts remained intracellularly compared to extracellular levels with absorbances of 0.457 and 0.137 respectively (P<0.05).

CONCLUSION: APC induced expressions of both *sodA* and *sodM*, resulting in increased total SOD activity in *S. aureus*. Higher *sodA* expression indicated stress induced intracellularly involving O_2^-, presumably leading to higher intracellular pools of H_2O_2. A concommittant decrease in *katA* expression and catalase activity possibly induced *ahpC* expression, which was increased the highest in APC-treated cells. Our findings suggest that in the absence of catalase, cells are propelled to seek an alternate

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pathway involving *ahpC* to reduce stress invoked by O_2^- and H_2O_2. Although APC reduced levels of ROS, significant amounts eluded its antioxidative action and remained intracellularly, which adds to oxidative stress in treated cells.

Keywords: allylpyrocatechol; catalase; superoxide dismutase; alkyl hydroperoxide reductase; reactive oxygen species; *Staphylococcus aureus*; *Piper betle*

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1 Introduction

Staphylococcus aureus causes a variety of diseases from mild skin infections to life-threatening pneumonias and septicaemias[1,2]. The host naturally presents a stressful, even hostile environment to which this pathogen has developed and demonstrates a high level of resistance. Within the host, it encounters significant oxidative and nitrosative stresses to which it has evolved many defense mechanisms[3]. Environmental stress significantly impacts bacteria, which alter cell physiology and gene expression patterns in ways that can and do influence antimicrobial susceptibilities[4]. Of significance is oxidative stress caused by O_2^- and H_2O_2, where failure to respond ultimately results in cell damage, potentially leading to cell death. *S. aureus* resolves this by concerted enzyme actions including catalase (*katA*), alkyl hydroperoxide reductase (*ahpCF*), and thioredoxin reductase (*trxB*) which are members of the PerR regulon[5]. In addition, superoxide dismutases (SODs) including *sodA* and *sodM*, which converts superoxide to H_2O_2 and O_2^-, play important roles in modulating oxidative stress resistance[6]. *S. aureus* possesses three SODs, two homodimeric forms SodA and SodM and a heterodimer SodA-SodM (encoded by *sodA* and *sodM* respectively) which are Mn-dependent enzymes[7].

The AhpR (AhpCF) system, together with catalase, constitutes a two-enzyme H_2O_2 scavenging system[8]. Together with AhpF, AhpC which catalyzes an nicotinamide adenine dinucleotide (NADH)-dependent reduction of alkyl hydroperoxides, has wide-spectrum activity against H_2O_2, organic peroxides and peroxinitrite, and also protects bacterial cells against reactive nitrogen intermediates[9]. AhpC provides residual catalase activity in a *katA S. aureus* mutant indicating its compensatory role in peroxide stress resistance; both AhpC and KatA are required for nasal colonization and resistance of *S. aureus* to dessication[10].

Various plants and plant-derived natural products, including phenolic compounds, have been shown to exert antimicrobial effects towards major pathogenic microorganisms including *S. aureus*[11,12]. *Piper betle* Linn. belongs to the family of Piperaceae which is widely found in Southeast Asia[13] and its leaves are widely used in traditional medicine for relief of various ailments. *P. betle* leaf extract has been shown to possess antimicrobial activity against several clinical isolates including *S. aureus* and *Escherichia coli*[14,15]. Crude ethanolic extract of *P. betle* shows antimicrobial properties against oral bacteria such as *Fusobacterium nucleatum* and *Streptococcus mutans*[16,17] and is suggested for use in the prevention of halitosis[18]. Allylpyrocatechol (APC) is a major phenolic constituent in ethanolic extract of *P. betle* which possesses antimicrobial and antioxidant activities. It has been previously shown to exert antioxidant activity against indomethacin-induced stomach ulceration in a rat model infection[19] and showed anti-inflammatory effect *via* inhibition of nuclear factor-kappaB (NF-κB) pathway in the lipopolysaccaride-induced murine macrophage cell line[20]. APC is the active compound of *P. betle* deemed responsible for protection against damage caused by photosensitization[21].

This study provides insight on the ability of APC to regulate gene expressions and activities of sentinel oxidative stress enzymes, namely, catalase, SOD and AhpC in *S. aureus*. Of significance, we showed APC exerting considerable oxidative stress internally within cells; this, coupled with reduction of catalase activity, most likely contributed to its antagonistic effect against *S. aureus*.

2 Materials and methods

2.1 Bacterial strain and materials

S. aureus (ATCC 25923) used in this study was stored in preservative beads at −80 ℃ until required. Strain confirmation tests including catalase and coagulase were previously performed and found positive. APC was obtained commercially from Sigma Aldrich (USA).

2.2 Inoculum preparation

S. aureus was cultured from frozen stock onto brain heart infusion (BHI) agar (Merck, Germany), grown overnight at 37 ℃ , and inoculated into fresh BHI broth overnight. One milliliter of overnight cell suspension was inoculated into fresh prewarmed BHI broth and grown to mid-exponential phase at 37 ℃ with agitation (100 r/min) for 3 h. The preculture was used to inoculate 250 mL