Research Article

Piper Betle Ethanolic Extract Reduces Neutrophil Scavenging Ability and Possibly Catalase Activity in *S.Aureus*

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ABSTRACT

P. betle leaves are known to have antimicrobial and antioxidant activities and are widely used in traditional medicine in Asian countries. Antimicrobial activities of ethanolic and aqueous extract of *P. betle* against *S. aureus* (ATCC 25923) were investigated by determination of Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) by the Antimicrobial Sensitivity Test (AST). In addition, hydrogen peroxide sensitivity test was performed to determine antioxidant activity of ethanolic extract of *P.betle* leaves against H_2O_2 with respect to its toxicity against *S.aureus*. Further, its effect on oxidative burst in neutrophils challenged with *S.aureus* was investigated using the chemiluminescence assay. Catalase, a 58.3kDa virulent associated proteinin *S.aureus* was isolated by serial gradient ammonium sulphate precipitations followed by separation on SDS-PAGE and NATIVE-PAGE. The effect of *P.betle* ethanolic extract on catalase activity was determined using ferric chloride and potassium ferricyanide procedure with H_2O_2 as specific substrate. Ethanolic extract of *P.betle* leaves showed significantly higher antimicrobial activity against *S.aureus* compared to the aqueous extract ($p<0.05$) with MICs values of 5mg/ml and 10mg/ml, respectively. Ethanolic *P.betle* leaf extract was found to detoxify hydrogen peroxide resulting in 13% survival of cells compared to 100% killing by H_2O_2. Interestingly, the extract itself effectively killed 64% of *S.aureus* cells within 30 minutes. A significant reduction in oxidative burst, which was measured by RLU in the chemiluminescence assay, was observed in treated neutrophils compared to the untreated sample ($p<0.05$) suggesting that *P.betle* ethanolic extract potentially scavenges Reactive Oxygen Species (ROS) produced by neutrophils. The protein of interest, catalase encoded by *katA* in *S.aureus* showed significant reduction after one hour treatment with *P.betle* ethanolic extract on SDS-PAGE analysis. Reduction in catalase activity was confirmed by the double staining method that was verified by a corresponding reduction in the protein. Data from this study suggest that the possible mechanism by which the ethanolic extract of *P.betle* inhibits *S.aureus* is by down regulation of an important virulent associated protein, catalase. Further work is required to quantitate the mRNA of *katA* expression following treatment with ethanolic of *P.betle* to confirm mechanism involved. This study potentially aids in the discovery of novel therapeutic targets in *S.aureus* leading to potentially the development of new antistaphylococcal drugs.

Keywords: *P.betle*, neutrophils, catalase activity, *S.aureus.*

INTRODUCTION

Medicinal plants are of proven value as potential therapeutics with the increase of resistant pathogens to commonly used antibiotics and emergence of new infectious diseases [1]. Extracts of *Piper betle* leaf are shown to be effective against several human pathogens such as *Streptococcus mutans*, *Bacillus cereus* and *Aeromonas hydrophilia*[2,3]. It also possesses antioxidant[4,5] properties although the mechanisms involved have not been elucidated.

S.aureus is a human pathogen that poses an increasing problem in treatment of related infections due to its ability to develop resistance to multiple antimicrobials with amazing efficiency. In the human host, neutrophil scavenging represents the major route of elimination of *S.aureus* during infections[6]. *S.aureus* in turn produces catalase as one of the major virulent mechanisms to overcome the oxidative stress environment presented in the host during oxidative bursts that ensue in challenged neutrophils[7,8].

In this study, we show that ethanolic *P.betle* leaf extract is a potent antioxidant that detoxifies H_2O_2 and significantly reduces neutrophil scavenging activity *in vitro* by reducing oxidative burst. *P.betle* ethanolic extract was also found to be directly lethal to *S.aureus* possibly by reduction of the important virulent associated protein, catalase. Further in depth studies are currently in pursuit to better explain these observations.

MATERIALS AND METHODS

Staphylococcus aureus (*S.aureus* ATCC 25923) was obtained from stock culture and maintained on blood agar. Working culture was propagated on sheep blood agar and species confirmation tests were performed including the catalase, coagulase and gram stain.

Standardized inoculum was prepared by first performing growth curve in BHI broth. A standard reference plot was generated to ensure that cells harvested for each tests were standardized inoculums (1×10^8 cells/ml) and at exponential phase.

Antimicrobial Susceptibility Testing (AST) was performed by the Kirby-Bauer disc diffusion method [9]. Penicillin (10U) was used as the referenced positive control.

Hydrogen peroxide resistance assay

S. aureus was grown in BHI overnight at 37°C, then pre-cultured in fresh BHI and grown until exponential phase cells were harvested and diluted to OD600 0.1 in BHI[12]. Cells were washed in phosphate buffered saline, diluted and plated onto BH agar for colony counts to determine percent of survival at 0 min. Cells were distributed as follows, Tube 1 containing cells resuspended in PBS with 7.5mM Hydrogen Peroxide (H_2O_2), Tube 2 containing cells resuspended in PBS with 7.5mM hydrogen peroxide (H_2O_2) and 5mg/ml *P.betle* ethanolic extract and Tube 3 containing cells resuspended in PBS and 5mg/ml *P.betle* ethanolic extract.

All tubes were incubated at 37°C and aliquots were sampled at 30, 60 and 90 min and immediately added to PBS containing 10mg/ml catalase. Serial dilutions were performed in sterile distilled water and aliquots of each sampling time were plated in triplicate onto BH agar and viability was assessed after overnight growth.

Neutrophils were harvested from freshly collected blood from healthy donors and prepared using gradient density centrifugation [11].

Chemiluminescence assay

S.aureus was opsonized and pellets were recovered in HBSS and light intensity (chemiluminescence) was measured at 37°C with 6 seconds integration time at 2 mins intervals using the Luminometer[12,13]. Reactive oxygen species (ROS) were detected in a total reaction volume of 250µL/well in a 96 well NUNC plate. Opsonized *S.aureus* was added last. Light emission was recorded in RLU (relative light unit) for 30 mins. Experiments were repeated in triplicate.

Treatment with *P.betle* leaves ethanolic extract. *S.aureus* cells were treated with MIC concentration (5mg/ml) of extract for 1,2 and 3 hours in the initial step. Cells were then lysed using lysostaphin, debris removed by centrifugation and PMSF and β-mercaptoethanol added. Cells were only treated for one hour with the extract for the AS serial precipitation steps.

Protein determination of the treated and untreated *S.aureus* samples were performed using the Bradford assay[10]. The measurements were referenced against the standard BSA graph to determine final protein concentration of each sample.

Ammonium sulphate (AS) gradient precipitation

Cytoplasmic extract from treated and untreated *S.aureus* cells were subjected to increasing serial ammonium sulphate (AS) gradient precipitation to remove unwanted proteins and isolate the 58.3kDa protein (catalase) for the double staining method that is specific for catalase activity. Proteins in the *S.aureus* cytoplasmic extract were solubilized in 20% increments beginning at 40% AS according to Kang et al. (2001).

The approximated 58.3kDa band appeared in the 60% AS resuspended pellet (P3) fraction (Fig. 5) obtained from the cytoplasmic extract of *S.aureus* cells treated for 1 hour with 5mg/ml of ethanolic *P.betle* extract. Resuspended pellet (P3) was dialysed against 50mM Tris HCL ph 7.5 overnight at 4^oC and separated on 7.5% non-denaturing gel (native-PAGE) in duplicate wells together with cytoplasmic extract from untreated *S.aureus* cells. After electrophoresis, the gel was halved so that each contained both the untreated cytoplasmic *S.aureus* extract as well as the dialysed P3 fraction. One half of the gel was stained in Coomassie Blue protein stain whilst the other half was stained by the double staining method of Wayne & Diaz (1986) for catalase activity. After stainings were performed, the two gel halfs were realigned to visualize both catalase activity and the corresponding protein bands.

Polyacrylamide Gel Electrophoresis (PAGE)

Gels were prepared according to formulations for SDS-PAGE and native - PAGE respectively[14]. Sample electrophoresis, staining with Commassie Blue and destaining procedures were performed accordingly.

Double staining for catalase activity was performed by method of Wayne & Diaz after the purified protein was loaded and separated on 7.5% native polyacrylamide gel[15].

RESULTS AND DISCUSSION

The Minimum Inhibitory Concentration (MIC) of ethanolic extract of *Piper betle* leaves was determined to be 5mg/ml. At all concentrations tested, the ethanolic extract was found to be significantly inhibitory ($p<0.05$) against *S.aureus* compared to the aqueous extract (Figure 1). MIC value of the ethanolic extract of *P.betle* (5mg/ml) was fourfold lower than the aqueous extract (20mg/ml). Consequently, the MIC concentration was used in all proceeding assays in this study.

A lowered inhibitory effect of the aqueous extract is probably due to the highly volatile and low solubility nature of the bioactive compounds in aqueous state therefore reducing the effective measurable inhibitory concentrations[16]. Water has limited ability to extract oil-based components from medicinal plants therefore bioactive compounds that were soluble only in organic solvents were not present in aqueous extract [17]. It is suggested that drying techniques may possibly give effect on the quality and quantity of active compounds presence in plant extract [18,19]. However neither the ethanolic nor aqueous extract was significantly inhibitory compared to Penicillin (10U). Also included in the test were the negative controls 10% DMSO and distilled water which did not show visible signs of inhibition.

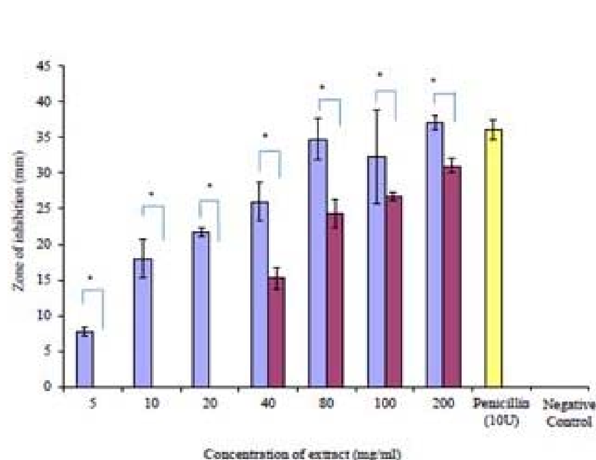

Figure 1: Antimicrobial sensitivity test comparing zones of inhibition (mm) between 5-200mg/ml aqueous ( ) and ethanolic ( ) extracts of *P.betle* leaf against *S.aureus* (1.0 x 10^8 cfu/ml). Positive control (Penicillin 10U) and negative controls (10% DMSO and distilled water for ethanolic and aqueous extract respectively). *p<0.05

The ethanolic extract was tested in its ability to detoxify hydrogen peroxide killing of *S.aureus* (Figure 2). As expected, all *S.aureus* cells were killed within 30 minutes when treated with 7.5mM H_2O_2, a suitable agent to induce oxidative stress challenge[20,21].

Presence of the extract significantly reduced toxicity of H_2O_2 where 13% cell survival was observed within 30 minutes and absolute killing achieved only after 60 minutes. The extract by itself was able to induce killing of *S.aureus* whereby 36% cell survival was observed within 30 minutes treatment and absolute killing achieved in 60 minutes albeit at a lower rate. Although the extract is protective effect against H_2O_2, it was found to be by itself significantly lethal against *S.aureus* resulting in 64% cell death after exposure to the ethanolic extract. It is suggested that the antioxidant potential of *P. betle* extract lies in its ability to scavenge the free radicals of H_2O_2[22,23].

Allylpyrocatechol (APC) an allyl-substituted catechol is the major phenolic constituent responsible for the antioxidant properties in ethanolic of *P.betle* extracts [24,25]. Moreover, compounds such as polyphenol like eugenol, chavicol, chavibetol and carvacrol are other active components in *P.betle* extracts are responsible to up-regulate its antioxidant effect [26]. Ethanolic extract demonstrated significant direct killing ability of *S.aureus*. The mechanism by which the extract exerts this effect is unknown although it is suggested that presence of ethanol in extract has antimicrobial effects on *S.aureus* by damaging its cell membranes [27]. However, a similar study using the killing assay showed that the growth of bacteria declined after treatment with *P.betle* extract, suggesting effective killing potential of*S.aureus*[28].

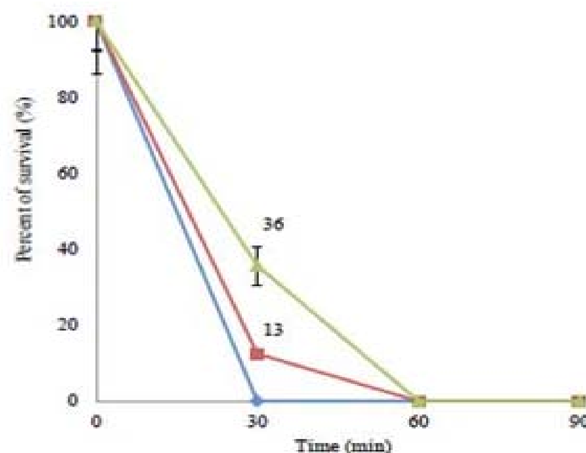

Figure 2: H_2O_2 sensitivity assay against 1x10^8 cells/ml of *S.aureus* challenged with 7.5mM H_2O_2 ( ), 7.5mM H_2O_2 + 5mg/ml *P.betle* ethanolic extract ( ) and 5mg/ml *P.betle* ethanolic extract ( ) at 30, 60 and 90 mins respectively.

Effect of the extract on neutrophil scavenging ability was determined using the chemiluminescence assay (Figure 3) that detects reactive oxygen species (ROS), particularly hydrogen peroxide, during oxidative bursts that accompany phagocytic events.

Neutrophils that were freshly harvested and stained with Trypan Blue showed 98% viability with baseline activity (negative control). Presence of 5mg/ml ethanolic *P.betle* extract showed significant reduction in measurable ROS compared to neutrophils that were challenged with only *S.aureus* (positive control) within the first 15 minutes of treatment. Two plausible explanations exist for this observation. First, the *P.betle* ethanolic extract acts to scavenge the ROS that are produced by the neutrophils during oxidative burst, thereby lowering detectable ROS leading to reduction in RLU reading. A decrease in chemiluminescence intensity derived from the luminol and superoxide anion radical generated from the enzyme reaction demonstrates the existence of antioxidant activity in test samples[29].

The presence of *P. betle* extract in treated liver fibrosis cell in rats (with *P.betle* extract) was shown to lower RLU value compared to untreated cells which showed high RLU [30]. Second, the significantly lowered RLU readings in treated samples may be due to reduced production of reactive oxygen species in the neutrophils thus reflecting reduction in oxidative burst.

The depth of the postulated mechanisms should therefore be further investigated. Clearly, the lowering of ROS by either mechanism reduces the ability of neutrophils to effectively impart damage to invading *S.aureus* cells. We then looked at an important component catalase, a 58.3kDa protein encoded solely by *katA* in *S.aureus* that enables it to overcome oxidative stress environments in the host.

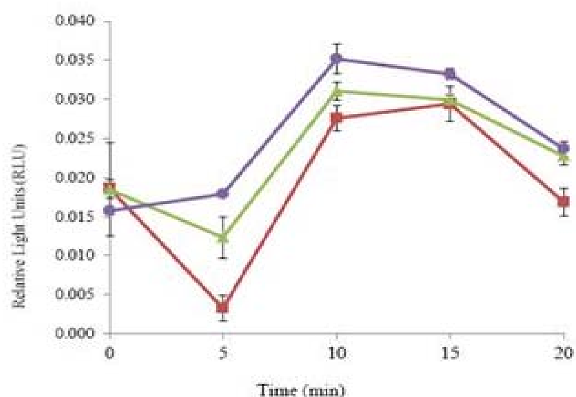

Figure 3: Measurement of Relative Luminescence Units (RLU) of neutrophils challenged with *S.aureus* in the presence and absence of *P.betle* ethanolic extract in 5 min intervals. Positive control (●), Negative control (■) and test sample (▲).

Cells were treated with MIC concentration of extract, lysed and cytoplasmic protein extract was obtained and separated on SDS-PAGE and compared to untreated cells. A significant reduction in density and numbers of cytoplasmic proteins was observed following exposure of cells to the ethanolic *P.betle* extract (Figure 4) with significant loss of proteins within 1hour of treatment. No additional protein loss was observed after one hour treatment with the extract, so proceeding treatments were performed using this time exposure.

A marked reduction was observed of proteins of molecular weights between 50-75kD.Of particular interest was the band approximating at 58.3kDa corresponding to catalase. To verify the band, cytoplasmic extracts from the treated and untreated samples were subjected to increasing serial ammonium sulphate (AS) gradient precipitations to remove unwanted proteins in addition to allow tracking of the catalase band for activity analysis (Figure 5). Although a considerable loss in proteins was visually observed after each purification step, the protein band representing catalase (58.3 kDA) was precipitated out in the pellet (P3) of the 60% AS fraction which appeared as a faint band. This is in accordance with previous study where *S.aureus* catalase precipitated out at the 60% AS concentration [31]. Moreover, no bands of the expected molecular weight of 58.3 kDa (catalase) were found in pellets or supernatants of the 40% and 80% AS precipitated fractions. The Bradford assay was performed on both treated and untreated cytoplasmic *S.aureus* extracts which confirmed an 86% total protein reduction in the cytoplasmic proteins of *S.aureus* cells treated with ethanolic *P.betle* extract for 1 hour compared to untreated cells. The absorbance (595nm) that was referenced on the standard BSA curve showed the protein concentrations in the treated and untreated samples to be 0.033 and 0.038 mg/ml respectively.

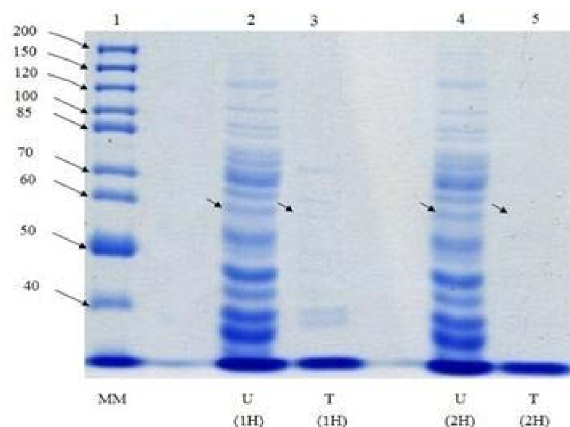

Figure 4: SDS-PAGE protein profile analysis of cytoplasmic extract of *S.aureus* cells treated (T) with 5mg/ml ethanolic *P.betle* extract compared to untreated (U) cells. Lane 1, MM - Molecular markers (Fisher Bioreagents, E-Z Run Prestain), Lane 2 untreated *S. aureus* at 1 hour, Lanes 3 *S.aureus* cells treated with *P.betle* ethanolic extract at 1 hour, Lane 4 untreated *S. aureus* at 2 hours, Lanes 5 *S.aureus* cells treated with *P.betle* ethanolic extract at 2 hours respectively. Arrow in line 2 and 4 shows targeted protein before treatments with extract at 1 and 2 hours, arrow in line 3 and line 5 shows decrement after 1 and 2 hours of treatment with *P.betle* extract.

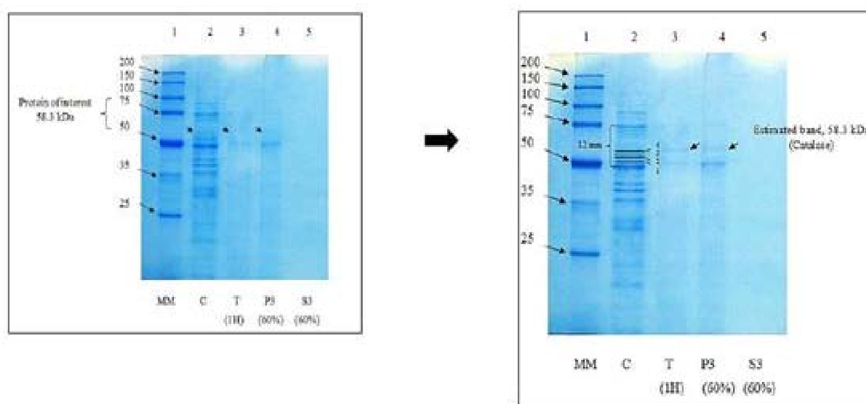

Figure 5: SDS PAGE cytoplasmic protein profile analysis of *S.aureus* cells treated with 5mg/ml ethanolic P.betle extract after saturation with 60% ammonium sulphate (AS). Lane 1 MM- Molecular marker (LONZA, Proseive), Lane 2 untreated (C) *S.aureus* cells, Lane 3 *S.aureus* cells treated for one hour (T) with *P.betle* extract, Lane 4 60% AS pellet resuspended in 1M Tris HCL ph 7.5 (P3) and Lane 5 60% AS supernatant (S3). Arrow in Lane 2 shows targeted protein before treatment with extract, arrow in Lane 3 shows decrement after one hour treatment with P.betle extract, arrow in Lane 4 shows targeted protein precipitated out in 60% AS (P3). Figure 16b Protein band estimation of 60% precipitation.

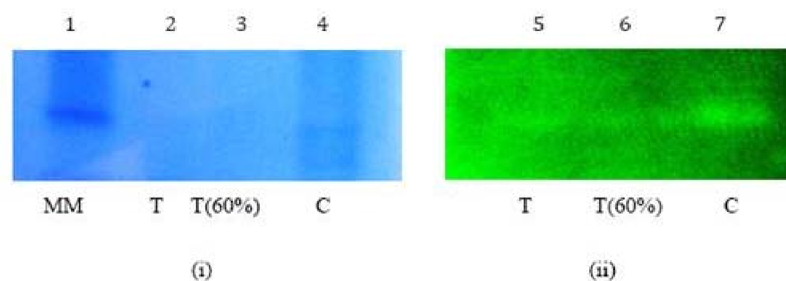

Figure 6: 7.5% Native-PAGE analysis to detect catalase activity in *S.aureus* Samples were run in duplicates on one gel, halved and stained with i) Commassie Blue. Lane 1, MM- Molecular markers. Lane 2, cytoplasmic extract of *S.aureus* cells after one hour treatment with *P.betle* extract. Lane 3, treated extract after precipitation with AS (60% saturation). Lane 4, untreated samples. ii) Double staining: Lane 5 duplicate of sample in Lane 2, Lane 6 duplicate of sample in Lane 3 and Lane 7, duplicate of sample in Lane 4.

Treatment of *S.aureus* with *P.betle* ethanolic extract significantly lowered its cytoplasmic protein density. Presence of catalase activity was verified by the double staining method that contains H_2O_2, a specific substrate for catalase, in addition to other reagents (Figure 6). An intense clearing against the green background that corresponds with catalase activity was observed in untreated cells (Lane 7) compared to treated cells (Lane 5). Correspondingly, a distinct band (Lane 4) was observed on the Coomassie stained gel that aligns with the band showing intense catalase activity. Similarly, a marked reduction in protein band intensity (Lane 2, arrow) on the Commassie gel correlates with the reduced catalase activity observed *S.aureus* cells treated with the ethanolic extract. Catalase activity was significantly higher in the non- treated *S.aureus* cells compared to those treated with *P.betle* ethanolic extract. This was verified by the Commassie protein stain on the corresponding gel whereby the band that was associated with catalase activity was clearly present as a dense protein band in the untreated sample but appeared faintly in the treated samples.

CONCLUSION

In this study the ethanolic extract of *P.betle* showed better potential as an antioxidant compared to antimicrobial activity against *S.aureus.* This is reflected by the high MIC value for the extract while as an antioxidant, it significantly reduced H_2O_2toxicity and decreased RLU in challenged neutrophils. Findings in this study suggest that *P.betle* reduces the killing efficiency of neutrophils that potentially allows *S.aureus* to evade an important initial host defense mechanism. It would be useful to determine the mechanism(s) or event(s) that occur with respect to the generation of ROS and other oxidative enzymes within the challenged neutrophils in the presence of the ethanolic extract.

However, observations from this study suggest that *P.betle* ethanolic extract probably does not exert its inhibitory effect against *S.aureus* by the phagocytosis mechanism associated neutrophils. Instead, ethanolic extract of *P.betle* most likely kills *S.aureus* by down regulating the expression of catalase that is an important virulence factor in the pathogenicity and survival of *S.aureus* in the human host. Our findings suggest that the *P.betle* ethanolic extract effectively lowers catalase activity in *S.aureus* by reducing production or expression of the protein, implying potentially a down regulation of or repressor effect on the *katA* gene. This may be a probable mechanism by which *P.betle* exerts its antimicrobial activity to allow direct killing of *S.aureus* that requires further investigations. Catalase expression allows survival within neutrophils as production of catalase corresponds to the production of ROS especially H_2O_2 inside the neutrophil in order to neutralize its effects[32]. Increasing catalase expression helps *S.aureus* survive elimination by neutrophils and contributes to infection and *S.aureus* strains that produced low levels of *katA* were found to be more sensitive to killing by theneutrophils[33,34]. Verification of the isolated catalase band and quantitation of *katA* mRNA transcripts following treatment of *S.aureus* with *P.betle* ethanolic extract are currently in progress. Identification of the major components of ethanolic extract of *P.betle* leading to its potential to regulate *katA* is being attempted. Other possible mechanisms by which the ethanolic of *P.betle* extract inhibits *S.aureus* are also being investigated.

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