RESEARCH ARTICLE

A Comparative Study of Antibacterial Activity of Citrus and Jabali Honeys with Manuka Honey

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Abstract: Honey is now being renowned as an alternative treatment due to its broad-spectrum antibacterial activity. The purpose of this study was to compare the antibacterial activity of Citrus honey (CH) and Jabali Honey (JH) with that of Manuka honey (MH) against *Pseudomonas aeruginosa*. The antibacterial, antibiofilm and antivirulence activities of CH, JH and MH against *P. aeruginosa* were investigated by agar well diffusion, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), time-kill curve, microtiter plate and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Agar inhibition assay showed that the CH, JH and MH at 20% have antibacterial activity with an inhibition zone of 14.1±0.1 mm, 12.2±0.2 mm and 10.9±0.1 mm respectively against *P. aeruginosa*. The results showed that the MIC values for CH and JH were 25% compared to MH (12.5%) and the MBC values for CH and JH were 50% compared with MH (25%) against *P. aeruginosa*. In addition, the MIC_{50} and MIC_{90} values for CH and JH were 25% and 50% respectively compared with MH (MIC_{50}: 12.5% and MIC_{90}: 25%) against *P. aeruginosa*. In time-kill curve, treatment *P. aeruginosa* with 2×MIC of MH, CH and JH for 9-hours resulted in reduction in colony-forming unit. The lowest concentration 20% of MH, CH and JH was found to inhibit and eradicate *P. aeruginosa* biofilm. RT-qPCR analysis revealed that the expression of all genes (*oprB*, *oprC*, *fleN*, *fleQ*, *fleR*, *lasR* and *lasI*) in *P. aeruginosa* were downregulated after exposure to all the tested honeys. Among the all-tested honeys, MH showed the highest total antibacterial, antibiofilm and antivirulence activities against *P. aeruginosa*. This study indicates that CH and JH has antibacterial and antibiofilm activities compared with that of MH due to a decrease in expression of essential genes associated with *P. aeruginosa*.
Keywords: *P. aeruginosa*, virulence genes, honey, antibacterial activity, gene expression.

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1 Introduction

Honey has good antibacterial activity against numerous microorganisms of many different genera and no honey-resistant phenotypes have yet emerged (Maddocks and Jenkins, 2013). However, the current and growing crisis of antibiotic resistance has revived interest in the use of honey, both as an effective agent in its own right and as a therapeutic lead to develop new methods of treatment (Carter et al., 2016). Honey has been used as a medicine throughout the history of the human race and has been employed to treat a wide variety of ailments (Blair and Carter, 2005). One of the most common and persistent therapeutic uses of honey has been as a wound dressing, almost certainly due to its antimicrobial properties (Carter et al., 2016; Pećanac et al., 2013). Honey has been reported to contain about 200 substances (Eteraf-Oskouei and Najafi, 2013). Honey is composed primarily of fructose, glucose, minerals, vitamins, amino acids, and enzymes (Eteraf-Oskouei and Najafi, 2013). The antimicrobial activity of honey may be attributed to multiple factors, including acidity, high osmolarity, and presence of hydrogen peroxide (H_2O_2) and non-peroxide components, such as methylglyoxal (Brudzynski et al., 2011; Mavric et al., 2008). The composition of honey and nutritional quality depend on botanical source, geographic location, bee species as well as processing and storage conditions (Elbanna et al., 2014; Machado De-Melo et al., 2018; Murean et al., 2022). Recently, several studies are focusing alternative antimicrobial strategies such as using plants and honeys products to treat bacterial infections (Bouacha et al., 2022; Maddocks and Jenkins, 2013; Sofowora et al., 2013). The use of traditional medicine to treat infection has been practiced since the existence of mankind and is one of the oldest traditional medicine which is important for the treatment of human ailments (Boorn et al., 2010). In addition, 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, virulence factor

production, as well as multicellular behaviors, such as biofilm formation, and quorum sensing (Abbas, 2014; Al-kafaween et al., 2020; Blair et al., 2009; Jenkins et al., 2014; Wang et al., 2012; Wasfi et al., 2016). In addition, since no data are available in the literature regarding the comparative of the antibacterial activity of CH and JH with that of MH on gene expression in *P. aeruginosa*. Therefore, the present study aimed to compare the antibacterial activity of CH and JH with that of MH against *P. aeruginosa* at planktonic, biofilm and molecular level.

2 Materials and Methods

2.1 Honey samples

Manuka honey (MH), Citrus honey (CH) and Jabali Honey (JH) were purchased from commercial supplier in Amman a state in Jordan. Stock samples 100% were stored in the dark bottles, labelled accordingly and stored at room temperature away from light source. The honey samples were prepared freshly for each experiment (Al-kafaween et al., 2020; Al-kafaween et al., 2021).

2.2 Test microorganisms and culture maintenance

One clinical sample of *Pseudomonas aeruginosa* isolated from diabetic foot ulcers and *P. aeruginosa* (ATCC 9027) was selected as references strain. *P. aeruginosa* were streaked on nutrient agar (NA) at 37°C for 24-hours. Subsequently, 2 to 3 colonies from agar plate were picked and grown in nutrient broth (NB) for 24 hours at 37°C. After incubation, 1.5 ml of culture was added to the 2 ml vial (20% glycerol final) and the mixture were inverted for 10 seconds. The samples were stored in the freezer at -80°C (Al-kafaween and Hilmi, 2022; Magharbeh et al., 2021; Lu et al., 2019; Roberts et al., 2012).

2.3 Determination of activity of honey on bacteria using the agar well diffusion assay

Sterile distilled water was used to dilute MH, CH and JH to achieve 80%, 60%, 40% and 20% (w/v) concentration of honey. The inoculum of *P. aeruginosa* was adjusted to be equal to 0.5 McFarland standard. A sterile cotton swab was then dipped into the bacterial suspension and streaked over the entire surface of Mueller Hinton Agar (MHA) plate. A sterile 9-mm cork borer was used to create wells on an agar plate. The wells of agar plate were labelled and were added with 150 μL of 100%, 80%, 60%, 40% and 20% (w/v) of MH, CH and JH. Wells with distilled water were served as negative control. The agar plate was incubated at 37°C for 24 hours. Digital venire calliper was used to measure the zones of inhibition. The assay was carried out in triplicate (Al-kafaween et al., 2020; Khleifat et al., 2022; Al-kafaween et al., 2021; Tarawneh et al., 2022; Tarawneh et al., 2021).

2.4 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of honey on bacteria

The minimum inhibitory concentration (MIC) value was determined using broth microdilution method. Briefly, the concentrations of MH, CH and JH; 75%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56 % (w/v) were freshly prepared with NB broth. The inoculum of *P. aeruginosa* was adjusted to be 0.5 McFarland standard as described previously. A 100 μL of culture was transferred into microtiter plate with 100 μL of MH, CH and JH. Broth medium only was used as negative control and inoculum alone was served as positive control. The plate was incubated for 24 hours at 37°C. Absorbance was measured by using the microtiter plate reader at 570 nm. The MIC_{50} and MIC_{90} values were determined by using the following formula as mentioned below. The MBC test was performed using streak plate method. A 30 μL from each well of the microdilution method (MIC assay) was taken and plated onto NA plate. The plate was incubated for 24 hours at 37°C. MBC was considered as the lowest honey concentration that produced no colony growth (Zainol et al., 2013; Al-kafaween et al., 2022).

2.5 Time-kill curve of *P. aeruginosa* against honeys

The effect of MH, CH and JH on the viability of the cells was determined by time-kill curve. By inoculating 100 μL of 1×10^6 CFU/mL of *P. aeruginosa* into 15 mL of NB with and without 2×MIC of MH, CH and JH. The samples were incubated at 37°C in a shaking water bath (100 rpm) for 9 hours. Then, the samples were collected every 3 hours up to 9 hours. The mean of Log_{10} CFU/ml over time were plotted for each sample. Subsequently, the log reduction (LR) was calculated by subtracting the Log_{10} CFU at zero time and the Log_{10} CFU at 9 hours of incubation (Al-kafaween et al., 2021; Zainol et al., 2013).

2.6 Microcolony formation

The inoculum of *P. aeruginosa* was adjusted to be 0.5 McFarland standard as described previously. A 200 of the culture was transferred into 16-mm glass coverslips in 24-well plates and incubated for 24 hours at 37°C. Then, 100 μL of planktonic cells were removed and 100 μL of 20%, 10%

$$\text{Growth inhibition (\%)} = \frac{1 - \text{OD (test well)} - \text{OD (corresponding negative control)}}{\text{OD (bacterial growth)} - \text{OD (sterility control)}} \text{ x } 100\%$$

$$\text{Biofilm inhibition (\%)} = \frac{OD_{570}\text{ (positive control)} - OD_{570}\text{ (individual or (combined) antimicrobial)}}{OD_{570}\text{ (positive control)}} \text{ x } 100\%$$

$$\text{Biofilm eradication (\%)} = \frac{OD_{570}\text{ (positive control)} - OD_{570}\text{ (individual or (combined) antimicrobial)}}{OD_{570}\text{ (positive control)}} \text{ x } 100\%$$

and 5% (w/v) of MH, CH and JH was added to the wells. Subsequently, the plate was incubated for 24 hours at 37°C. Inoculum alone was used as positive control. Coverslips were washed with PBS and stained with 0.1% (w/v) crystal violet. Images were viewed by light microscopy at 1000x magnification (Al-kafaween et al., 2021; Al-kafaween and Hilmi, 2022).

2.7 Biofilm inhibition assay

To determine the concentrations of MH, CH and JH required to inhibit *P. aeruginosa* biofilm. Different concentrations; 80%, 60%, 40%, 20%, 10% and 5% (w/v) of MH, CH and JH were freshly prepared by using NB broth. Inoculum of *P. aeruginosa* was adjusted to be 0.5 McFarland standard as described previously. A 100 μL of each diluted honey was dispensed into 96-well plates and inoculated with 100 μL of inoculum. Wells with honey was served as corresponding negative control and inoculum without added honey was used as positive control. The plate was incubated for 24 hours at 37°C. After incubation time was done, the media were removed and the plate was washed with PBS to remove planktonic cells. The plate was stained with 200 μL of 0.1% crystal violet for 3 minutes. Then, the plate was rinsed under running tap water and dried at room temperature before solubilizing the biofilm with 95% of ethanol. The absorbance was measured by using a microplate reader at 570 nm. Percentage of biofilm inhibition was calculated by following formulas as mentioned below. The experiment was performed in triplicate (Huwaitat et al., 2021; Al-kafaween et al., 2021; Tarawneh et al., 2022; Tarawneh et al., 2021).

2.8 Biofilm eradication assay

Different concentrations of MH, CH and JH; 80%, 60%, 40%, 20%, 10% and 5% (w/v) were freshly prepared by using NB broth. Inoculum of *P. aeruginosa* was adjusted to be 0.5 McFarland standard as described previously. Then, 100 μL of the culture was transferred into wells of microtiter. The plate was incubated at 37°C for 24 hours. After biofilms were formed, 50 μL of planktonic cells were removed and replaced with 50 μL of MH, CH and JH; 80%, 60%, 40%, 20%, 10% and 5% (w/v). Then, the plate was incubated for overnight at 37°C. Biofilm without honey treatment was served as positive control, broth alone was employed as sterility control, and honey alone was served as corresponding negative control. Then, the media were removed and the plate washed with PBS to remove free-floating planktonic bacteria. The plate was drained and stained with 100 μL of 0.1% crystal violet for 5 minutes. Finally, the plate was rinsed with distilled water and dried at room temperature before solubilizing the biofilm with 95% of ethanol. Absorbance was determined at 570 nm wavelength using microplate reader. The percentage of biofilm eradication was calculated by following formulas as described below. The experiment was carried out in triplicate (Huwaitat et al., 2021; Al-kafaween et al., 2021; Tarawneh et al., 2022; Tarawneh et al., 2021).

Table 1. Gene-specific primers of *P. aeruginosa* used for RT-qPCR analysis

Gene name	Amplicon Size (bp)	Annealing temp (°C)	Direction	Primer sequence(5' → 3')
oprB	140	54	Forward	TGACGACGACAAGACAGGAC
			Reverse	GGTCGTTGGAAAGGTTCTTG
oprC	105	55	Forward	GCCTGAACATCCTCACCAAC
			Reverse	CGGTGAGCTTGTCGTAGGTT
fleN	137	56	Forward	GAGCCGTATACGAGGCATTC
			Reverse	GTGTTGGACCAGTCGTTCG
fleQ	134	54	Forward	AAGGACTACCTGGCCAACCT
			Reverse	CCGTACTTGCGCATCTTCTC
fleR	109	55	Forward	ACAGCCGCAAGATGAACCT
			Reverse	TGGATGGCGTTGTCGAGTT
lasR	129	54	Forward	CGGTTTTCTTGAGCTGGAAC
			Reverse	TCGTAGTCCTGGCTGTCCTT
lasI	129	54	Forward	ATGATCGTACAAATTGGTCG
			Reverse	GTCATGAAACCGCCAGTCG
rpoD *	146	53	Forward	GCGACGGTATTCGAACTTGT
			Reverse	CGAAGAAGGAAATGGTCGA

Note: Reference gene

2.9 RNA extraction for RT-qPCR

Inoculum of *P. aeruginosa* was adjusted to be 0.5 McFarland standard as described previously. A 100 μL of the culture was dispensed into 96-well plate with 100 μL MIC of MH, CH and JH. The plate was incubated for 24 hours at 37°C. Inoculum without honey treatment was used as positive control. The mixture was resuspended in 1 ml of PBS, vortexed and centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and the pellet was washed in phosphate buffered saline. The total RNA of treated and untreated samples were extracted using SV Total RNA Isolation System (Promega, UK). After RNA extracted, the total RNA was quantified by using a NanoDrop. Subsequently, RNA purity levels were assessed at 260/280 and 260/230 absorbance ratio. Only sample ranged between 1.8 and 2.1 being accepted for conversion to cDNA. The samples were adjusted to 100 ng/ μL to convert to cDNA. The RNA was stored at -80°C. Total RNA samples were converted to cDNA by using a cDNA conversion kit (Promega, UK). Following conversion to cDNA, samples were stored at -20°C for further experiment. Sequences of the primers for *P. aeruginosa* were used as shown in Table 1. Lyophilised and desalted oligonucleotides were reconstituted

using sterile ultra-pure water. Oligonucleotides of *P. aeruginosa* were optimised and the efficiencies were tested prior to gene expression analysis. The RT-qPCR mastermix for each reaction was prepared based on the manufacturers instructions (Promega, UK). The following PCR protocol was used: One cycle of denaturation at 95°C for 2 minutes, 40 cycles of amplification at 95°C for 15 seconds and 40 cycles of annealing at 60°C for 1 min. Densitometry was performed by using the Applied Biosystems StepOne Software v2.3. To determine the level of relative gene expression, a modified $2^{-\triangle\triangle}$ Ct method was used. The experiment was performed in triplicate (Al-kafaween et al., 2021; Jarrar et al., 2019; Jarrar et al., 2022; Livak and Schmittgen, 2001; Schmittgen and Livak, 2008; Wasfi et al., 2016; Yadav et al., 2012).

2.10 Statistical analysis

For all assays, all experiments were performed in triplicate. All data were expressed as mean ± standard deviation. Independent student t-test from (SPSS version 20) was used to compare between treated and untreated groups. The statistical analyses performed were considered significant when $P<0.05$.

3 Results

3.1 Determination of activity of honey on bacteria using the agar well diffusion assay

Agar well-diffusion assay shows the zone of inhibition for *P. aeruginosa* after being treated with MH, CH and JH. All the tested honeys were observed to have antibacterial activity against *P. aeruginosa*. Agar inhibition assay demonstrated that the lowest concertation of MH, CH and JH at 20% has antibacterial activity against *P. aeruginosa* with an inhibition zone of 14.1±0.1 mm, 12.2±0.2 mm and 10.9±0.1 mm respectively. In general, MH, CH and JH showed a measurable antibacterial activity against *P. aeruginosa* with different values (Table 2).

Table 2. Antibacterial activity (Inhibition Zone(mm)±SD) of all the tested honeys against *P. aeruginosa*

Honey samples	100%	80%	60%	40%	20%
Manuka honey (MH)	26.2±0.5	24.2±0.3	22.5±0.2	21.6±0.1	14.1±0.1
Citrus honey (CH)	23.5±0.4	21.6±0.5	20.8±0.3	19.5±0.3	12.2±0.2
Jabali Honey (JH)	21.2±0.3	19.5±0.4	18.7±0.3	17.4±0.3	10.9±0.1

3.2 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of honey on bacteria

The MIC values for MH, CH and JH against *P. aeruginosa* were 12.5%, 25% and 25% (w/v) respectively. The MIC_{50} values for MH, CH and JH against *P. aeruginosa* were 12.5%, 25% and 25% (w/v) respectively. The MIC_{90} values for MH, CH and JH against *P. aeruginosa* were 25%, 50% and 50% (w/v) respectively. The MBC values for MH, CH and JH against *P. aeruginosa* were 25%, 50% and 50% (w/v) respectively (Figure 1 and Table 3).

Table 3. MIC, MIC_{90}, MIC_{50} and MBC of MH, CH and JH against *P. aeruginosa*

Honey samples	MIC % (w/v)	MIC_{50} % (w/v)	MIC_{90} % (w/v)	MBC % (w/v)
Manuka honey (MH)	12.50%	12.50%	25%	25%
Citrus honey (CH)	25%	25%	50%	50%
Jabali honey (JH)	25%	25%	50%	50%

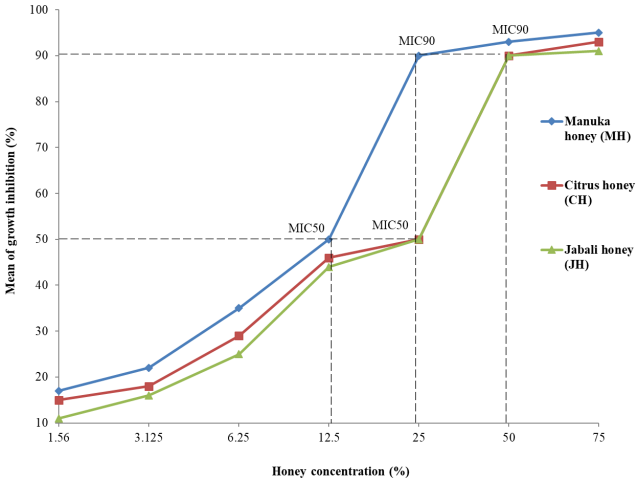


Figure 1. Growth inhibition of *P. aeruginosa* following treatment with MH, CH and JH

3.3 Time-kill curve of *P. aeruginosa* against honeys

The total number of *P. aeruginosa* cells significantly decreased when exposed to 2×MIC of all the tested honeys. Therefore, after treatment with 2×MIC of MH, CH and JH, *P. aeruginosa* resulted in a 1.3-Log_{10}, 1.1-Log_{10} and 0.7-Log_{10} reduction in CFU/ml compared to untreated cells at 3-hours incubation ($P<0.05$) respectively. In addition, after exposure to 2×MIC of MH, CH and JH, *P. aeruginosa* resulted in a 2.5-Log_{10}, 1.8-Log_{10} and 1.5-Log_{10} reduction in CFU/ml compared to untreated cells at 6-hours incubation ($P<0.05$) respectively. *P. aeruginosa* incubated with 2×MIC of MH, CH and JH demonstrated that the greatest bactericidal activity at 9 h incubation with ≥2.9-Log_{10} for MH and ≥2.5-Log_{10} for CH and JH (Figure 2).

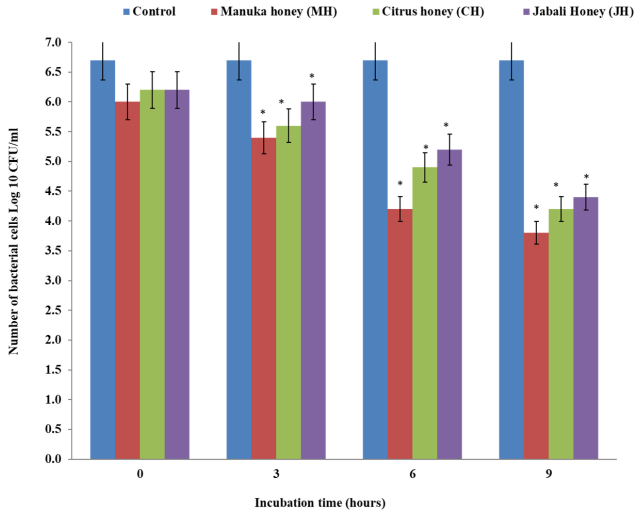
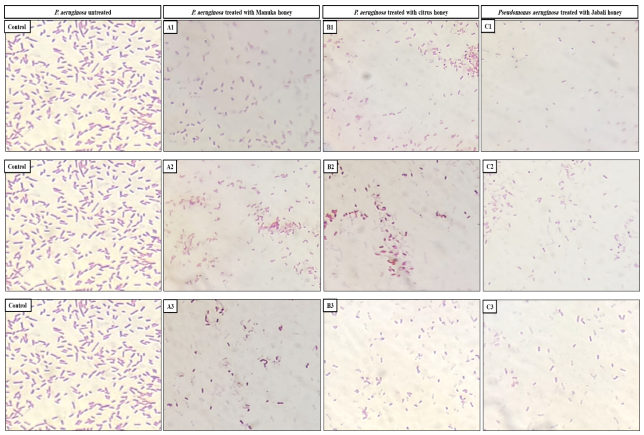


Figure 2. Time-kill studies of *P. aeruginosa* after exposed to MH, CH and JH. Asterisks; *P<0.05 indicate statistically significant difference between treated and control samples.

3.4 Microcolony formation

MH, CH and JH were found to disrupt microcolony formation in *P. aeruginosa* at 5%, 10%, and 20% concentrations of MH, CH and JH compared to control. Light microscopy verified that all the tested honeys disrupted the microcolony formation of almost all planktonic cells in *P. aeruginosa* and showed the bacterial cells to be associated loosely with each other in a background of planktonic cells (Figure 3).

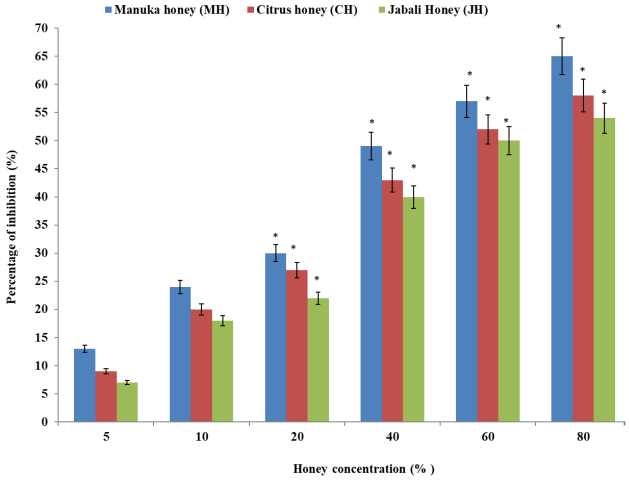


Biofilm treated with Manuka honey (A1; 5%, A2; 10%, and A3; 20%). Biofilms treated with Citrus honey (B1; 5%, B2; 10%, and B3; 20%). Biofilms treated with Jabali Honey (C1; 5%, C2; 10%, and C3; 20%). Images were viewed by light microscopy using oil immersion at 1000×magnification.

Figure 3. Disruption of microcolony development of *P. aeruginosa* after exposure to MH, CH and JH.

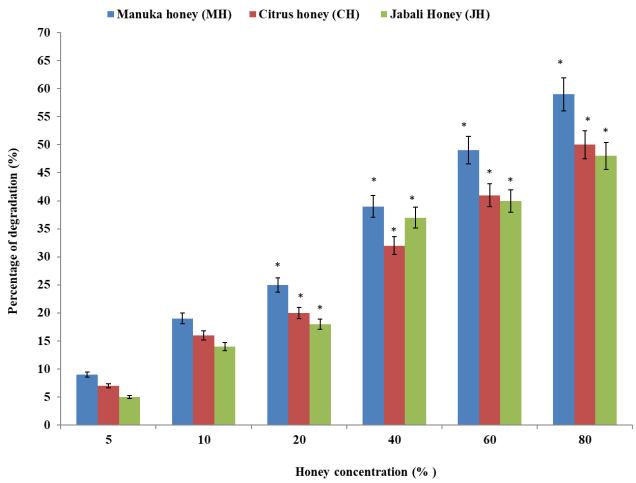
3.5 Biofilm inhibition assay

Growth of *P. aeruginosa* biofilm in the presence of 80%, 60%, 40% and 20% (w/v) of MH, CH and JH was significantly (P<0.05) reduced relatively to the untreated cells. However, the greatest inhibition of *P. aeruginosa* biofilm was found to be 65%, 58% and 54% after exposure to 50% (w/v) concentration of MH, CH and JH respectively. The lowest concentration of MH, CH and JH that inhibited *P. aeruginosa* forming a biofilm was found to be 5% (w/v). Furthermore, it was observed that the susceptibility testing of MH, CH and JH concentration below the MIC has decreased the growth of *P. aeruginosa* biofilm (Figure 4).



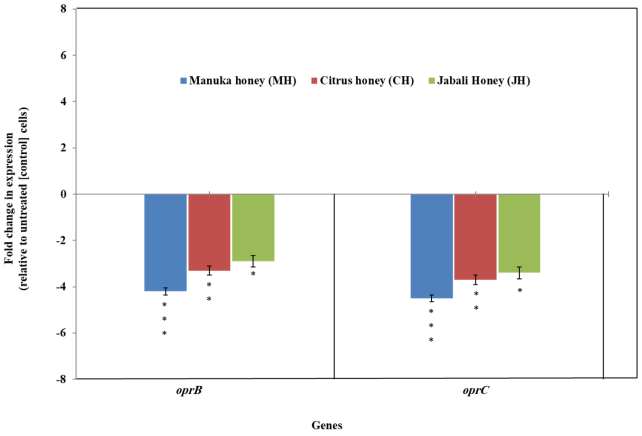
Asterisks; *P<0.05 indicate statistically significant difference between treated and control samples.

Figure 4. Effect of MH, CH and JH on biofilm formation by *P. aeruginosa*.



Asterisks; *P<0.05 indicate statistically significant difference between treated and control samples.

Figure 5. Effect of MH, CH and JH on established *P. aeruginosa* biofilms.



Mean values of fold changes (± SD) are shown in relation to untreated *P. aeruginosa* cells. Asterisks; *P<0.05; **P≤0.01; and ***P≤0.001 indicate statistically significant difference in the expression of each gene between treated and untreated samples.

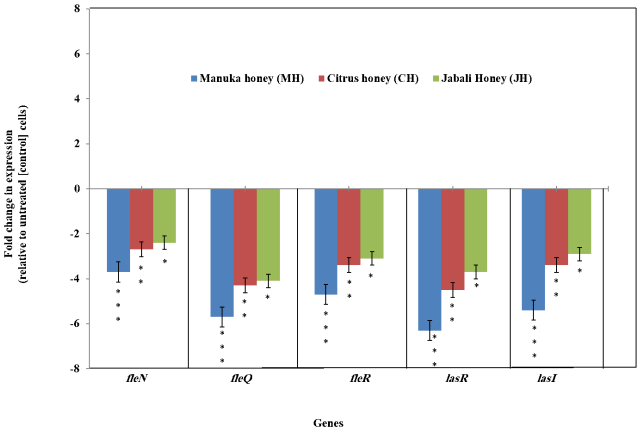
Figure 6. Changes in gene expression profiles of *P. aeruginosa* after being treated with MH, CH and JH as determined by RT-qPCR

3.6 Biofilm eradication assay

MH, CH and JH were significantly (P<0.05) able to eradicate the formation of biofilm by *P. aeruginosa* compered to untreated. The biofilm formation by *P. aeruginosa* was reduced to 25%, 20%, and 18% after being treated with 20% (w/v) of MH, CH and JH respectively. Similarly, MH, CH and JH were able to reduce 39%, 32%, and 37% the biofilm formation by *P. aeruginosa* after exposed to 40% (w/v) of MH, CH and JH respectively. In addition, MH, CH and JH were able to reduce the biofilm formation by *P. aeruginosa* to 49%, 41%, and 40 % after treated with 60% (w/v) of MH, CH and JH respectively. The greatest eradication of biofilm formation by *P. aeruginosa* was found to be 59%, 50% and 48% after exposure to 80% (w/v) of MH, CH and JH respectively. The lowest concentration 5% and 10% (w/v) of MH, CH and JH were found to eradicate biofilm formation by *P. aeruginosa* (Figure 5).

3.7 Gene expression profiling

In the present study, RT-qPCR was used to assess and compare the expression of seven genes in *P. aeruginosa* that have been previously shown to be involved in the flagella regulon proteins, biofilm formation, motility and virulence of the microorganism after exposure to MIC of MH, CH and JH. In the current study, RT-qPCR results showed that all genes in *P. aeruginosa* were downregulated following exposure to MH, CH and JH. Furthermore, different degrees of down-regulation were observed. Two major genes *oprB* and *oprC* associated with the outer membrane protein in *P. aeruginosa* showed the significant reduction (P<0.05, P≤0.01 and P≤0.001) of gene expression following treatment with MIC of MH, CH and JH. The expression of *oprB* decreased in a 4.2-fold, 3.3-fold and 2.9-fold following treatment with MH, CH and JH respectively. Whereas, the expression of *oprC* decreased in a 4.2-fold, 3.3-fold and 2.9-fold after exposure to MH, CH and JH respectively compared to untreated cells (Figure 6 and Figure 8).



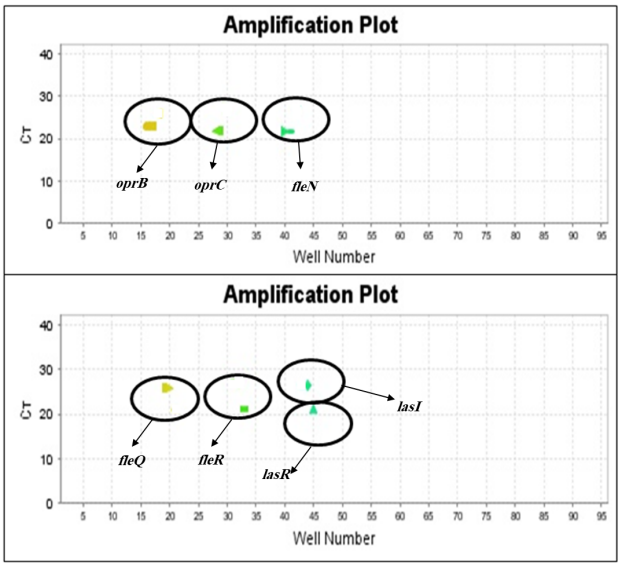
Mean values of fold changes (± SD) are shown in relation to untreated *P. aeruginosa* cells. Asterisks; *P<0.05; **P≤ 0.01; and ***P≤0.001 indicate statistically significant difference in the expression of each gene between treated and untreated samples.

Figure 7. Changes in gene expression profiles of *P. aeruginosa* after being treated with MH, CH and JH as determined by RT-qPCR

Five investigated flagella and biofilm formation genes: *fleN*, *fleQ*, *fleR*, *lasR* and *lasI* of *P. aeruginosa* showed the significant reduction (P<0.05, P≤0.01 and P≤0.001) in gene expression after exposure to MIC of MH, CH and JH. The *fleN*, *fleQ*, *fleR*, *lasR* and *lasI* genes in *P. aeruginosa* were downregulated to 3.7-fold, 5.7-fold, 4.7-fold, 6.3-fold and 5.4-fold respectively after treated with MH. When *P. aeruginosa* exposed to CH, the *fleN*, *fleQ*, *fleR*, *lasR* and *lasI* genes were downregulated in a 2.7-fold, 4.3-fold, 3.4-fold, 4.5-fold and 3.4-fold respectively. In addition, when *P. aeruginosa* treated with JH, the *fleN*, *fleQ*, *fleR*, *lasR* and *lasI* genes were downregulated in a 2.4-fold, 4.1-fold, 3.1-fold, 3.7-fold and 2.9-fold respectively (Figure 7 and Figure 8).

4 Discussion

Antibacterial activity of honey has been broadly discussing among researchers worldwide. It is postulated to be closely on several factors such as, osmolarity, pH and other major constituents such as phenolic acids and flavonoids (Abu Baker et al., 2018; Jibril et al., 2019; Zainol et al., 2013). Study by (Alkhyat and Al-Maqtari, 2014) showed that 25% concentration of honey exhibited lower antibacterial action. Similarly, study by (Sherlock et al., 2010) showed that Manuka honey and Chilean honey exhibited higher antibacterial action at 25% concentration (Sherlock et al., 2010). MIC is the lowest concentration of honey solution required to inhibit 99% of bacterial growth and MBC is the lowest



P. aeruginosa (*oprB*, *oprC*, *fleN*, *fleQ*, *fleR*, *lasR*, and *lasI*).

Figure 8. Amplification of an cDNA target genes is plotted against Ct value.

concentration of honey required to kill at least 99% of the tested bacterial strains (Zainol et al., 2013). In the current study, the MIC values were 12.5%, 25% and 25% (w/v) for MH, CH and JH respectively against *P. aeruginosa* and the MBC values were 25%, 50% and 50% (w/v) for MH, CH and JH respectively against *P. aeruginosa*. Previous studies demonstrated that the MIC and MBC values for Algerian honey, Manuka honey and Egyptian clover honey were 20% and 25% respectively against *P. aeruginosa* (Abbas, 2014; Bouacha et al., 2018; Shenoy et al., 2012; Schmittgen and Livak, 2008). Study by (Roberts et al., 2012) reported that the MIC and MBC values for Manuka honey were 12% and 16% against *P. aeruginosa* (Roberts et al., 2012). MIC_{90} and MIC_{50} are defined as the lowest concentration of the antimicrobial at which 90% and 50% of the isolates were inhibited respectively. Study by (Zainol et al., 2013) showed that the MIC_{50} value for Tualang honey against *P. aeruginosa*, *S. aureus* and *E. coli* was between 10% to 12.5% concentration of honey and MIC_{90} value was between 15-20% concentration of honey (Zainol et al., 2013). Limitations of some antibacterial assay such as agar well-diffusion test were reported including the insensitivity in detecting low level of antimicrobial activity, variation in the experimental conditions and permeability of non-polar components (Bang et al., 2003; Allen et al., 1991; Molan, 1992; Molan, 1999). The time kill curve was used to determine the bactericidal or bacteriostatic activities of antimicrobial. Total cell count is defined as the total number of both dead and living cells in the sample, whereas total viable count (TVC) is defined as the number of living cells (Singleton, 2004). In this study, the total viable count (TVC) of *P. aeruginosa* were decreased after exposed to 2×MIC of MH, CH and JH at 3, 6 and 9 hours. A study by (Henriques, 2006) reported that Manuka honey decreased the number of *P. aeruginosa* cells to 3-log reduction within 24 hours (Henriques, 2006). Previous study showed that Manuka honey decreased the number of *P. aeruginosa* cells to 6-log reduction (Roberts, 2014). Previous study revealed that Polish honey reduced 4-log of the number of *S. aureus* and *S. epidermidis* cells (Grecka et al., 2018). Study by (Bouacha et al., 2018) showed that Algerian honey had destroyed the number of *E. coli* and *P. aeruginosa* cells after 24 hours (Bouacha et al., 2018). In this study, MH, CH and JH were found to inhibit biofilm formation by *P. aeruginosa*. This indicates that biofilm formation was possibly inhibited at the beginning of the attachment stage. This statement can be applied in this study which supports the effectiveness of honey in reducing biofilm biomass. The presence of lysozyme is able to breakdown the established biofilm by digesting the bacteria (Glinski and Jarosz, 2001). The flavonoid pinocembrin which is believed present in honey is a very unique antibacterial factor (Ng et al., 2014). Flavonoid pinocembrin is an antioxidant that is able to kill bacteria and thus might contribute to the reduction of biofilm biomass (Ng et al., 2014). As demonstrated by RT-qPCR, a number of genes *oprB*, *oprC*, *fleN*, *fleQ*, *fleR*, *lasR* and *lasI* have been previously shown to be involved in the process of outer membrane protein, biofilm formation and motility in *P. aeruginosa* (Al-kafaween et al., 2020). The current results revealed that all selected genes in *P. aeruginosa* were downregulated following exposure to MH, CH and JH. Study by (Roberts et al., 2012; Roberts et al., 2015) reported downregulation of multiple genes involved in microcolony, motility and biofilm formation in *P. aeruginosa* following exposure to Manuka honey (Roberts et al., 2012;Roberts et al., 2015). Previous study reported that the expression of *algD*, *oprF*, *fleN*, *fleQ*, *fleR*, *fliA*, and *fliC* in *P. aeruginosa* were decreased in gene expression after exposure to Sidr honey (Al-kafaween et al., 2021). Study by (Roberts, 2014) showed that *fliA*, *fliC*, *flhF*, *fleN*, *fleQ* and *fleR* genes in *P. aeruginosa* were reduced in gene expression after treated with Manuka honey (Roberts, 2014). Previous study showed that the expression of *lasI* and *rhl* genes in *P. aeruginosa* were reduced after treated with local Iraqi honey (Ahmed and Salih, 2019). It was noticed that all these studies that mentioned above are in agreement with our results. This indicates that the honey-induced alterations in the expression of this group of genes are most probably due to particular molecules contained in honey and not only due to their sugar content. Previous study suggested that the osmotic action of sugar combined with hydrogen peroxide and bee-derived antibacterial peptide defensin-1 is crucial for the antibiofilm activity of honey (Proaño et al., 2021). The similarly or divergence of results might be due to several reasons such contain different level of active compounds including phenolic acids and flavonoids (Alzahrani et al., 2012; Jibril et al., 2019; Moussa et al., 2015). This could be due to the presence of unique organic antibacterial factors obtained by honey which depend on nectars botanical origin, different

batch of honey and technical variations involved (Abu Baker et al., 2018; Jibril et al., 2019).

5 Conclusion

This is the first attempt study to compare the impacts of CH and JH with that of MH on *P. aeruginosa* at planktonic, biofilm and molecular levels. A reduction of *P. aeruginosa* cell growth in both planktonic and biofilm was observed after exposure to all the tested honeys. Comparing all the tested honeys against planktonic and biofilm cultures, MH had a higher effect on *P. aeruginosa*. In this study, the results indicate that the CH and JH may represent promising antibacterial, antibiofilm and antivirulence agents for treatment and modulation of infections caused by *P. aeruginosa* compared with MH. The use of a natural product such as honey may be used in clinical practice, to prevent or even treat *P. aeruginosa* infection. This study suggests that each honey could have a crucial derivatives compound that have the ability to effectively inhibit the biofilm of *P. aeruginosa*.

Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

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