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EFFECT OF APC ON KILLING OF STAPHYLOCOCCUS AUREUS BY OXIDATIVE STRESS AGENTS

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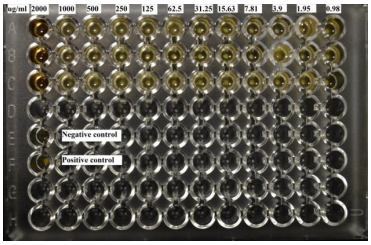
Graphical abstract



Abstract

Allylpyrocatechol (APC) which consists of benzene ring with hydroxyl groups, is a major phenolic constituent in *Piper betle* L. leaves extract. Antimicrobial activity of APC against *S. aureus* (ATCC 25923) was tested by determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) using the broth microdilution method. In addition, oxidative stress resistance assay was performed to determine the antioxidant activity of APC against hydrogen peroxide (10 mM), diamide(10 mM) and methyl viologen (10 mM) in relation to its toxicity against *S. aureus*. MIC and MBC values of APC against *S. aureus* were both 2000 µg/ml. Exposure of *S. aureus* cells to hydrogen peroxide, diamide and methyl viologen for 1 hour caused reduction of cells with percentage of survival of 87%, 15% and 10% respectively. Treatment of *S. aureus* cells with APC was found to increase killing compared to treatment with oxidative stress agent alone. Further research is recommended to investigate the effect of oxidative stress induced by hydrogen peroxide, diamide and methyl viologen on the production of reactive oxygen species (ROS) and antioxidant enzyme activities in *S. aureus* to elucidate the mechanism involved. This study potentially leads to discover and develop new antibiotic agents against all types of MRSA and in particular, novel phytochemicals of antimicrobial potential from natural products.

Keywords: Allylpyrocatechol, oxidative stress

1.0 INTRODUCTION

Recently, the focus on discovery of therapeutic agents has shifted towards natural products. Various plants and their bioactive compounds have been shown to have antimicrobial effects against major pathogenic microorganism including *Staphylococcus aureus* [1] .

S. aureus is a gram positive bacteria. Although antimicrobial use has greatly contributed to lowering mortality and morbidity associated with infectious disease, many organisms have developed resistance. Staphylococci which are included in the microbiota of the skin's normal flora, are especially prone to developing resistance [2]. Data from our preliminary studies suggested that ethanolic extract of *Piper betle* L. has high antimicrobial activity against *S. aureus* [3]. Our studies also reported a positive correlation between antimicrobial activities of plant and their antioxidant effects, suggesting the potential action of antioxidants in inhibiting bacterial growth. Phenolic compounds such as allylpyrocatechol have been shown to possess both antimicrobial and antioxidant activities [4], [5].

Stress is one of significant environmental impact on bacteria, which alters cell physiology and gene expression patterns in ways that can and do influence antimicrobial susceptibility [6]. Stress may elicit a

variety of adaptive mechanism that not only protect bacteria from the offending stress, but also manifest changes in the cellular mechanism that impact innate antimicrobial susceptibility.

Thus exposure of organism to reactive oxygen or nitrogen species (oxidative stress), nutrient or heat stress for example, will have implications on bacterial susceptibility to a variety of antimicrobials through their initiation of stress responses. Hence, it may positively impact of antimicrobial resistance determinants or stimulate physiological changes that compromise antimicrobial activity.

The limited number of antimicrobial classes and common occurrence of antimicrobial resistance have also reinforced the urgent need to discover new compounds targeting novel cellular mechanisms not yet targeted by currently used antibiotics [7]. Plant-derived natural products, including phenolic compounds have shown anti-staphylococcal activities [8], [9]. Allylpyrocatechol (APC) is one of the major phenolic constituents of *Piper betle* Linn. leaves which possesses a wide range of pharmacological properties including antioxidant, antimicrobial and anti-inflammatory activities [4], [11], [12] .It consists of two hydroxyl groups as a component of its chemical structure which confers overall polarity to the compound (Figure 1). However, the mechanism of action of allylpyrocatechol on *S. aureus* is still not fully understood.

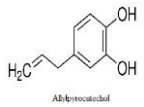


Figure 1 Chemical structure of APC

Hydrogen peroxide is widely used as a biocide and is specifically important in applications where it is decomposed into non-toxic by-product [13]. Methyl viologen or commonly known as paraquat, is a nonselective herbicide, whereas diamide is generally used as a chemical probe to study the effect of changes in thiols oxidation [14], [15]. These chemicals have been found to induce oxidative stress by generation of oxidants and cause toxicity to aerobic cells [16]. This study will provide insight on the effectiveness of allylpyrocatechol (APC) to detoxify the damaging effect of these oxidative stress agents against *S. aureus*.

2.0 EXPERIMENTAL

2.1 Bacterial Strain and Materials

The *S. aureus* strain used in this study was a methicillin-sensitive reference strain, ATCC 25923. It was stored in preservative beads at -80°C until required. Allylpyrocatechol was obtained commercially from Sigma Aldrich (USA). Catalase from bovine liver, methyl viologen, diamide and hydrogen peroxide were also purchased from Sigma Aldrich (USA).Brain heart infusion (BHI) agar and broth from Merck (Germany).

2.2 Inoculum Preparation

S. aureus from stock culture was grown on blood agar at 37°C for 18 to 24 hours. A single colony of growth was inoculated into 5 mL of BHI broth and incubated for 18 hours at 37°C. After 18 hours incubation, pre-culture is made by transferring 1 ml of bacterial suspension into 10 ml BHI broth. The broth is incubated to exponential phase for 2 hours at 37°C with agitation and then used to inoculate 250 ml BHI broth to a starting OD_{600} of 0.05 and incubated for 2 hours again to a final OD_{600} of 0.5 containing 1 x 10^8 cfu/ml at exponential phase.

2.3 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC of the APC against S. *aureus* was tested by the broth microdilution method as recommended by Clinical Laboratory Standardization Institute (CLSI) guideline [17]. Two fold serial dilutions of APC were obtained in sterile BHI broth in amounts of 100 µl per well in a 96 well microtiter plate. 100 µL aliquots of inoculum suspension (1 x 10^8 cfu/ml) was added into each well resulting in a final inoculums concentration of 5 x 10^5 cfu/ml per well. Final concentrations of APC range from 0.98 to 2000 µg/mL. The microtiter plate was incubated aerobically at 37°C for 24 hours. All experiments were carried out in triplicate. The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) was regarded as MIC. The MBC was determined by subculturing the test dilution in microtiter plate on to a fresh drug-free solid medium (Blood agar) and incubated further for 18 to 24 hours. The highest dilution that yielded no single bacterial colony on a solid medium was taken as MBC.

2.4 Hydrogen Peroxide Resistance Assay

1 ml of the exponential phase *S. aureus* culture was washed twice in PBS, diluted and transferred onto BHI agar in triplicate for colony counts to determine the percentage of survival at time of 0. For the test sample, 1 ml aliquot from the same culture was diluted in PBS (1 x 10^8 cfu/ml) was mixed with 1 ml of APC (2 mg/mL) and 1 ml of 10 mM H_2O_2. Two controls were set up as follows; tube containing 2 ml of diluted culture (1 x 10^8 cfu/ml) and 1 ml of APC (2 mg/ml), and another control tube containing 2 ml of diluted culture (1 x 10^8 cfu/ml) and 1 ml of H_2O_2. All tubes were mixed thoroughly and incubated at 37°C. 50 µL aliquots from each tube were sampled at 1 hour intervals for 3 hours and immediately added to 450 µL PBS containing 10 mg/ml catalase. Serial dilutions were performed and 25 µl from each sampling was

plated in triplicate onto BHI agar. The plates were incubated at 37°C for 24 hours. The amount of killing was determined by colony counts of surviving bacteria on BHI agar plates and the percentage of cell survival at each time point was plotted.

2.5 Diamide and Methyl Viologen Resistance Assay

S. aureus cells treated with APC were challenged separately with methyl viologen (paraquat) (10 mM) and diamide (10 mM). Cells were prepared in three different tubes, test sample containing cells (1 x 10^8 cfu/ml) with 1 ml 10mM diamide or methyl viologen and 1 ml of APC (2 mg/ml). Control tube consisted of cells (1 x 10^8 cfu/ml) with 1 ml 10mM diamide or methyl viologen. Another control tube consisted of cells (1 x 10^8 cfu/ml) and 1ml APC (2 mg/ml). All tubes were incubated at 37°C and 50 µL aliquots were sampled at 1, 2 and 3 hours. Viable bacteria were determined in triplicate by serial dilution and plating the samples onto BHI agar. The experiment was recorded in a representative dataset

3.0 RESULTS AND DISCUSSION

3.1 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal concentration

MIC is defined as the first well in a serial dilution test that shows no visible bacterial growth. Results in Table 1 and Figure 2 show clear well indicating no growth in well containing APC at 2000 µg/ml. Other wells were turbid indicating growth. From the data, MIC of APC against *S. aureus* (ATCC 25923) was determined to be 2000 µg/ml. MBC of APC was determined to be 2000 µg/ml (Figure 3). The MIC value was used in all proceeding assays in this study.

Table 1 MIC of APC (0.98 - 2000 µg/ml) against *S. aureus* (ATCC 25923)

Concentration (µg/ml)	Result
0.98	Turbid
1.95	Turbid
3.9	Turbid
7.81	Turbid
15.63	Turbid
31.25	Turbid
62.5	Turbid
125	Turbid
250	Turbid
500	Turbid
1000	Turbid
2000	Clear

Concentration of extract µg/ml)

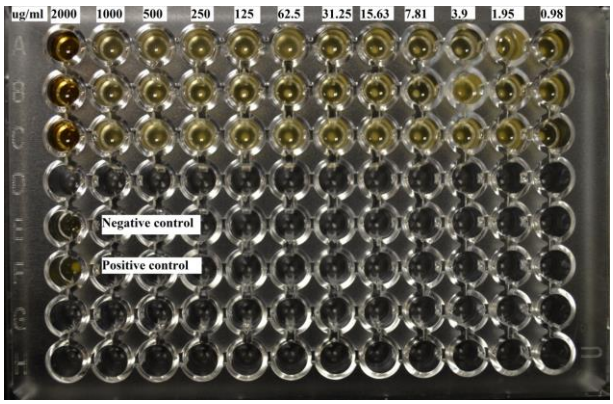


Figure 2 Microtiter plate containing mixture of various concentration of APC (0.98 - 2000 µg/ml) with inoculum suspension for MIC assay in triplicate after 24 hours incubation at 37°C. Negative control, BHI broth and positive control, inoculum suspension

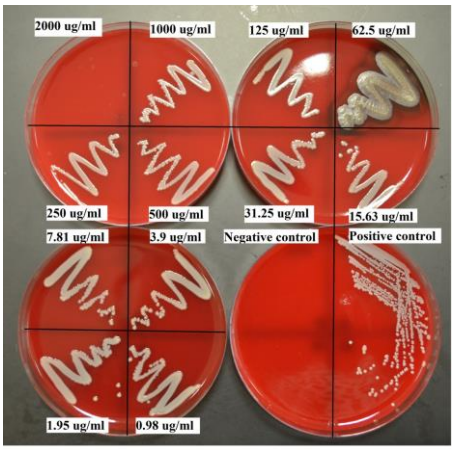


Figure 3 Subculture of various concentration of APC (0.98 µg/ml - 2000 µg/ml) with inoculum suspension from MIC microtiter well. Negative control, BHI broth and positive control, inoculum suspension. The plate was observed visually after 24 hours incubation at 37°C

Purity checks on the *S.aureus* culture was performed to rule out contamination that possibly occurred during the test. A pure growth of bacteria with the typical characteristics of *S.aureus* colonies was observed in all purity checks, i.e. purity check of inoculum prior to MIC assay. Purity check of BHI broth in diluting APC shows no growth. This indicated that no contamination occurred before; during and after the MIC assay was performed. The purity check is crucial to prevent false results reported from mixed cultures of organism.

The structure of APC is simple, less complex and small compounds. In addition, APC was originated from natural product. These could provide more benefits over the current antibiotics used in clinical because it might have a prolonged time for bacteria to mutate and become resistant, especially in extract. Active compounds could have fewer side effects compared to the synthesized antibiotics as it

was produced from plant materials. This will be useful for its development as new antibiotic for staphylococcal infections.

3.2 Hydrogen Peroxide Sensitivity Assay

Hydrogen peroxide sensitivity assay was performed to determine the ability of antioxidant properties of APC in reducing the toxic effects of H_2O_2 to *S. aureus*. The viability of *S. aureus* cells was determined upon challenge with 10 mM H_2O_2, 10 mM H_2O_2 with 2 mg/ml APC, and 2 mg/ml APC in separate test tubes.

As shown in Figure 4, percentage of cells that survived intreatment with H_2O_2, H_2O_2 with APC and APC only after 1 hour were 87%, 2.4% and 8.9% respectively. Addition of APC increased killing of *S.aureus* by H_2O_2 with only 2.4% cell that survived. All cells were killed after 2 and 3 hours of treatment with APC and hydrogen peroxide.

Hydrogen peroxide is a source of ROS including singlet oxygen, superoxide radicals (O_2^-) and hydroxyl radicals (.OH) that are highly reactive and very toxic for microorganisms [18]. The exact mechanism of H_2O_2 in producing lethal products for many microorganisms has not been completely elucidated. However, due to its ability to produce the above mentioned ROS with strong oxidative properties that can cause damaging effects to nucleic acids, enzymes and membrane constituents [19].

Treatment of cells with APC alone is significantly different from H_2O_2 killing ($p<0.05$). Results have shown that cells treated with APC displayed an impaired ability to replicate after 1 hour of exposure. Thus, APC is more effective to inhibit *S.aureus* in comparison with H_2O_2 with increase in 78.1% killing.

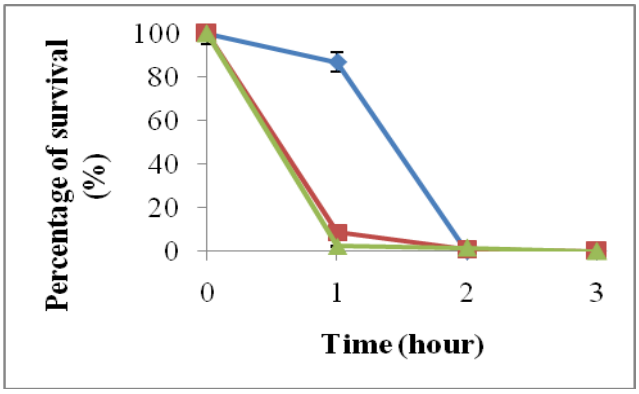


Figure 4 Hydrogen peroxide (H_2O_2) sensitivity assay against 1x10^8 cells/ml of *S.aureus* challenged with 10 mM H_2O_2 (◆) , 10 mM H_2O_2 + 2 mg/ml APC (▲) and 2 mg/ml APC (■) at 1, 2 and 3 hours respectively.

3.3 Diamide Sensitivity Assay

The effect of diamide on the *S. aureus* cells survival was determined in the presence of APC (2 mg/ml) (Figure 5). 15% of cells were survived after 1 hour treatment with diamidef, whereas 5% and 10% of cells survival after 1 hour treatment with diamide with APC and APC only, respectively. Similar pattern of percentage killings were found in all treatments after two hours. After 3 hours treatment, diamide with APC showed total inhibition to *S. aureus* with no cell survival. Diamide and APC by themselves had only 5% survival of *S. aureus* after 3 hours of treatment.

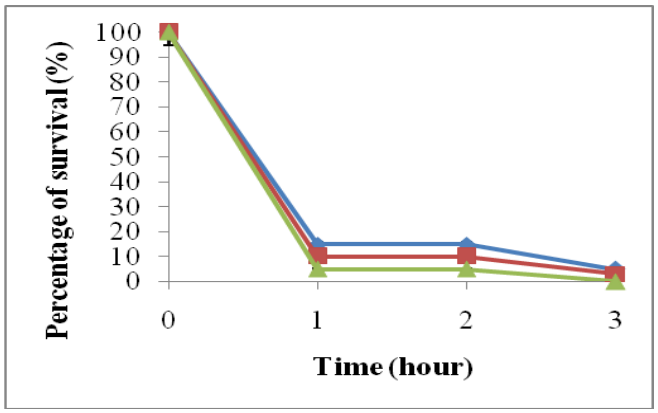


Figure 5 Diamide sensitivity assay against 1 x10^8 ells/ml of *S.aureus* challenged with 10 mM diamide (◆) , 10 mM diamide + 2 mg/ml APC (▲) and 2 mg/ml APC (■) at 1, 2 and 3 hours respectively.

Cells treated with APC showed significant difference in the percentage of survival ($p<0.05$) in comparison to diamide killing. Diamide commonly oxidizes small thiols and rapidly decreases the intracellular gluthathione pool [15]. It penetrates the cell and causes oxidative stress by oxidizing intracellular thiols [20]. This may account for the distinctive pattern of percentage of bacteria survival that led to diamide sensitivity. The strain was more sensitive to the treatment of diamide together with APC. There is a reversible inhibition of cell growth when *E. coli* is exposed to diamide at concentration of 0.3 and 0.6 mM. The bacteriostatic effect of diamide may be due to the oxidation of glutathione to glutathione disulfide and the formation of mixed disulfides of glutathione with proteins [21].

3.4 Methyl Viologen Sensitivity Assay

The toxicity of methyl viologen (paraquat) to *S. aureus* cells was also determined in the presence of APC (2 mg/ml). Exposure of *S. aureus* cells to methyl viologen resulted in 10% of cell survival, which was reduced to 8% and 6% after 2 and 3 hours treatment, respectively. APC by itself had lower percentage (8%) of cells survival in comparison to methyl viologen (10%) after 1 hour of treatment meanind of APC was more effective in killing of the bacteria. The reduction of percentage to 6% and 4% after 2 and 3 hours respectively was observed in the treatment with APC. However, the treatment of methyl viologen with APC has reduced the number of cells survived to 7%. At 2 hour of treatment, 5% of cells were survived and it was

totally inhibited after 3 hours of treatment. The difference effect of APC and methyl viologen in killing *S. aureus* is significant ($p<0.05$).

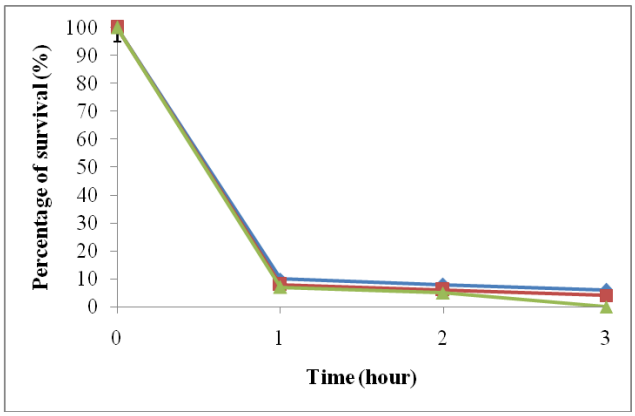


Figure 6 Methyl viologen sensitivity assay against $1x10^8$ cells/ml of *S.aureus* challenged with 10 mM methyl viologen (■) , 10 mM methyl viologen + 2 mg/ml APC (▲) and 2 mg/ml APC (◆) at 1, 2 and 3 hours respectively

Treatment of *S. aureus* cells with methyl viologen and APC produced a progressive decrease in the number of cells that survived after 1 hour. Mechanism of methyl viologen toxicity can be explained through radical formation and lipid peroxidation via microsomal NADPH- cytochrome c reductase. Methyl viologen undergoes a single electron reduction with NADPH as a source of electrons. Upon aerobic oxidation of reduced methyl viologen by molecular oxygen, superoxide radicals are formed which may non-enzymatically dismutate to singlet oxygen[22]. Other study have demonstrated that the production of the superoxide anion involved in methyl viologen toxicity, however bacteria containing elevated SOD levels that can detoxify the superoxide anion, were resistant to methyl viologen toxicity [23]. High concentration of this agent is required to inhibit bacteria of human origin. Gram positive organisms have been shown to be more resistant to methyl viologen [24].

Bacterial cells have acquired the suitable protective mechanisms in order to maintain the lowest possible levels of ROS inside the cell. Bacteria occupy enzymatic mechanisms to eliminate the damaging effects of oxidative stress, such as catalase, superoxide dismutase [19], [25] and alkylhydroperoxide reductase [26]. In the present study, the effect of oxidative stress induced by hydrogen peroxide, diamide and methyl viologen on killing of *S. aureus* cells was enhanced in the presence of APC. APC is the major phenolic constituent that is responsible for antioxidant properties in *P. betle* L. leaf extract [5], [27]. APC displayed more effective killing on *S. aureus* in comparison to the oxidants, similarly, previous findings showed that an ethanolic *P. betle* leaf extract demonstrated significant direct killing ability of *S. aureus* [3]. Increased sensitivity of the cells to APC through an unknown mechanism is yet to be determined.

Previously, a variety of oxidants has been used to study oxidative stress. The data presented in this study show that oxidative stress causes a broad range of cellular insults that have profoundly different and very specific physiological consequences for the cell. The cells itself have many functions that are uniquely required for resistance to only one ROS. Therefore, there is a complex cell processes needed for maintenance of cell viability in the face of different oxidants.

4.0 CONCLUSION

In conclusion, it is possible to state that APC more effectively reduces the number of viable *S. aureus* cells compared to hydrogen peroxide, diamide and methyl viologen. APC has a greater inhibitory effect than the oxidants used in killing assay of *S.aureus*. Mechanism outlined for the oxidants may serve as a model of toxic mechanism for the investigation of the toxicity of APC and other antimicrobial agents. Better understanding of the correlation between *S. aureus* mechanism against ROS and the remainder of the cell's metabolism can lead to more inventive methods for combating this pathogen.

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