Antibacterial properties of selected Malaysian Tualang honey against *Pseudomonas aeruginosa* and *Streptococcus pyogenes*

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ABSTRACT

Background and Objectives: Tualang honey (TH) is a Malaysian multifloral jungle honey. In recent years, there has been a marked increase in the number of studies published in medical databases regarding its potential health benefits. The study aimed to investigate the effect of TH against *Pseudomonas aeruginosa* and *Streptococcus pyogenes*.

Materials and Methods: The effect of TH on both bacteria was investigated using MIC, MBC, growth curve, time-kill curve, scanning electron microscopy (SEM) and RT-qPCR.

Results: The MIC of TH against *P. aeruginosa* and *S. pyogenes* was 18.5% (w/v) and 13% (w/v) respectively and MBC was 25% (w/v) for both bacteria. Spectrophotometric readings of at least 90% inhibition yielded MIC_{90} values of TH, 18.5% (w/v) and 15% (w/v) for *P. aeruginosa* and *S. pyogenes* respectively. A time–kill curve demonstrated a bactericidal with a 4-log reduction estimated within 8 hours. Using SEM, loss of structural integrity and marked changes in cell shape were observed. RT-qPCR analysis showed that TH reduced the pattern of gene expression in both bacteria, with a trend toward reduced expression of the virulence genes of interest.

Conclusion: This study suggests that TH could potentially be used as an alternative therapeutic agent for microbial infection particularly against these two organisms.

Keywords: Antibacterial activity; Tulang honey; Scanning electron microscopy; Differential expression

INTRODUCTION

Honey is one of the oldest traditional medicines that has been highly reputed and widely used for the treatment of several human diseases for thousands of years ago (1). This reputation has continued up to the present day, leading to the emergence of a relatively new branch of alternative medicine, called "apitherapy", which focuses on medical applications of honey and other bee products (2, 3). Nowadays, different types of honey have been used in many countries as an alternative to pharmaceutical products for treating contaminated, infected, and burn wounds (3-6). This is attributed to the effectiveness of these honeys in inhibiting or killing a broad spectrum of bacteria (7-9). The antimicrobial activity of honey may be attributed to several factors, including high osmolarity, acidity, in addition to the presence of hydrogen peroxide (H_2O_2) (10) and non-peroxide components, such as methylglyoxal (11). In addition to exerting direct antimicrobial effects, some honey varieties have been implicated in the differential expression of a number of genes essential for bacterial survival and virulence, including those involved in stress tolerance (12), virulence factor production (13), as well

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as multicellular behaviors, such as biofilm formation (14, 15), and quorum sensing (16). To the best of our knowledge, only three published studies to date have focused on the honey-induced expression patterns in *P. aeruginosa* and *S. pyogenes* (15, 17, 18). Honey's composition (and hence its antimicrobial activity) is dependent on the environmental and geographical locations from which the original nectar was collected (19, 20). This is attributed to natural variations in floral sources and climatic conditions at different locations (19). Therefore, several researchers have explored the therapeutic effects of honeys obtained from diverse geographical areas worldwide (21-23). Several studies have addressed different aspects of honey varieties, including their physicochemical properties, chemical composition, antibacterial activity, and therapeutic usefulness (24-30). However, it is not yet known whether these antibacterial activities, as well as any possible and anti-virulence activities possessed by these honeys could be attributed to alteration of bacterial gene expression. Therefore, the objectives of the present study were threefold: (i) to evaluate the direct antimicrobial activity of TH of the most common Malaysian honey against *P. aeruginosa* and *S. pyogenes* (ii) to study the effects of TH on the structure of *P. aeruginosa* and *S. pyogenes* (iii) to estimate the impacts of this honey on the expressions of virulence-related genes (in other words, to evaluate the anti-virulence potential of this honey).

MATERIALS AND METHODS

Honey sample. Tualang honey (TH) samples was purchased from Kuala Terengganu a state in Malaysia and were used throughout this study. Stock samples 100% were stored in the dark bottles, labelled accordingly and stored at room temperature away from light source. Then, honey samples have been prepared freshly for each experiment and syringe filter (pore size 0.45 μm, diameter 25 mm) was used in this study (31). To ascertain the security of the purchased product, honey was presented to the bee hunters based on their geographical hunting area to identify the purity, reliability, and quality of the honey.

Bacterial strain and culture conditions. Standard reference strains of Gram-negative bacteria *P. aeruginosa* (ATCC 10145) and Gram-positive bacteria *S. pyogenes* (ATCC 19615) were purchased from American type tissue culture (ATCC) and were used throughout this study. The bacteria samples were cultured and maintained on nutrient agar (NA; Oxoid, UK) and incubated at 37°C unless otherwise stated (32).

Minimum inhibitory concentration and minimum bactericidal concentration. The minimum inhibitory concentration (MIC) of the TH was determined using the broth dilution method in sterile 96-well microtiter plates. Fifty percent (w/v) stock solution of TH was prepared by weighing 10 g of the honey and bringing the volume up to 20 ml using Nutrient broth (NB). Further dilutions were done to obtain honey concentrations of 6.25%, 7.5%, 8.75%, 9.25%, 10%, 11.25%, 12.5%, 13%, 15%, 16.5%, 17.5%, 18.5%, 20%, 22.5% and 25% (w/v). A few single bacterial colonies from an overnight culture on nutrient agar (NA) were inoculated into peptone water to achieve a turbidity of 0.5 McFarland ($\approx 1 \times 10^8$ CFU/ml). Plates were incubated for 24 hours at 37°C and turbidity measured at 570 nm in a microplate reader (Tecan Infinite 200 PRO, Austria) Herein, the most common way to assess microbial growth in solution is the measurement of the optical density between 600 nm and 620 nm. In this study, we measured the turbidity at 570 nm, this wavelength was chosen from previous studies (31-33) and based on absorbance detection mode and basically determines which portion of light passes through a sample, more specifically through a suspension of microorganisms). Inoculum only was used as a positive control, broth only was served as a negative control and honey without inoculum was used as corresponding negative control. Wells with the lowest concentration without growth were recorded as the MIC. The minimum bactericidal concentration (MBC) was determined by taking a loopful of the culture medium from each test well (from the broth MIC assay) that showed no apparent growth and sub-culturing on fresh nutrient agar (NA) plates. After incubation at 37°C for 24 hours, the MBC was read as the least concentration showing no growth on the NA plates (33). The growth inhibition for the test wells at TH dilution was determined by the formula as mentioned below (34).

$$\text{Percentage of inhibition (\%)} = \frac{[1-(\text{OD test well-OD corresponding negative control well})\times100\%}{(\text{OD viability control well - OD broth only well})]}$$

Experiments were performed in triplicate.

Growth kinetics curves. To determine the effects of TH on the growth curves of *P. aeruginosa* and *S. pyogenes*, the inoculum concentration was adjusted to 0.5 McFarland using a spectrophotometer. Cells were grown and treated in 96-well microtitre plates with MIC, half-MIC and quarter-MIC. The plate was incubated at 37°C for 24 hours. At 60 minutes intervals, the absorbance of wells were measured at 570 nm using a microplate readers (Tecan Infinite 200 PRO, Austria). The experiment was performed in triplicate for each bacteria tested (32).

Time-kill curve. An overnight broth culture of each test organism in 15 ml NB was prepared by inoculating a colony from a pure culture and incubating at 37°C for 24 hours. After incubation, each culture was diluted with NB to an optical density of 0.5 at 600 nm. 60 ml of sterile NB was added to conical flask labelled as control for each isolate. A 60 ml of appropriate of TH solution (MIC) was prepared in another conical flask and labelled as test for each isolate. A 60 ml of appropriate of TH solution (2 × MIC) was prepared in another conical flask and labelled as test for each isolate. Then 6 ml from the diluted overnight culture was transferred into each flask. The mixture was then incubated at 37°C with shaking (100 rpm). One ml from each flask was removed into a microcuvette and absorbance was recorded at 570 nm 1 hour up to 8 hours (0, 1, 2, 3, 4, 5, 6, 7 and 8 hours). Finally, total viable count for each collected specimen was determined as follows: 30 μl of the specimen was diluted in a decimal dilution series from 10^{-1} to 10^{-10} using NB broth, plated on nutrient agar and were cultured for 24 hours at 37°C for determination of total bacterial count in each well. A graph mean of Log10 CFU/ml versus time was plotted for determination of total bacterial count in each well (35). The experiment was performed in triplicate for each bacteria tested.

Scanning electron microscopiy (SEM). The effects of MIC TH on the morphology of *P. aeruginosa* and *S. pyogenes* was examined by SEM (JEOL 6360LA, Japan). Bacterial cultures were centrifuged (3500 rpm, 10 min) after 8 hours incubation with MIC TH at 37°C. Pellets were collected and were fixed overnight with 2.5% (v/v) glutaraldehyde in 0.01 M phosphate buffer solution (PBS). The samples were washed twice with 0.01 PBS for 5 minutes followed by deionized water for 10 minutes. All samples were dehydrated with ascending concentrations of ethanol for 10 minutes and subjected to critical point drying. The sample was then subsequently coated with platinum, placed onto the copper stage holder and examined by SEM (36).

RNA Extraction for RT-qPCR. Initially, Mid-exponential phase *P. aeruginosa* and *S. pyogenes* cells were diluted 1:1 with NB, and NB containing MIC TH. Meanwhile, inoculum only without honey was served as a positive control. Subsequently, the samples were incubated for 8 hours in a shaking (100 rpm) at 37°C. After 8 hours incubation, 1 ml of sample treated and untreated were extracted and centrifuged at 13,000 rpm for 2 minutes. The supernatant was discarded and the pellet was washed with PBS. The pelleted cells were resuspended in 50 μl of lysozyme (Sigma-Aldrich, UK), and 50 μl mutanolysin (Sigma-Aldrich, UK) and then incubated at room temperature for 15 minutes. Total RNA from treated and untreated samples were extracted using kit SV Total RNA Isolation System (Promega, UK). Total RNA concentrations were examined using Implen NanoPhotometer® NP80. RNA purity levels were assessed using the 260/280 absorbance ratio, with only sample ratios between 1.8 and 2.1 being accepted for conversion to cDNA. Total RNA samples were converted to cDNA following the manufacturer's instructions kit (Promega, UK). Samples were diluted to 100 ng/μl using ultra-pure water. For each reaction, qPCR mastermix was prepared by following the manufacturer's instructions (Promega, UK) and PCR primers were used as shown in Tables 1 and 2 (15, 17, 32, 37, 38). The following PCR protocol was used: denaturation at 95°C for 2 minutes in one cycle, amplification at 95°C for 15 seconds in 40 cycles and a final elongation annealing at 60°C for 1 min in 40 cycles. Densitometry was performed using the Applied Biosystems StepOne Software v2.3. To determine and calculate the level of gene expression, a modified $2^{-\Delta\Delta}$ Ct method was used (15, 32). The experiments were performed in triplicate.

Statistical analysis. Data was expressed as mean ± standard deviation. Independent student t-test from (SPSS version 20) was used to compare between honey-treated and control groups. The significant was set at $P<0.05$.

Table 1. Gene specific primers of *P. aeruginosa* used for RT-qPCR analysis (37, 38).

Gene name	Amplicon Size (bp)	Direction	Primer sequence (5' → 3')
algD	144	Forward	CGCCGAGATGATCAAGTACA
		Reverse	AGGTTGAGCTTGTGGTCCTG
oprF	101	Forward	CTGGACGCCATCTACCACTT
		Reverse	CTGTCGCTGTTGATGTTGGT
fleN	137	Forward	GAGCCGTATACGAGGCATTC
		Reverse	GTGTTGGACCAGTCGTTCG
fleQ	134	Forward	AAGGACTACCTGGCCAACCT
		Reverse	CCGTACTTGCGCATCTTCTC
fleR	109	Forward	ACAGCCGCAAGATGAACCT
		Reverse	TGGATGGCGTTGTCGAGTT
oprB	140	Forward	GACGACGACAAGACAGGAC
		Reverse	GGTCGTTGGAAAGGTTCTTG
oprH	102	Forward	CTCGACAAGGTGATCGACAA
		Reverse	GGTGTCGGAGATGTTCTCGT
fliA	192	Forward	CTCCAATTGAGCCTCGAAGA
		Reverse	TTCGTTGTGACTGAGGCTGG
fliC	121	Forward	GCTTCGACAACACCATCAAC
		Reverse	AGCACCTGGTTCTTGGTCAG
*rpoD**	146	Forward	GCGACGGTATTCGAACTTGT
		Reverse	CGAAGAAGGAAATGGTCGAG

* Reference gene for *P. aeruginosa*

Table 2. Gene specific primers of *S. pyogenes* used for RT-qPCR analysis (15, 17, 32).

Gene name	Amplicon Size (bp)	Direction	Primer sequence (5' → 3')
Sof	873	Forward	ACTTAGAAAGTTATCTGTAGGG
		Reverse	TCTCTCGAGCTTTATGGATAG
SfbI	960	Forward	AACTGCTTTAGGAACAGCTTC
		Reverse	CCACCATAGCCACAATGCT
scpA	622	Forward	GCTCGGTTACCTCACTTGTCC
		Reverse	CAATAGCAGCAAACAAGTCACC
ftsY	97	Forward	TCGAAAATTCTTTGGCCTGT
		Reverse	ATCAAACGTGTTGTGCCAGA
*emm*13	373	Forward	CGCCAGGGTTTTCCCAGTCACGAC
		Reverse	AGCGGATAACAATTTCACACAGGA
*glr**	797	Forward	ATGGATACAAGACCAATTGG
		Reverse	TCATAAGGTGACATGCTCCAC

* Reference gene for *S.pyogenes*

RESULTS

Minimum inhibitory concentrations and minimum bactericidal concentrations. Under visual inspection, the MIC and MBC for TH against *P. aeruginosa* were 18.5% (w/v) to 25% (w/v) respectively, while the MIC and MBC for TH against *S. pyogenes* were 13% (w/v) and 25% (w/v) respectively. When tested against *P. aeruginosa* and *S. pyogenes*, TH had equal MBC value (25% (w/v). Spectrophotometric readings of at least 90% inhibition (MIC90) gave MIC values for TH 18.5% (w/v) and 15% (w/v)

against *P. aeruginosa* and *S. pyogenes* respectively. (Fig. 1 and Table 3).

Growth kinetics curves. Growth kinetic curves of untreated *P. aeruginosa* and *S. pyogenes* demonstrated that under the experimental conditions, exponential phase occurred between 1 and 11 hours. *P. aeruginosa* and *S. pyogenes* samples treated with (MIC) TH demonstrated zero growth. Treatment with (half-MIC) TH resulted in an increased lag phase, decreased exponential phase and lower absorbance values during stationary phase. Conversely, treatment with (quarter-MIC) TH was observed to have a similar lag phase and exponential phase to untreated samples. However, the rate of exponential growth during the end of this growth phase appeared greater, resulting in higher absorbance values during early stationary phase, subsiding below that of untreated samples with the onset of death phase (Fig. 2).

Time kill curve. The time-kill curve clearly shows an increase in number of *P. aeruginosa* and *S. pyogenes* cells without TH treatment. However, MIC TH resulted in 2.36-$\log_{10}$ and 2.48-$\log_{10}$ reduction in CFU/ml of *P. aeruginosa* and *S. pyogenes* cells respectively compared to the starting inoculum at 8 hours incubation. Therefore, *P. aeruginosa* and *S. pyogenes* incubated with $2 \times$ MIC TH in 3.51-$\log_{10}$ and 3.64-$\log_{10}$ reduction in CFU/ml respectively compared to the starting inoculum at 8 hours incubation. Although, the mean difference in number of colony forming units between non-honey and honey treated were statistically significant ($P < 0.05$). The MIC and MBC were confirmed by growth kinetics curves and time–kill studies, where cells exposed to tualang and TH were found to lose viability with time, yet the numbers of untreated cells increased.
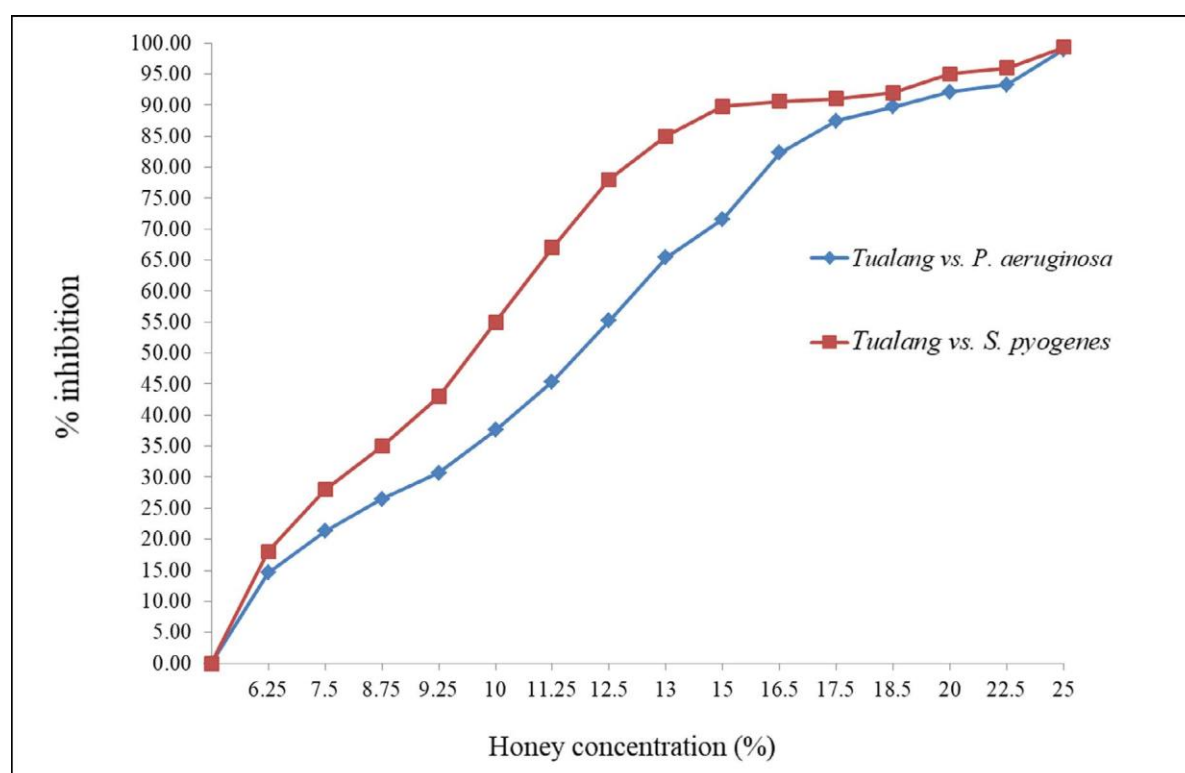

Fig. 1. Inhibition of the growth of *P. aeruginosa* and *S. pyogenes* caused by TH at different concentrations

Table 3. MIC values (%) determined by visual inspection and spectrophotometric measurement and MBC values (%) of TH

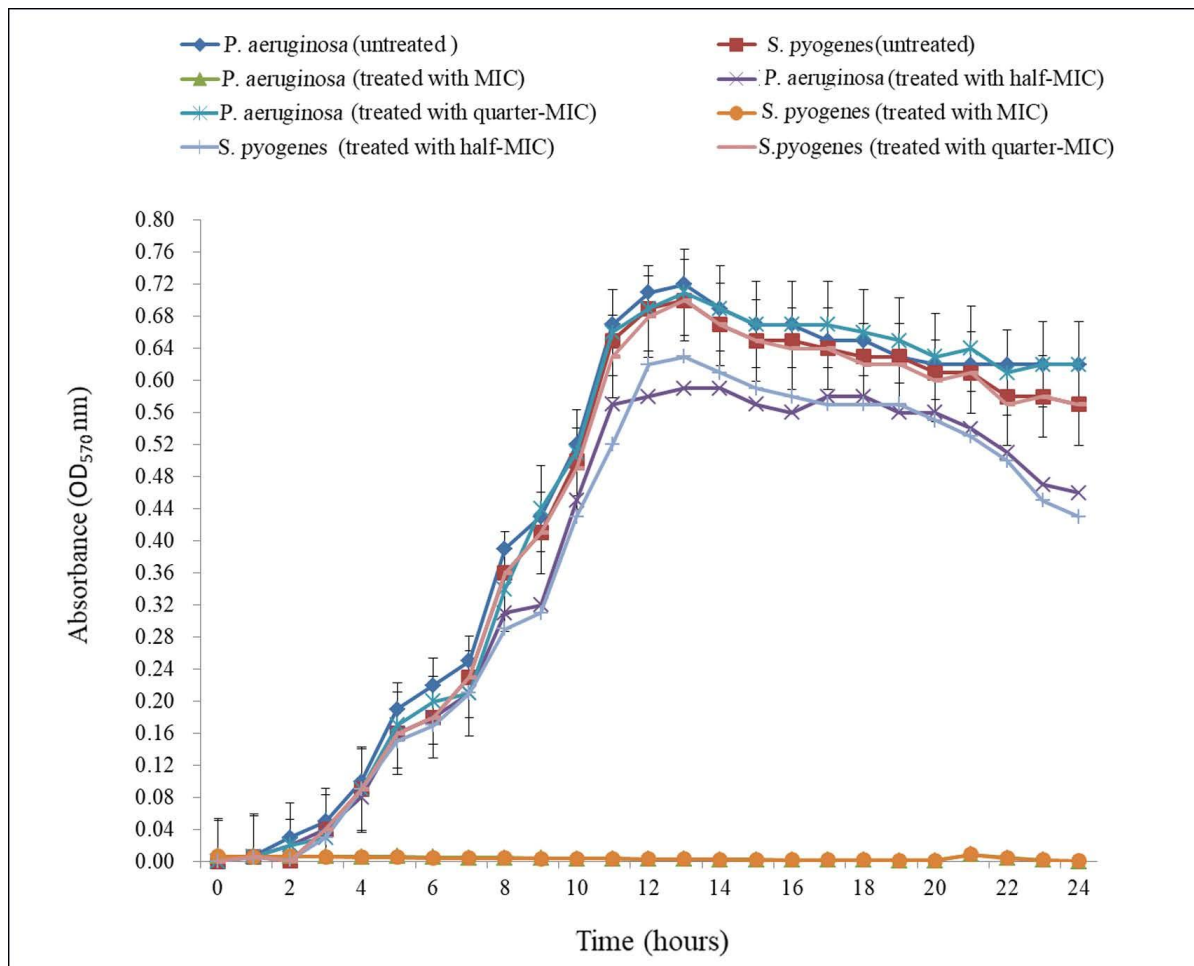| Microorganism	Visual MIC (%)	Spectrophotometric MIC90 (%)	MBC (%)
P. aeruginosa	18.5	18.5	25
S. pyogenes	13	15	25

Fig. 2. Growth kinetics curve of *P. aeruginosa* and *S. pyogenes* cells grown with (MIC, half-MIC and quarter-MIC) and without TH

The time estimated to achieve a 4 log reduction of both bacteria incubated with MIC and $2 \times$ MIC (w/v) TH was 8 hours (Fig. 3).

Scanning electron microscopy (SEM). SEM micrograph of untreated cells of *P. aeruginosa* incubated with broth only was observed to have a regular rod shaped cell with a smooth surface and normal cell size. However, after 8 hours incubation with MIC TH this bacterium appeared enlarged and elongated. Honey-treated cells also appeared to be shortened and to have distorted shapes. In addition, clumping and cell aggregation increased. This suggests that the effect of TH on *P. aeruginosa* is not due exclusively to the sugars contained in honey (Fig. 4).

SEM micrograph of *S. pyogenes* incubated with broth alone (no honey) was found to have a conventional cocci shape after 8 hours incubation in liquid media. In SEM micrographs of MIC TH treated cells,

cell size and shape appear to be different to untreated cells. However, cell size increased after exposure to TH. In addition, morphological changes, in which the lysed cells started to clump together as compared to typical spherical morphology of *S. pyogenes* (Fig. 5).

Effects of TH on the mRNA expression of *P. aeruginosa*. In the present study, RT=qPCR was used to evaluate and compare the impacts of exposure of *P. aeruginosa* and *S. pyogenes* cells to TH (at MIC; for 8 h) on the expression of genes that have been previously shown to be involved in virulence of the microorganisms. As revealed by the t-test (SPSS version 20), there was a significant overall difference ($P < 0.05$) in the expression of each of the tested genes among the different groups. All genes were down-regulated following exposure to TH under study though different degrees of down-regulation
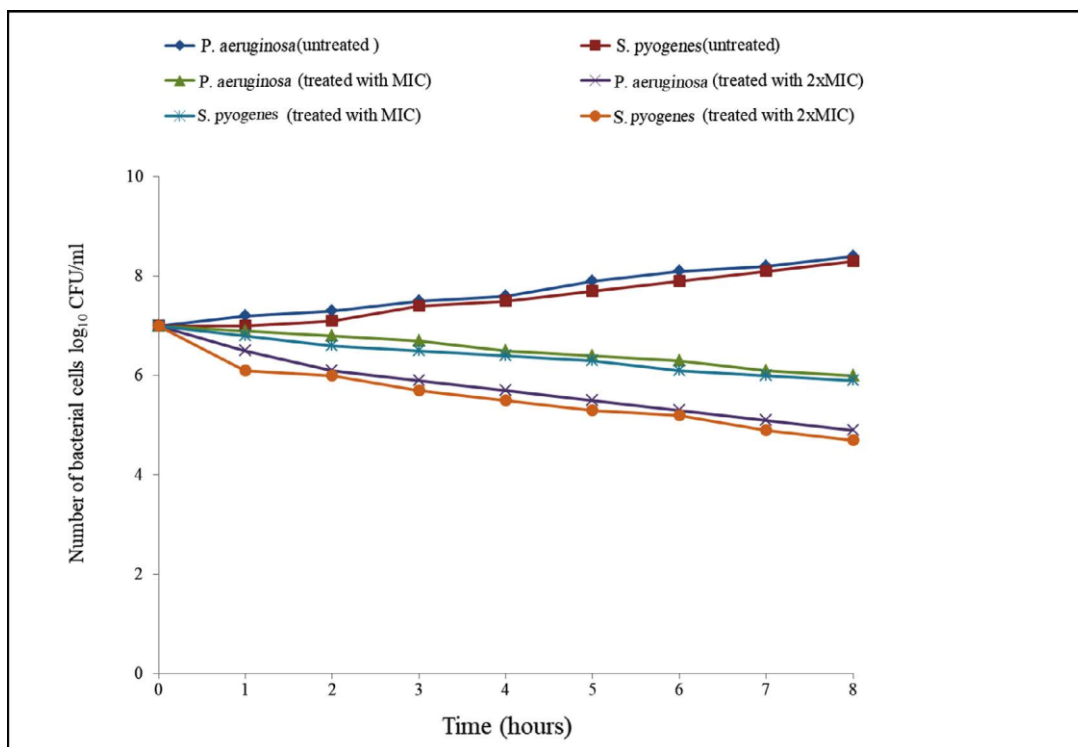
Fig. 3. Culturability of *P. aeruginosa* and *S. pyogenes* samples grown with (MIC and 2 × MIC) and without TH
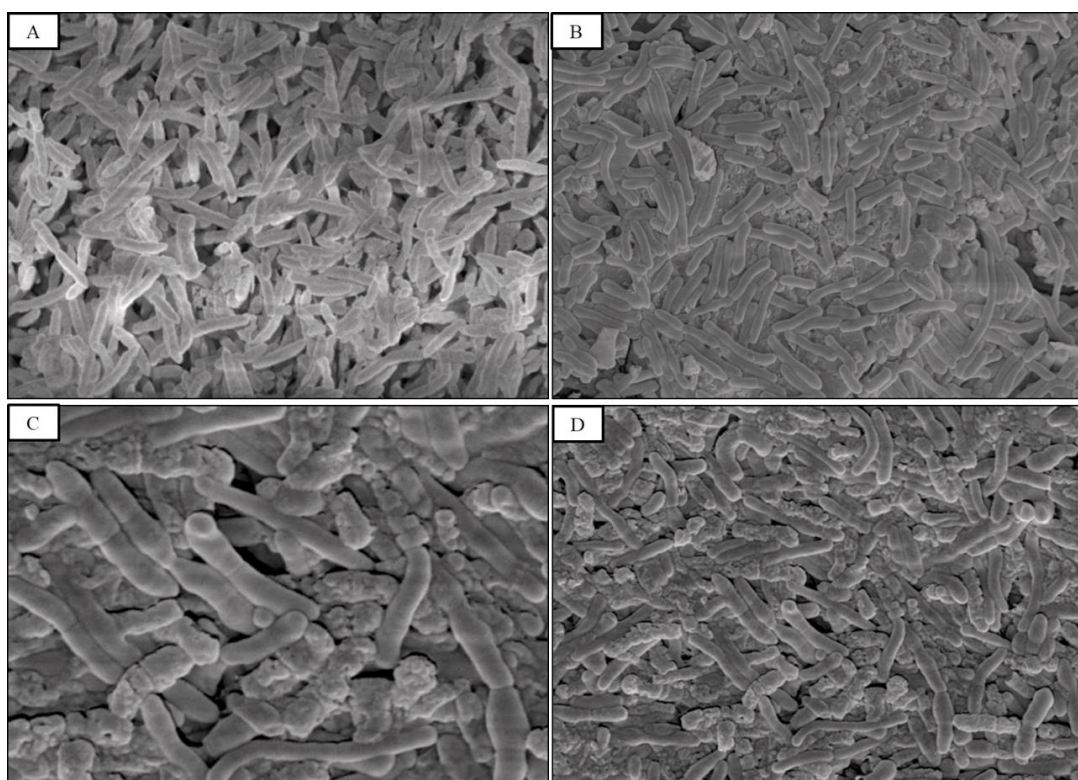

Fig. 4. SEM micrographs of *P. aeruginosa* cells exposed to MIC TH at 10,000× magnification. *P. aeruginosa* incubated with broth only (A and B). *P. aeruginosa* incubated with MIC TH (C and D).
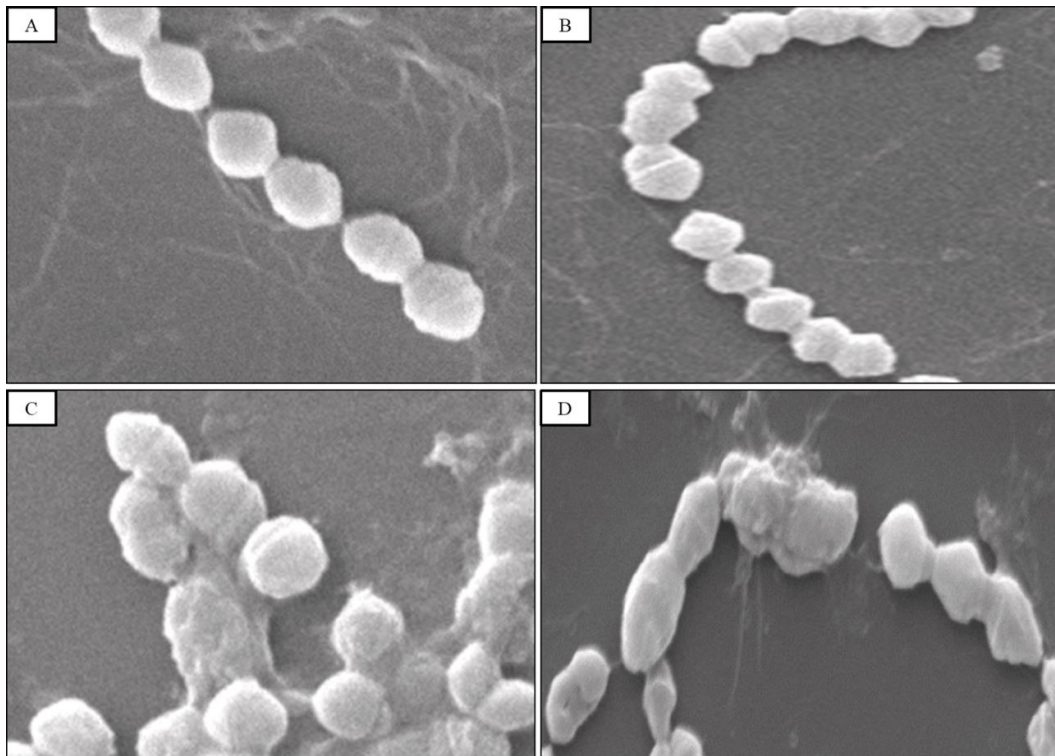
Fig. 5. SEM micrographs of *S. pyogenes* cells exposed to MIC TH at ×10,000 magnification. *S. pyogenes* incubated with broth only (A and B. *S. pyogenes* cells incubated with MIC TH (C and D).

were observed following exposure to MIC TH. Two (*algD* and *oprF*) microcolony forming-associated genes investigated showed a statistically significant reduction (P <0.05) in gene expression with 0.33-fold change and 0.31-fold change following treatment with MIC TH respectively. Five (*fleN, fleQ, fleR, fliA* and *fliC*) of the flagellum-associated genes investigated showed a statistically significant reduction (P <0.05) in gene expression following treatment with MIC TH. This suggests that TH inhibits flagellum gene expression by impacting on both regulatory (*fleN, fleQ, fleR* and *fliA*). Following treatment with MIC TH there was a significant reduction (P <0.05) in gene expression of *oprB* and *oprH* that involved in the outer. The fold change of mRNA transcripts of *oprB* and *oprH* that was 0.22-fold and 0.43-fold following treatment with MIC TH respectively (Fig. 6).

Effects of TH on the mRNA expression of *S. pyogenes*. The major genes encoding the surface adhesins *sof, sfbl, scpA, ftsY* and *emm*13 in *S. pyogenes* showed different degrees of down-regulation in response to MIC TH. Following treatment with MIC TH there was a significant reduction (P <0.05) in the expression of mRNA transcripts for *sof, sfbl, scpA,*

ftsY and *emm*13 in *S. pyogenes* (Fig. 7).

DISCUSSION

The therapeutic potential of uncontaminated, pure honey is grossly underutilized. It is widely available in most communities and although the mechanism of action of several of its properties remain obscure and needs further investigation, the time has now come for conventional medicine to lift the blinds off this traditional remedy (39). This study was carried out to evaluate the efficacy of TH in the inhibition of *P. aeruginosa* and *S. pyogenes*. The antibacterial potency of each honey was determined by MIC and MBC, growth curve, time-kill study, SEM and RT-qPCR. Each experiment provided some insight into possible inhibitory mechanisms of TH on both bacteria. Tualang honey (TH) is readily available in Malaysia, but its quality and floral origin have yet to be determined and standardized. In this study, we found that TH has variable broad-spectrum activities against *P. aeruginosa* and *S. pyogenes*. We chose the broth dilution method for this study because it generates more quantitative and precise results compared to the
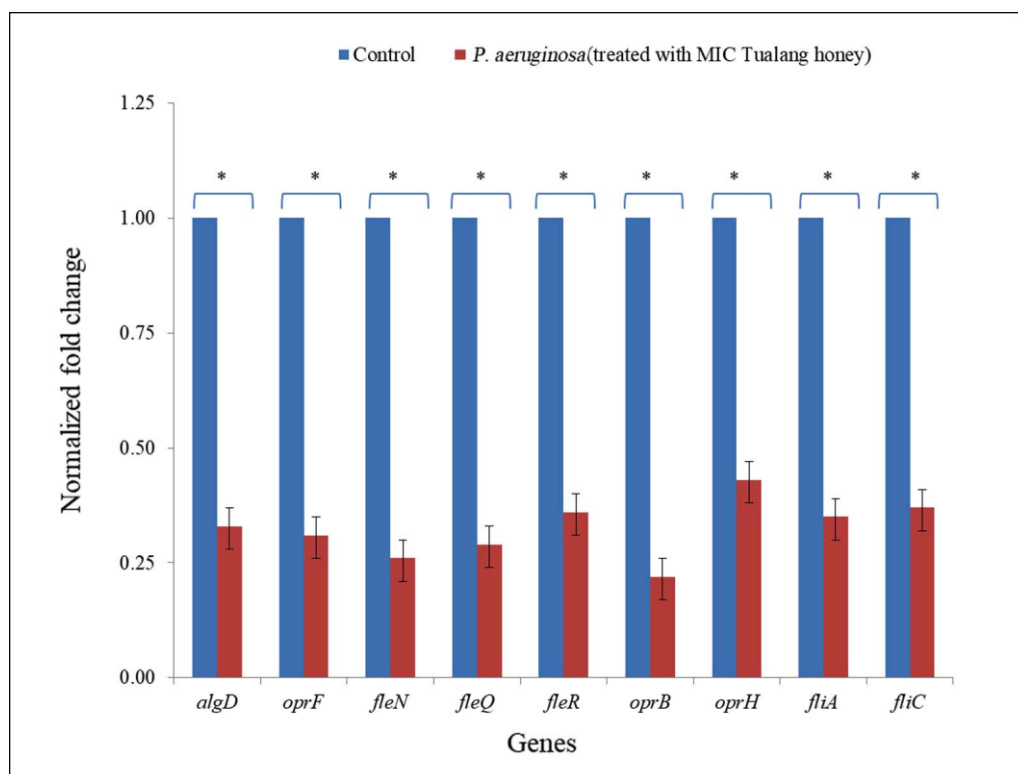
Fig. 6. mRNA expression of genes in *P. aeruginosa* following treatment with MIC TH. Results are expressed as the mean fold change (control standardised to 1.0) with error bars (* *P* <0.05).
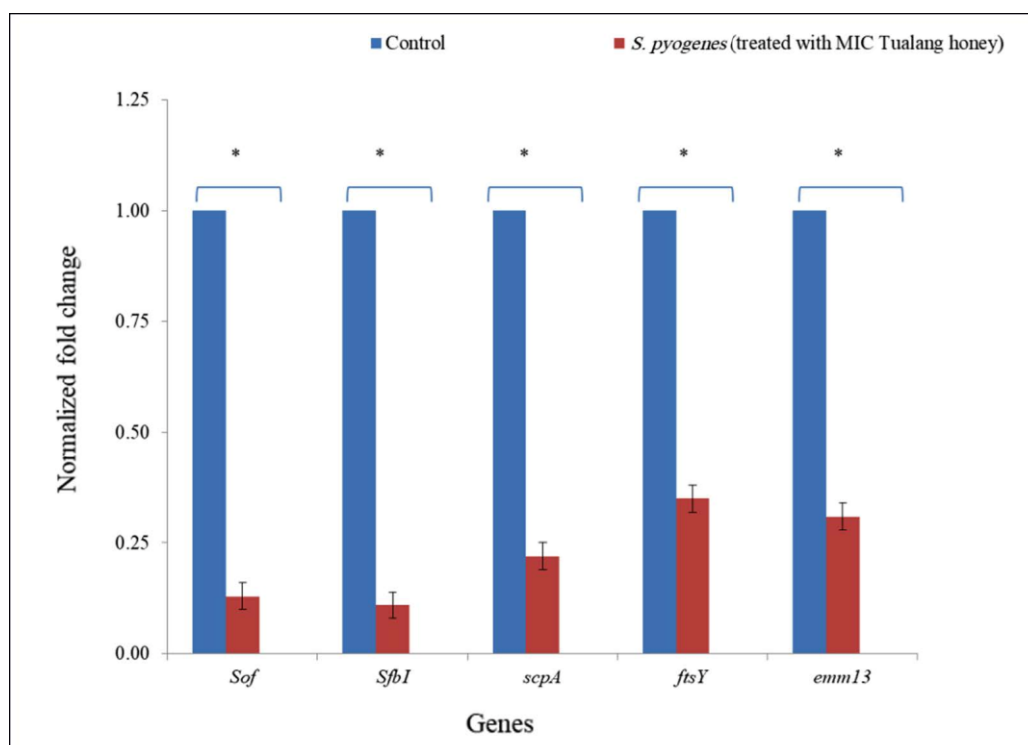

Fig. 7. mRNA expression of genes in *S. pyogenes* following treatment with MIC TH. Results are expressed as the mean fold change (control standardised to 1.0) with error bars (* *P* <0.05).

agar well diffusion method. Moreover, the MIC values determined by the broth dilution method might be accurate (indicating higher activity) than those obtained using the agar well diffusion method, as diffusion rates of active constituents in agar may be slower than those in broth (40). We also performed spectrophotometric assays using microtiter plates; it is a simple and rapid method, it has a greater sensitivity than the standard well and disc diffusion methods and the results are highly reproducible (41). Spectrophotometry can detect inhibitory levels below those recorded for well or disc diffusion assays (41). Historically, both MIC and MBC values have been used as indicators of bacteriostatic or bactericidal activity for antimicrobial compounds. The TH used in this study had MIC values of 18.5% and 13% (w/v), for *P. aeruginosa* and *S. pyogenes* respectively and had MBC value of 25% (w/v) for both bacteria. Thus, more effective treatment is needed to treat *P. aeruginosa* and *S. pyogenes*. Analysis of the inhibition of bacterial growth caused by different honey concentrations revealed the differences in the pattern of inhibition exhibited by both bacteria. The observed differences might reflect how each type of bacteria reacts to honey treatment. Growth analysis confirmed MIC concentrations and identified disparate growth curves for both bacteria treated with sub-inhibitory concentration of TH, suggesting the sugar and antimicrobial fractions are affected differently by the dilution factor. Decreased growth was observed in bacteria treated with TH at half-MIC, indicating the antimicrobial fraction, whilst diluted, still retained inhibitory activity. Time-kill curves studies are used to measure the killing rate of organism by TH using time and concentration. A bactericidal activity is defined as *in vitro* activity of 3 log reduction in the CFU/ml or 99.9% killing over a specific period of time (42). Killing measurement was made in this study by the actual decrease in viable counts at 8 h for both bacteria. There was significant differences between treated and untreated TH sample (*P* <0.05). The loss of structural integrity in *P. aeruginosa* and *S. pyogenes* treated with TH was observed and cells under SEM images were short, distorted and displayed cell surface abnormalities. Previous study is in agreement with our finding showed that loss of structural integrity and marked changes in cell shape of *P. aeruginosa* after exposure to manuka honey (36). Previous study revealed that Trigona honey had decreased cell density, and cells appeared curved of

P. aeruginosa and rough, holes and crevices of *S. pyogenes* (33). The analysis of gene expression found differential expression of *algD, oprF, fleN, fleQ, fleR, fliA* and *fliC* genes of *P. aeruginosa* when TH was applied, suggesting the impact of TH on numerous aspects of the flagellar regulon. In turn, the differential suppression of *fliC* and *fliA* occurred. Hence, the repression of flagella-associated genes allows TH to mediate the de-flagellation of *P. aeruginosa*, which leads to decreased motility, adherence and virulence. In addition, it is likely that the reduced expression of the three outer membrane proteins (*oprB, oprH* and *oprF*) caused the reduced survivability of *P. aeruginosa*. As demonstrated by RT-qPCR, the expression of *sof, sfbl, scpA, ftsY* and *emm*13 genes had decreased. These genes are the major genes encoding the surface adhesins in *S. pyogenes*, the decreased expression of these genes in *S. pyogenes* following the TH treatment might show that the TH restricts the growth and biofilm formation of *S. pyogenes*. Among the physicochemical parameters of honey, the acidity and the osmolarity represent the principal factors responsible for the antimicrobial activity of honey. However, there are other factors that are closely related to the antimicrobial capacity of honey such as the hydrogen peroxide content, and other non-peroxide components such as methylglyoxal, the antimicrobial peptide bee defensin-1, polyphenols and other compounds from the bees (43).

CONCLUSION

TH exhibited variable activities against both bacteria. In some cases, it showed equivalent or better activities against Gram-positive bacteria than Gram-negative bacteria. The potency of TH against certain microorganisms suggests its potential to be used as an alternative therapeutic agent for certain medical conditions, particularly wound infection. Together, our results revealed that the tested TH has the potential to be effective inhibitors of *P. aeruginosa* and *S. pyogenes*. Differential gene expression in response to TH exposure exhibited downregulation of several virulence genes of *P. aeruginosa* and *S. pyogenes*. The obtained results indicate that the TH under study may represent promising antibacterial and anti-virulence agents for treatment and modulation of infections caused by *P. aeruginosa* and *S. pyogenes*. Further investigation into its effect on

cellular and molecular targets is warranted, and its clinical efficacy must be confirmed.

ACKNOWLEDGEMENTS

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