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Original Article

UPREGULATION OF TRXB EXPRESSION BY PIPER BETEL LINN ETHANOLIC EXTRACT SUGGESTS INDUCTION OF OXIDATIVE STRESS WHICH POTENTIALLY CONTRIBUTES TO KILLING OF STAPHYLOCOCCUS AUREUS *IN VITRO*

ROSLINAH MOHAMAD HUSSAIN*, FARAH KHALID ADAM HO[1], MAIMUNAH MUSTAKIM[1], HAMDAN SUHAIMI [2]

Medical Laboratory Technology Department, Level 17, FSK6, Faculty of Health Sciences, Universiti Teknologi MARA (UiTM),Puncak Alam Campus, 42300 Puncak Alam, Selangor Malaysia.
Email: roslinah561@salam.uitm.edu.my

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ABSTRACT

Objective: In this study, mRNA expressions of thioredoxin reductase B and catalase (encoded by *trxB* and *katA* respectively) in *S. aureus* were quantitated in response to *P. betel* ethanolic extract to determine its regulatory potential on oxidoreductases that may affect the organism's ability to survive oxidative damage within the host.

Methods: *P. betle* ethanolic extract was prepared using the rotary evaporator and predetermined MIC (5mg/ml) for *S. aureus* (ATCC25923) was used in gene expression analyses that were performed using real-time PCR. Standardized bacterial inoculum (1 x10^8 cfu/ml) harvested at mid exponential phase were used in all assays. Total cytoplasmic protein concentrations were performed using the Bradford assay as per manufacturer's instruction.

Results: *trxB* was expressed 3.95 fold higher in *S. aureus* cells treated with the extract ($p<0.05$) whereas expression of *katA* was only 0.0083 compared to untreated cells**.** A corresponding increase (87%) in total cytoplasmic protein was observed in cells treated with the extract.

Conclusion: Our observations suggest that *P. betle* ethanolic extract exerts oxidative stress on *S. aureus* causing upregulation of *trxB*. Further, it down regulates expression of *katA* (catalase) which may contribute to reduced ability of the organism to survive damage caused by phagocytic cells.

Keywords: *Piper betle*, *S. aureus*, Thioredoxin reductase B, *trxB*, Catalase, *Kata*, RT-PCR.

INTRODUCTION

Persistence of multidrug-resistant pathogens is problematic as the efficacy of current antibiotics continues to diminish. Thus there is an urgency in the discovery and development of antimicrobial compounds with novel mechanisms of action for emerging and recurrent infectious diseases [1]. In the search for novel therapeutic agents, researchers have focused on medicinal plants as alternative sources. *Piper betel* Linn is an edible plant that has been used traditionally as a medicinal remedy amongst people in India, China, Indonesia, Vietnam, Thailand and Malaysia [2].

It is used as an antiseptic and contains an essential oil which possesses antibacterial, antiprotozoan, and antifungal properties [3] and has a stronger antibacterial effect [4] against food borne pathogens [5]. It also has been proven to possess various pharmacological activities including antimicrobial, antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory properties [6]. However, the underlying mechanisms that are responsible for these beneficial properties are yet to be fully realized.

Staphylococcus aureus is a tenacious pathogen causing a wide range of infections that continues to be problematic as they persist to be refractory to treatment with many antibiotics. Thioredoxin reductase and catalase were chosen in this study as both proteins play an important role in shielding the organism from numerous oxidative damaging mechanisms that are presented by the human host. *S. aureus* possesses thioredoxin reductase B (encoded by *trx B)* which is an essential protein involved in protein repair that protects it from oxidative or disulfide stress presented by host defense agents [7]. Expression of *trxB* is enhanced under conditions of oxygen or disulfide stress and is indispensable in *S. aureus* under conditions of normal aerobic growth [8].

Catalase is one of the major virulence associated factors that is encoded by a single gene *katA* in *S. aureus*. It is responsible for oxidative stress resistance which defends ingested staphylococci from the toxic effect of H_2O_2 produced within phagosomes in neutrophils and macrophages [9].

We previously demonstrated *P. betle* ethanolic extract capable of direct killing of *S. aureus*. This study attempts to glean information which may shed light on its inhibitory mechanism. *P. betel* ethanolic extract was used to identify effects on expressions of thioredoxin reductase (*trxB)* and catalase (*katA*) in *S. aureus* in attempt to better understand its mode of action possibly leading to identification of novel therapeutic targets for its development as an antimicrobial agent.

MATERIALS AND METHODS

Piper betel ethanolic extraction (PEE)

Piper betel Linn leaves were speciated at the Forest Research Institute Malaysia (PID 521113-29). And ethanolic extract was prepared according to Voravuthikunchai & Mitchell (2008) with modifications [10].

Powdered leaves (100 g) were soaked in ethanol for 7 days at room temperature, filtered and evaporated under reduced pressure (60°C) in a rotary evaporator (Eyela OSB-2100). The extract was stored at 4^0C in the dark to prevent degradation of photosensitive bioactive compounds.

Preparation of standardized inoculum of *S. aureus* (ATCC 25923)

S. aureus (ATCC 25923) was maintained on 5% SBA and confirmed by conventional tests. For all assays, cells were grown in BHI broth and harvested at an exponential phase at a standardized inoculum of (1 x 10^8 cells/ml).

Treatment of *S. aureus* with PEE (MIC)

Treated cells were mixed with 5mg/ml extract and incubated for 1 hour in a shaking incubator. Extract volume for untreated cells was replaced with distilled water. 500 µL of 10% DMSO solution was added to both samples.

***katA* and *trxB* expression analyses**

Isolation of total RNA and cDNA synthesis

Total genomic DNA from each sample was isolated using Rneasy Protect Bacteria Mini Kit (Qiagen, USA) according to manufacturer's instructions. cDNA synthesis was performed using Quanti Tect Reverse Transcription Kit (Qiagen, USA) according to manufacturer's instructions. RNA purity and concentration were confirmed using a biophotometer (Eppendorf, USA).

Primer and probe design for target genes *trxB*, *katA* and housekeeping gene (16S rRNA)

Complete *trxB* and *katA* sequences of *Staphylococcus aureus* ATCC 25923 were accessed from NCBI Gen Bank database. For probe design, sequences were each aligned using Sci Tools Real Time PCR software (Integrated DNA Technologies, USA) to identify unique regions was bound suitable for amplification of the respective genes.

Primers and probes were selected using the ABI Primer Express program (Applied Biosystems, USA) (Table 1).

Specificity of primers and probes were confirmed using primer blast and nucleotide Blast Tool (NCBI). The probe was labeled with 6-carboxy-fluorescein (FAM) at the 5′ end (reporter) and with the 6-carboxy-tetramethylrhodamine (TAMRA) at the 3′ end (quencher). All the steps above were repeated for the designated reference gene (16S rRNA) for selection of forward and reverse primers and probe (Table 2).

Reverse transcription with elimination of genomic DNA for quantitative, Real-time PCR using quantitect reverse transcription kit (Qiagen)

Genomic DNA elimination reactions were prepared using gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-free water in 14u final reaction volume as per manufacturer's instructions. The mixtures were incubated for 2 minutes at 42 °C placed immediately on ice.

Table 1: Primers and probes designated for gene *trxB* and *katA* of *S. aureus* ATCC 25923).

Primers and probes	Sequence (5′- 3′ end)	Reference gene
16S rRNA-f	GGTCTGTAACTGATGTG	16S rRNA
16S rRNA-r	GTGGACTACCAGGGTATCTAATCCT	
16S rRNA-probe	5'- /56-FAM/TCCCCACGC/ZEN/T TTCG/3IABkFQ/ -3'	

Table 2: Primers and probes designated for 16SRNA in *S. aureus* ATCC 25923

Primers and probes	Sequence (5′- 3′)	Target gene
trxB-f	AATTATCGGTGCAGGTCCAG	*trxB*
trxB-r	TTCGAAACCAGGGAAGTTCTC	
trxB-probe	CCAGGCGGTCAAATGGCTAATACAGA	
katA-f	GCGTTTGCACCAACTAATATTATTCCA	*katA*
katA-r	GCGCATCGCCATATGAGAATAAAC	
katA Probe	CCCTTGCAACATTTTG	

Real-time PCR

Reactions were performed in 20 µL final reaction volume 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 reaction plate according to manufacturer's recommendations (3 min. at 95°C, 5 sec. at 95°C and 30 sec. at 60°C for 40 cycles). PCR reaction for each gene was performed in replicate and gene expressions were analyzed using Livak method and level of significance determined by paired t-test.

Relative quantification by using Livak method or ΔΔCt

Data generated in the bar graph were derived using the Livak or C_t method ($^{-\Delta\Delta Ct}$) [11] also known ΔΔCt as defined by the following formula. Manual calculations were also performed using this formula to verify comparison of expression levels of targeted genes between treated and untreated cells.

$$2^{-\Delta\Delta Ct} = 2^{-[(Ct\ target - Ct\ HKG) - (Ct\ target - Ct\ HKG)]}$$

Sample (Treated) — Sample (Untreated)

RESULTS

Species confirmation

The organism was microscopically identified as gram positive cocci in clusters after gram stain, catalase positive with rapid gas bubbles production, and coagulase thereby positively identifying *S. aureus*.

trxB* expression in *S. aureus

The effect of *P. betle* ethanolic extract on expression of *trxB* in *S. aureus* was investigated to determine changes in the targeting gene. Expression of the gene of interest, *trxB* was determined after treatment (in triplicate) with 5mg/mL *Piper betle* ethanolic extract for 1 hour. The relative expression of *katA* gene in treated and untreated samples with 16S rRNA act as reference gene was determined using the $2^{-\Delta\Delta C_T}$ (Livak method).

The amplification efficiencies for both genes (*trxB* and 16S rRNA) were 95%. According to the amplification graphs (Figures 2 and 3), *trxB* in treated cells amplified lower than untreated sample with indicating a lowered expression of the gene in treated cells to untreated cells (Figure 2b).

First RT-PCR Run

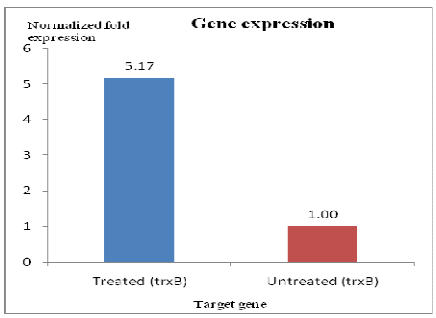

Fig. 1: Gene fold-expression of *trxB* in *S. aureus* in cells and treated with MIC (5mg/mL) of ethanolic extract of *P. betle* for 1 hour and untreated cells for the first RT-PCR run. 16S rRNA included as reference.

Table 3: Quantification data of target gene (*trxB*) and reference gene 16S rRNA in untreated and treated samples for the first RT-PCR run

Samples	Genes	
	Target (*trxB*)	Reference (16S rRNA)
Untreated	21.25 (Ct)	19.79 (Ct)
Treated	22.47 (Ct)	23.38 (Ct)

Relative quantification of *trxB* expression using Livak method or ΔΔCt

ΔCt (untreated) = Ct (target, untreated) – Ct (reference, untreated)

= 21.25 – 19.79

= 1.46

ΔCt (treated) = Ct (target, treated) – Ct (reference, treated)

= 22.47 – 23.38

= - 0.91

ΔΔCt= ΔCt (treated) – ΔCt (untreated)

= (- 0.91) – 1.46

= - 2.37

Normalized expression ratio = $2^{-\Delta\Delta Ct}$

= $2^{-(-2.37)}$

= 5.17

Therefore, treated cells (test sample) have expressed *trxB* gene at a 5.17-fold higher level than untreated cells (control sample) for the first RT-PCR run.

Second RT-PCR run

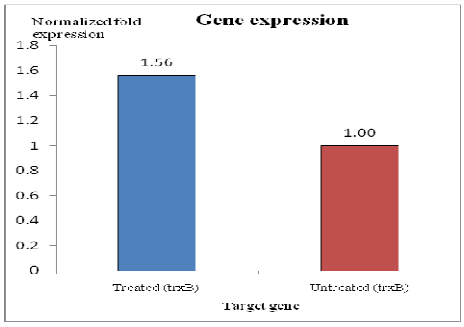

Fig. 2: Gene fold-expression of *trxB* in *S. aureus* in cells and treated with MIC (5mg/mL) of ethanolic extract of *P. betle* for 1 hour and untreated cells for the second RT-PCR run. 16S rRNA included as reference

Table 4: Quantification data of target gene *trxB* and reference gene 16S rRNA in untreated and treated samples for the second RT-PCR run

Samples	Genes	
	Target (*trxB*)	Reference (16S rRNA)
Untreated	21.855 (Ct)	21.375 (Ct)
Treated	21.115 (Ct)	21.275 (Ct)

Relative quantification of *trxB* expression using livak method or ΔΔCt

ΔCt (untreated) = Ct (target, untreated) – Ct (reference, untreated)

= 21.855-21.375

= 0.48

ΔCt (treated) = Ct (target, treated) – Ct (reference, treated)

= 21.115-21.275

= - 0.16

ΔΔCt= ΔCt (treated) – ΔCt (untreated)

= (-0.16) – (0.48)

= -0.64

Normalized expression ratio = $2^{-\Delta\Delta Ct}$

= $2^{-(-0.64)}$

= 1.56

Therefore, treated cells (test sample) have expressed *trxB* gene at a 1.56-fold higher level than untreated cells (control sample) for the second RT-PCR run.

Third RT-PCR run

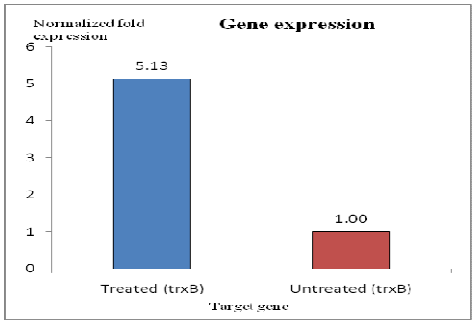

Fig. 3: Gene fold-expression of *trxB* in *S. aureus* in cells and treated with MIC (5mg/mL) of ethanolic extract of *P. betle* for 1 hour and untreated cells for the third RT-PCR run. 16S rRNA included as reference

Table 5: Quantification data of target gene *trxB* and reference gene 16S rRNA in untreated and treated samples for the third RT- PCR run

Samples	Genes	
	Target (*trxB*)	Reference (16S rRNA)
Untreated	20.49 (Ct)	22.89 (Ct)
Treated	21.10 (Ct)	25.86 (Ct)

Relative quantification of *trxB* expression using livak method or ΔΔCt

ΔCt(untreated) = Ct (target,untreated) – Ct (reference, untreated)

= 20.49 – 22.89

= -2.40

ΔCt(treated) = Ct (target, treated) – Ct (reference, treated)

= 21.10 – 25.86

= - 4.76

ΔΔCt= ΔCt(treated) – ΔCt(untreated)

= (- 4.76) – (-2.40)

= - 2.36

Normalized expression ratio = $2^{-\Delta\Delta Ct}$

= $2^{-(-2.36)}$

= 5.13

Therefore, treated cells (test sample) have expressed *trxB* gene at a 5.13-fold higher level than untreated cells (control sample) for the third RT-PCR run.

Ct (cycle threshold) values were obtained from quantification data of the amplification curve generated by the real time PCR data. *Ct or threshold cycle is the cycle number at which the detectable signal is achieved. The Ct values of the target gene in each sample were used to calculate the gene expression ratio using the Livak (ΔΔCt) method for each run. Treated cells showed increased *trxB* expression at 5.17, 1.56, and 5.13-fold higher than in untreated cells (Table 4). The mean value of gene expression for the treated samples showed expression of *trxB* gene at a 3.95-fold higher level than in untreated samples (Figure 4).

Table 6: Ct values and normalized expression ratio data for the overall three RT-PCR runs of target gene, *trxB* for untreated and treated *S. aureus* with PEE

Real Time PCR run	Target gene (*trxB*)		Reference gene (16S rRNA)		Normalised expression ratio, Livak method (ΔΔCt)	
	Treated sample (Ct)	Untreated sample (Ct)	Treated sample (Ct)	Untreated sample (Ct)	Target (*trxB*) Treated sample	Target (*trxB*) Untreated sample
First	22.47	21.25	23.38	19.79	5.17	1.00
Second	21.115	21.855	21.275	21.375	1.56	1.00
Third	21.10	20.49	25.86	22.89	5.13	1.00
					3.95	1.00

Mean value for the gene expression of three runs

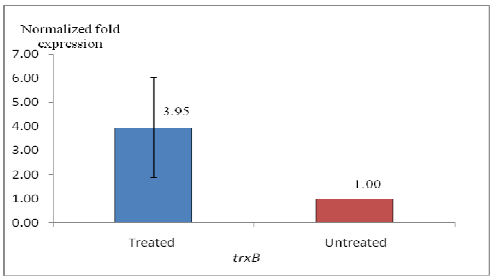

Fig. 4: Overall mean normalized fold expression ratio of *trxB* expression from the first, second, and third Real Time PCR runs of target gene in *S. aureus* after treatment with MIC (5mg/mL) of *P. betle* ethanolic extract for 1 hour

katA expression in *S. aureus*

The effect of *P. betle* ethanolic extracts on expression of *katA* in *S. aureus* was also investigated to determine changes in the targeting gene. Expression of the gene of interest, *katA*, which encodes catalase in *S. aureus* was determined after treatment with 5mg/mL *Piper betle* ethanolic extract for 1 hour. The relative expression of *katA* gene in treated and untreated samples with 16S rRNA act as reference gene was determined using the $2^{-\Delta\Delta C_T}$ (Livak method). The amplification efficiencies for both genes (*katA* and 16S rRNA) were 95%. According to the amplification graph (Figure 3a), *katA* in treated cells amplified lower than untreated sample with indicating a lowered expression of the gene in the treated cells to untreated cells (Figure 3b).

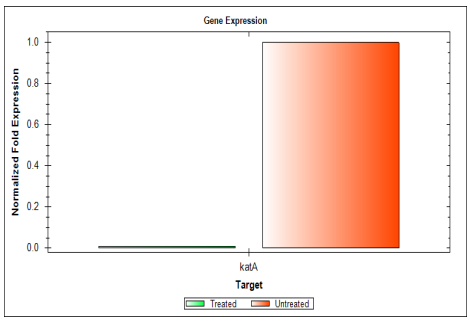

Fig. 5: Mean normalized fold expression ratio of *katA* of Real Time PCR runs of target gene in *S. aureus* after treatment with MIC (5mg/mL) of *P. betle* ethanolic extract for 1 hour

The Ct values katA gene in samples treated and untreated with the extract were used to calculate the gene expression ratio using the Livak (ΔΔCt) method for one run. Normalized fold gene expression of *katA* gene for the treated sample was 0.008344 which is considerably lower compared to that in untreated sample (Figure 5). Although the RT-PCR expression analyses for the targeted gene were performed in triplicate, here we incorporate data from only one run as the remaining runs were compromised by contamination in the negative control which showed > 1.0 value on the amplification curve). However, the general trend of downregulation of the *katA* gene by the extract was observed in all runs (Figure 6).

Relative quantification of *katA* expression using Livak method or ΔΔCt

$2^{-\Delta\Delta Ct} = 2^{-[(Ct\ target - Ct\ HKG) - (Ct\ target - Ct\ HKG)]}$

$= 2^{-[38.71-13.62) - (28.36-10.17)]}$

$= 2^{-(25.09-18.19)}$

$= 2^{-(6.9)}$

$= 2^{-6.9}$

$= 0.0083$

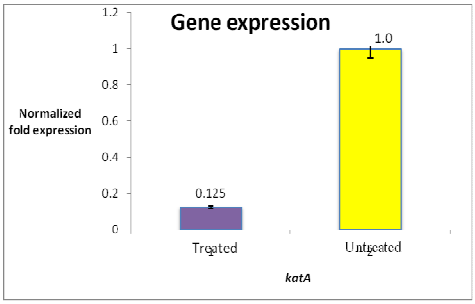

Fig. 6: Mean normalized fold expression ratio of *katA* of Real Time PCR runs of target gene in *S. aureus* after treatment with MIC (5mg/mL) of *P. betle* ethanolic extract for 1 hour (data compromised due to contamination of negative control)

Protein quantification using brad ford technique

Total protein contents were determined in untreated and *S. aureus* cells were treated with 5mg/mL of extract for 2 hours using the Bradford Assay. A protein curve was plotted using BSA as reference (Figure 7). The unknown x (protein concentration) were determined

from the known value of y (A595). Absorbance (A_{595}) values of treated (T) and untreated samples (U) were 0.334 and 0.383 which corresponded with protein concentrations of 0.164 mg/mL and 0.188 mg/ml respectively. From calculation data (Table 5), an 87% reduction in cytoplasmic protein contents was observed in *S. aureus* after treatment with PEE.

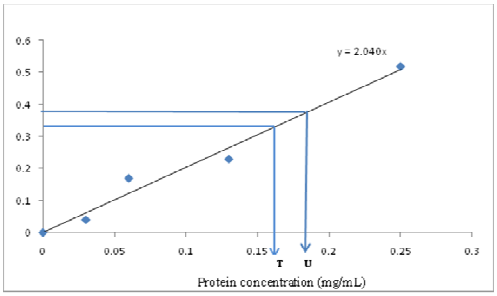

Fig. 7: Standard BSA protein curve with concentrations of cytoplasmic protein in *S. aureus* (mg/mL) for untreated cells (control) and treated cells with 5mg/mL of *P. betle* ethanolic extract for 2 hours

Table 7: Comparison of protein concentration (mg/mL) of untreated *S. aureus* cells and treated *S. aureus* cells with 5mg/mL of *P. betle* ethanolic extract for 2 hours Calculation of unknown x (protein concentration) using equation y = 2.040x

Sample	Absorbance (A_{595}) y-axis	Protein concentration (mg/mL) x-axis
Untreated cells (U)	0.334	0.188
Treated cells (T)	0.383	0.164

Percentage of protein reduction after treatment with *P. betle* ethanolic extract

Protein in treated cells/ Protein in untreated cells x 100 = % of protein reduction

Therefore, 0.164/0.188 x 100 = 87%

DISCUSSION

Expressions of *trxB* and *katA*, encoding thioredoxin reductase B and catalase respectively, in *S. aureus* in response to *Piper betel* ethanolic extract were investigated to determine its regulatory effect towards targeted genes. The chosen concentration of the extract (5mg/mL) used for mRNA quantitation of gene expressions was based on our previous report of MIC for *Piper betle* Linn ethanolic extract against *S. aureus*.

The thioredoxin system is ubiquitious in living organisms and consists of thioredoxin and thioredoxin reductase which are thiol-disulfide reductases that uses NADPH as reducing power [12]. Generally the thioredoxin system maintains the redox balance in the cell system and acts as a major protectant of cytosolic proteins from aggregation or inactivation as a result of oxidative damage from formation of intra- or inter-molecular disulfide. Thioreoxin reductase (TrxB) is a flavoenzyme that catalyzes the reduction of thioredoxin and is essential in *S. aureus* due to absence of an alternative thiol-redox system [13]. Exposure of microorganisms to oxidative stress agents are known to increase *trxB* expression. For instance, treatment of *Bacteroides fragilis*, an anaerobe, with diamide, H_2O_2 or exposure to oxygen induces expression of *trxB* [14]. Increasing *trxB* expression contributes to ability of *S. aureus* to resist damage caused by stressful oxidative conditions presented by the host. *P. betle* extracts have been established to possess antioxidant activities [15-17]. As a novelty, we show that *P. betle* ethanolic extract upregulates *trxB* in *S. aureus* suggesting that the extract imparts significant oxidative stress on the organism. Unresolved disulphide bond formation destroys proteins and deactivates enzymes, culminating in loss of function and cell death. It is uncertain if the extract causes sufficient oxidative stress in the organism to be directly inhibitory. Further investigations are required for verification of this causative effect and more importantly, identification of the bioactive component(s) is warranted.

S. aureus produces a single major catalase encoded by *katA* which protects it from damaging effects of H_2O_2 that is generated within phagosomes of neutrophils and macrophages [18]. A majority of pathogenic strains of *S. aureus* are catalase positive although catalase-negative strains have been isolated from wounds, septicemia and catheters in patients worldwide [19]. Based on the RT-PCR amplification data, we observe that *katA* expression in cells treated with *P. betle* ethanolic extract amplified significantly lower compared to untreated cells indicating down regulation of *katA* by the extract. This finding verifies our previous observation where MIC of the ethanolic extract significantly reduced both catalase production and activity in the organism [20]. It would be interesting to determine if these effects are replicated *in vivo* and how clearance of the organism by neutrophils and macrophages may be affected.

The amplification efficiency of the RT-PCR reactions in this study were 95 percent which indicate that the amplification of the reactions were almost ideal. By using this method, the differences in mRNA steady state levels between two types of samples (treated and untreated cells) were quantified. Relative quantification describes a real-time PCR experiment in which the expression of a gene of interest in one sample (treated) is compared to expression of the same gene in another sample (untreated). The results are expressed as fold change (increase/upregulated or decrease/downregulated) in expression of the treated in relation to the untreated. A normalized gene (housekeeping gene), 16S rRNA-processing protein from *Staphylococcus aureus* was used as reference for experimental variability for quantification. Two methods of relative quantification analysis were performed to calculate fold change. Fold changes were automatically generated after each gene expression analysis was completed using real-time PCR and fold expressions were then verified manually using the mathematical formula described by the Livak (ΔΔCt) method that requires 100 percent amplification efficiencies to validate our findings. Data obtained in this study were statistically analyzed using t-test (SPSS Version 18.0). As can be seen from the output, significant difference exists in expression of the targeted gene between treated and untreated samples. The treated samples significantly showed higher expression of *trxB* gene after being treated with *Piper betle* ethanolic extract, $p<0.05$.

The significant reduction in total protein concentrations after treatment with *P. betle* extract has been observed repeatedly and suggests that upregulation of *trxB* did not compensate for the total loss of proteins that probably involved other pertinent enzymes such as catalase amongst others which strongly suggest the regulatory effects of *P. betle* ethanolic extract. Protein concentrations were measured using the Bradford assay and the result was accepted since the protein concentration detected was within the linear assay range for BSA (125–1,000 µg/ml) [21]. Protein degradation from sample preparation was minimized during sample preparation by using phenylmethane sulfonyl fluoride (PMSF) that serves as a protease inhibitor that inhibits the degradation of proteins by deactivating the enzyme activity. Furthermore, all protein manipulations were conducted at controlled temperature to prevent protein degradation. In addition, 2-mercaptoethanol which acts as reducing agents kept the protein in its reduced form thus facilitating downstream analysis of *trxB* and aids in the breakdown of disulfide bonds in tertiary and quaternary protein structures.

CONCLUSION

P. betle ethanolic extract significantly upregulates *trxB* which encodes the essential oxidoreductase enzyme that plays a sentinel role in maintenance of protein secondary structures and enzyme activity in *S. aureus*. Treated cells show the significant increase in *trxB* expression compared to untreated cells ($p<0.05$), suggesting ability of the extract to induce oxidative stress. In addition, we show the extract capable of down regulating expression of catalase that protects the organism from damaging effects of H_2O_2. Taken together

our observations suggest a probable method by which *P. betle* ethanolic extract induces direct killing of *S. aureus* cells. Our finding supports earlier observation where *P. betle* ethanolic extract directly killed *S. aureus* cells *in vitro*, albeit at a lower potency compared to H_2O_2. It may be possible that an overwhelming oxidative stress impacted by the extract, together with reduced ability to neutralize peroxide toxicity could not be over comed by the increase in thioredoxin reductase B activity thus potentially causing cell death. Further investigations involving recognition of the phytoactive component(s) responsible for the observed effects and *in vivo* studies are required to verify our observations.

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Disclosure policy

"The author(s) declare(s) that there is no conflict of interests regarding the publication of this paper."

CONFLICT OF INTERESTS

Declared None

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