JOURNAL OF APITHERAPY, 2019
VOL 5, NO. 2 PAGE 10–17
10.5455/Ja.20190407083601

ORIGINAL ARTICLE

Open Access

Effect of *Trigona* honey on *Escherichia coli* cell culture growth: *In vitro* study

Mohammad Abdulraheem Al Kafaween[1], Abu Bakar Mohd Hilmi[1], Rao Sanaullah Khan[2], Mabrouka Bouacha[3], Malik Amonov[4]

[1]Faculty of Health Sciences, University Sultan Zainal Abidin, Terengganu, Malaysia

[2]School of Food and Agricultural Sciences, University of Management and Technology, Lahore, Pakistan

[3]Laboratory of Biochemistry and Microbiology, Department of Biochemistry, Faculty of Sciences, University of Badji Mokhtar, Annaba, Algeria

[4]Faculty of medicine, Universiti Sultan Zainal Abidin, Terengganu, Malaysia

ABSTRACT

Aim: This study aimed to investigate the antibacterial activity of *Trigona* honey against *Escherichia coli.*

Methods: The antibacterial activity of honey was examined by agar well diffusion assay, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill curve assay. Bacterial strains were cultivated in microtiter plates with varying concentrations of honey (10%, 20%, 30%, 40%, and 50% w/v) for specific incubation time (24, 48, and 72 hours) at 37°C.

Results: Agar well diffusion assay showed that *Trigona* honey had the highest antibacterial activity against *E. coli* with 18.2 ± 0.6 mm. The MIC value against *E. coli* was 10% (w/v) and MBC was 30% (w/v). In time-kill curve, *Trigona* honey has inhibited *E. coli* in a 4 $\log_{10}$ at 18 hours, and total viable counts were killed after 24 hours. It was found that even ≥30% *Trigona* honey dilution interfered significantly with *E. coli* cell culture growth. Moreover, it was found that a difference of more than 10% honey concentration between the treatments was considered significant to produce inhibitory effects. This study has shown that *Trigona* honey has significant inhibitory effects on *E. coli* growth *in vitro*. *Trigona* honey may be used as an alternative to antibiotics in controlling infections caused by *E. coli*. However, further investigation is required to strengthen this argument.

ARTICLE HISTORY

Received April 07, 2019
Accepted July 16, 2019
Published August 01, 2019

KEYWORDS

Trigona honey; *Escherichia coli*; inhibitory effects; minimum inhibitory concentration; minimum bactericidal concentration; time-kill curve

Introduction

Antibiotics have significantly reduced the mortality associated with infectious diseases during the 20th century. Unfortunately, their massive and repeated use has led to the emergence of bacteria resistant to these drugs. Today, bacterial resistance to antibiotics has become a worrying reality; the increasing evolution of bacterial resistance associated with a decrease in the stock of antibiotics is one of the essential motivations for research and the introduction of new antibacterial agents [1]. Alternative antimicrobial strategies are urgently needed and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as honey [2,3]. Honey has a long history of medicinal use that continues to prevent microbial infections to this day. It is also recognized as a topical antimicrobial agent effective in the treatment of burns and infected wounds [4–7]. The therapeutic properties of honey could be due to various factors, and the floral source of honey plays an important role in the biological properties of honey [8,9].

Honey has been used for thousands of years as a food, as a medicine, and has been incorporated into cosmetic products. A large number of different cultures have widely used honey as a medicine for many disorders such as chronic wounds and ulcers [10–14].

Trigona honey is produced by *Trigona* bees without stings grown in uncultivated bushland in

Contact Mabrouka Bouacha ✉ mohammadalkafaween25@yahoo.com Laboratory of Biochemistry and Microbiology, Department of Biochemistry, Faculty of Sciences, University of Badji Mokhtar, Annaba, Algeria.

Malaysia and Indonesia. *Trigona* honey is generally brighter in appearance than ordinary forest honey and has a distinctive "bush" taste, i.e., a mixture of sweet and sour with a hint of fruity taste [15]. It has been reported that honey has more than 100 distinct compounds with different biological functions [16], the main antibacterial compound in most types of honey is hydrogen peroxide [17,18]. However, perhaps the antibacterial activity of *Trigona* honey is due to phenolic compounds [15,19,20]. These compounds have non-sticky properties that could be used to control bacterial growth and biofilm formation [21–23].

The properties and compositions of honey depend on its geographical floral origin, season, environmental factors, and beekeeping management [7,24,25]. It is recognized that some chemical changes occur when nectar is transformed into honey. These changes are mainly due to the enzymatic activity of bee enzymes deposited in honey by bees. These enzymes are invertase, which hydrolyzes sucrose into glucose and fructose, amylase or diastase, and glucose oxidase, which generates gluconic acid and hydrogen peroxide from glucose in dilute honey. The other enzymes present in honey are catalase and acid phosphatase [26,27]. Honey is used in some hospitals, particularly for the clinical treatment of ulcers, bedsores, burns, and surgical wounds [28]. The antibacterial properties of honey can be particularly useful against bacteria that have developed resistance to several antibiotics [11,29,30]. The antibacterial properties of honey referred to acidity, the activity of non-hydrogen peroxide, the high osmotic effect, and the presence of phytochemical components [31,32]. The high osmotic effect of honey due to its high sugar content also plays a role in reducing the rate of bacterial growth [7]. In addition to the high osmotic effect of honey, its acidity could also reduce the rate of bacterial growth. The acidity of honey, which is in the pH range 3.2 to 4.5, creates an environment unfavorable to bacterial growth [7,33]. Numerous reports and clinical studies have demonstrated the antimicrobial activity of honey against *Escherichia coli*, *Salmonella entercolitis, Shigella dysenteriae*, *Mycobacterium*, *Staphylococcus aureus*, *Enterococci*, *Candida albicans*, and *Streptococcus pyogenes* [34–36].

In this study, the antibacterial effect of *Trigona* honey on *E. coli* growth was evaluated in several concentrations of *Trigona* honey. We selected *E. coli* because it is a common bacterium used in microbiological laboratory tests; its structure and physiology are well-known. In addition, many strains of this bacterium have been implicated in diseases such as gastroenteritis and urinary tract infections. Antibiotic resistance and biofilm formation by pathogenic strains of *E. coli* are considered a major concern, especially in hosts with weakened immune systems [37].

Materials and Methods

Honey samples

Medical grade sterile honey from the stingless bee *Trigona* was obtained from a local pharmacy at Kuala Terengganu, Malaysia. The honey was sterilized using gamma-irradiation 25 kGy). The samples were kept at room temperature and protected from sunlight [23,38,39].

Bacterial growth

Escherichia coli ATCC 25922 was used for this study. By using a sterile loop, bacteria were streaked across the nutrient agar medium and incubated at 37°C for 24 hours. The bacterial culture was prepared by picking up 1–2 morphologically identical colonies from the stock culture and suspended in 20 mL of sterile Brain Heart Infusion in a sterile conical flask. The inoculum was adjusted to 0.5 McFarland standard (approximately to $1–2 \times 108$ CFU/ml) and it incubated at 37°C for 24 hours [31,39–43].

The effect of Trigona honey on growth of E. coli

Five different concentrations of honey 50%, 40%, 30%, 20%, and 10% were prepared with inoculums as shown in Table 1, and 150 µl of each concentration were pipetted into a 96-well plate. A 200 µl of honey was used as a corresponding negative control, 200 µl of inoculum was used as a positive control, and 200 µl of broth was used as a sterility control. The plates were incubated for 1, 2, and 3 day at 37°C. Absorbance was measured each day (1st, 2nd, and 3rd day) by using the microtiter plate reader (Tecan Infinite 200 PRO, Austria) at 570 nm. This test was performed in triplicate [38,42,43].

Table 1. *Trigona* honey diluted with inoculum.

Treatments	Stock Honey (g)	Volume of inoculums (ml)	Final honey Concentration (% w/v)
A	0.2	1.8	10
B	0.4	1.6	20
C	0.6	1.4	30
D	0.8	1.2	40
E	1.0	1.0	50

Minimum inhibitory concentration

Micro broth dilution was employed for the determination of the minimum inhibitory concentration (MIC) of the honey. Following the method of [31,39,60], with minor modifications. Working bacteria culture was prepared as previously described, adjusted to be equal to 0.5 McFarland standards. MHB broth was used to prepare 50%, 40%, 30%, 20%, and 10% (w/v) concentrations of honey. Initially, the first well added with 200 µl of every honey dilution was used as dilution sterility controls. 100 µl of bacterial culture was mixed with 100 µl of each honey dilution in other wells. While a well with 200 µl of bacterial culture was used as the assay growth control. Also, a well containing 200 µl broth only was labeled as assay sterility control well. Plates were incubated at 37°C for 24 hours, and the presence/absence of visible growth was noted for each well. Also, the absorbance of the wells was read at 570 nm using microtitre plate reader (Tecan Infinite 200 PRO, Austria) [23,31,38,39,44–46].

Minimum bactericidal concentration

Minimum Bactericidal Concentration (MBC) was conducted using wells that appeared to have no growth (no turbidity) by visual inspection and were streaked onto nutrient agar plates using sterile 100 µl loops. The plates were incubated at 37°C for 24 hours. After incubation, any growth on the plates was marked as positive and no growth was marked as negative. The plates with the lowest concentration of honey showing no growth were recorded as the MBC [23,31,38,39,44–47].

Agar well diffusion assay

A prepared 0.5 McFarland *E. coli* suspension was streaked evenly on the surface of Muller Hinton agar (MHA) by using a sterile cotton swab. After that, wells with 7-mm diameter were prepared on the agar with a sterile cork borer. Each well was then filled with 70 µl of 50%, 40%, 30%, 20%, and 10% concentration of *Trigona* honey. Following 24 hours of incubation at 37°C, the diameters of the zone of inhibition for each sample were then recorded in millimeter (mm). Assays were completed in triplicate and an average value was obtained [31,39,44,45].

Time-kill curve

An overnight broth culture of *E. coli* in 5 ml of MHB was prepared by inoculating a colony from pure culture and incubating at 37°C for 24 hours. The first tube was inoculated with 0.6 g of honey and 1.4 ml of a broth culture of the test bacterium in an initial concentration of approximately 10^7 CFU/ml and the second tube was filled with 2 ml of inoculum used as a positive control. The tubes were incubated at 37°C. Broth aliquots were collected at different time points, serially diluted in saline solution, plated on nutrient agar media, and grown for 24 hours at 37°C to determine the colony-forming units (CFUs) in each tube. Finally, a graph of $\log_{10}$ total viable count (TVC) versus incubation time was plotted to allow the exponential growth phase to be identified [39,48].

Statistical analysis

Repeated measures analysis of variance (ANOVA) was applied on the collected data using "Statistical Package for Social Science version 21" The differences of mean values within groups (time effect) were analyzed by using pairwise comparisons with the assumption of compound symmetry as given by Mauchly's test of sphericity. A separate ANOVA for checking the treatment effect was performed with *Post-hoc multiple comparisons* to reveal the differences in mean values among groups.

The level of significance was set at 0.05 with two-tailed fashion. The assumptions of normality and homogeneity of variance were applied to check the fit of the model.

Results

Effect of Trigona honey on E. coli growth

As shown in Table 2, there are no significant differences in mean growth rate values between day one, two, and three ($F = 0.30$, $p = 0.745$). It was found that *E. coli* growth was five times higher in 10% honey compared to the other concentrations ($F = 551.37$, $p < 0.001$).

The differences in mean values among groups with regard to time (time-treatment interaction) were analyzed by using ANOVA. There were significant differences between mean growth values in day 1 and day 2 (Table 3). It was also observed that the treatments become non-significant even after 72 hours of incubation (Table 3).

Profile plot (Fig. 1) for the adjusted mean (estimated marginal means) of *E. coli* cell culture for Days 1, 2, and 3 revealed that at 30% honey concentration, days of incubation become irrelevant.

It can be inferred from this study that honey at a concentration of 30% (w/v) may be the most suitable treatment to inhibit the growth of *E. coli*

cells. Similar findings have been reported by [38] wherein 20%–40% dilutions have shown a marked inhibitory effect on bacterial growth. Moreover, it is observed that 40% and 50% treatment on the third day (Table 3) becomes non-significant in producing inhibitory effects.

MIC and MBC determination

In Table 4, the results obtained show that *Trigona* honey inhibited the growth of *E. coli* at 10% (w/v) and it has a bactericidal effect at the dilution of 30% (w/v).

Agar well diffusion

Agar well diffusion assay was summarized in Table 5. The table shows the zone of inhibition for *E. coli* after treated with *Trigona* honey. *Trigona* honey exhibits greater inhibition on *E. coli* cultures, which is related to its dilution.

Time-kill curve

The time-kill curve clearly shows an increase in the number of E.coli cells without honey treatment (Fig. 2). However, a reduction in the number of *E. coli* was observed, which showed the decreased 2 $\log_{10}$ reductions in TVCs at 6 hours (Table 6). At 12 hours, *E. coli* was decreased 3 $\log_{10}$reduction in TVCs, and at 18 hours, it was decreased by 4 $\log_{10}$ reductions. *Escherichia coli* was killed after 24 hours ($\log_{10}$CFU/ml = 0, $p = 0.001$).

Table 2. Overall mean difference of growth rate (cell culture) among groups (Treatment effect).

Comparison	Mean difference (95% CI)	*p*-value
50% versus 40%	−0.05 (−0.09, −0.01)	0.018
50% versus 30%	−0.09 (−0.13, −0.05)	0.001
50% versus 20%	−0.18 (−0.22, −0.14)	<0.001
50% versus 10%	−0.24 (−0.27, −0.20)	<0.001
50% versus control	−0.37 (−0.41, −0.33)	<0.001
40% versus 30%	−0.04 (−0.08, −0.003)	0.035
40% versus 20%	−0.13 (−0.17, −0.09)	<0.001
40% versus 10%	−0.19 (−0.23, −0.15)	<0.001
40% versus control	−0.33 (−0.37, −0.29)	<0.001
30% versus 20%	−0.09 (−0.13, −0.05)	0.001
30% versus 10%	−0.15 (−0.19, −0.11)	<0.001
30% versus control	−0.28 (−0.32, −0.25)	<0.001
20% versus 10%	−0.06 (−0.10, −0.02)	0.007
20% versus control	−0.20 (−0.23, −0.16)	<0.001
10% versus control	−0.14 (−0.18, −0.10)	<0.001

F-stat (df) = 551.37 (5), *p*-value < 0.001.

The time-kill curve is used to determine the bactericidal or bacteriostatic activity of antimicrobials. It is analyzed by plotting log $_{10}$ CFU/ml versus time. Total cell count is defined as the total number of both dead and living cells in the sample, whereas the TVC is defined as the number of living cells [50]. To maintain and minimize the impact of time-kill variables, several factors should be considered when performing time-kill studies. These variations affect the results and its interpretation. These factors are first, the initial or starting inoculum of 10^4 to 10^7 CFU/ml should be applied. Second, the samples should be incubated at 37°C. Third, the assay should be continued up to 24 hours [51]. In

Table 3. Comparison of mean cell culture among different groups based on time (time-treatment).

	Comparison	Mean difference (95% CI)	*p*-value
Day 1	50% versus 40%	−0.03 (−0.15, 0.08)	>0.95*
	50% versus 30%	−0.04 (−0.15, 0.08)	>0.95*
	50% versus 20%	−0.13 (−0.24, −0.10)	0.033
	50% versus 10%	−0.13 (−0.25, −0.02)	0.026
	40% versus 30%	−0.01 (−0.12, 0.11)	>0.95*
	40% versus 20%	−0.09 (−0.21, 0.02)	0.131
	40% versus 10%	−0.10 (−0.22, 0.02)	0.097
	30% versus 20%	−0.09 (−0.20, 0.03)	0.187
	30% versus 10%	−0.09 (−0.21, 0.02)	0.136
	20% versus 10%	−0.01 (−0.12, 0.11)	>0.95*
Day 2	50% versus 40%	−0.07 (−0.16, 0.04)	0.335*
	50% versus 30%	−0.10 (−0.20, −0.002)	0.045
	50% versus 20%	−0.15 (−0.25, −0.05)	0.006
	50% versus 10%	−0.28 (−0.38, −0.18)	<0.001
	40% versus 30%	−0.04 (−0.14, 0.06)	>0.95*
	40% versus 20%	−0.09 (−0.19, 0.01)	0.104
	40% versus 10%	−0.21 (−0.31, −0.11)	0.001
	30% versus 20%	−0.05 (−0.15, 0.05)	0.948*
	30% versus 10%	−0.18 (−0.28, −0.08)	0.002
	20% versus 10%	−0.13 (−0.23, −0.03)	0.014*
Day 3	50% versus 40%	−0.05 (−0.15, 0.06)	>0.95*
	50% versus 30%	−0.13 (−0.23, −0.02)	0.017
	50% versus 20%	−0.26 (−0.36, −0.16)	<0.001
	50% versus 10%	−0.30 (−0.40, −0.19)	<0.001
	40% versus 30%	−0.08 (−0.19, 0.02)	0.152*
	40% versus 20%	−0.21 (−0.32, −0.11)	0.001
	40% versus 10%	−0.25 (−0.35, −0.15)	<0.001
	30% versus 20%	−0.13 (−0.24, −0.03)	0.014*
	30% versus 10%	−0.17 (−0.27, −0.07)	0.004
	20% versus 10%	−0.04 (−0.14, 0.07)	>0.95*

*Non-significant ($p > 0.001$).

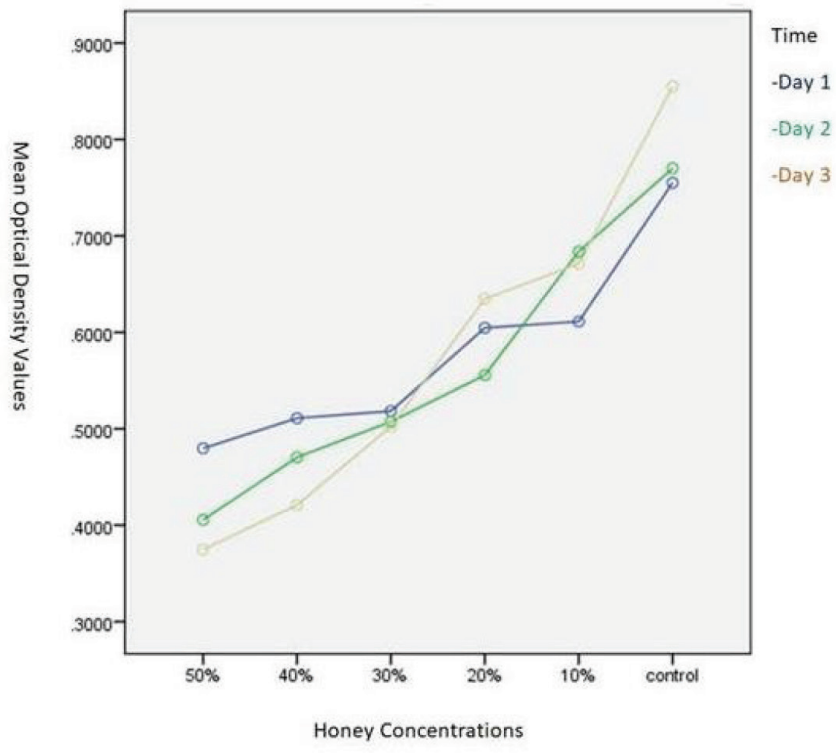

Figure 1. Mean values of *E. coli* cell culture (measured as OD) incubated at 37°C.

Table 4. MIC and MBC determination.

Strain	MIC % (w/v)	MBC% (w/v)
E. coli	10	30

Table 5. Inhibition zone (mm) (Mean ±SD), *n* = 3.

Test bacteria	The concentration of Honey (%w/v)				
	50	40	30	20	10
E. coli	18.2 ± 0.6	17.2 ± 0.4	15.2 ± 0.3	13.5 ± 0.4	9.0 ± 0.2

this study, all these conditions were applied in the time-kill assays. The $\log_{10}$ CFU/ml for treated *E. coli* was noticed at 12 hours almost half of *E. coli* was killed ($\log_{10}$ CFU/ml = 3.2). Also, at 24 hours, almost 100% of *E. coli* was killed.

Discussion

In this study, we have demonstrated that all concentrations of Trigona honey were able to decrease the growth rate of *E. coli*. This study demonstrated that there was considerable variation between the concentrations of honey and that indication of activity derived solely from the mean size of the zone of inhibition to determine the relative activity of honey. Antibacterial activities of honey have been broadly discussed among researchers worldwide. It is strongly related to several factors such as osmolarity, pH, and other major constituents such as phenolic acids and flavonoids [49]. The MIC value of Trigona honey against *E. coli* was 10%, whereas the MBC value was 30% (w/v). A study by Zainol et al. [31] showed that MIC and MBC of Kelulut honey against *E. coli* were at 20% concentration of honey. Previous studies showed that the MIC of stingless honey was 4% to >10% (w/ v) for Gram-positives bacteria, 6% to >16% (w/ v) for Gram-negative bacteria, and 6% to >10% (w/ v) for Candida spp. [55,56]. Currently, the studies conducted by [12,57] have been reported that MIC of Manuka honey against S. pyogenes was at 20% concentration and MBC was at 25% concentration. Previous studies showed that MIC of Kelulut honey, Algerian honey, and Manuka honey against Pseudomonas aeruginosa was at 20% concentration and MBC was at 25% concentration [31,39,58,59]. These variations affect the results and its interpretation. Firstly, the initial or starting inoculum of 10^4 to 10^7 CFU/ml should be applied. Secondly, the samples should be incubated at 37°C. Thirdly, the assay should be continued up to 24 hours [51]. Previous studies showed that stingless bee Trigona carbonaria decreased

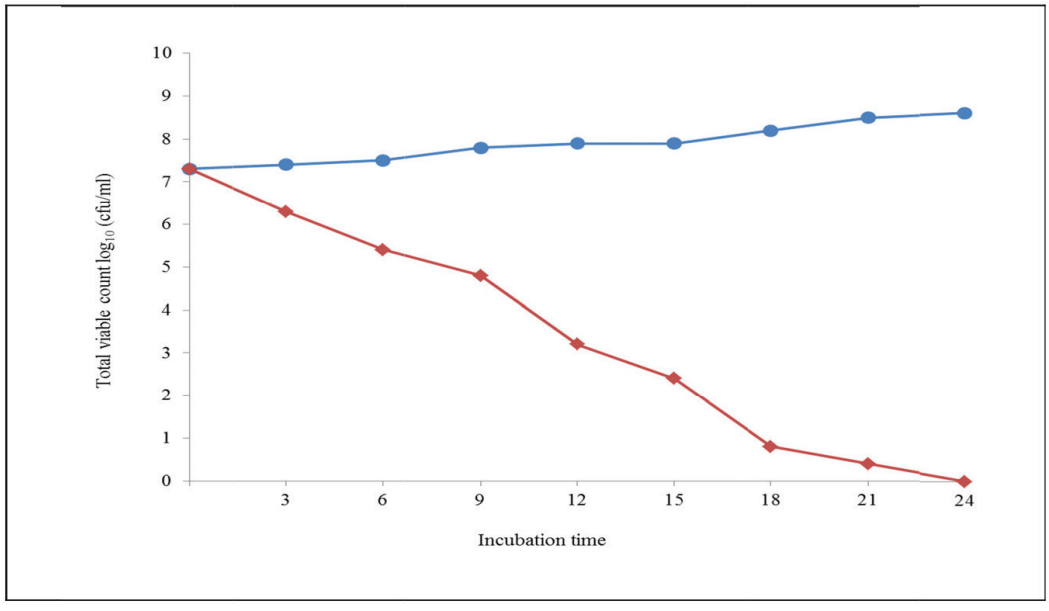

Figure 2. Time-kill curve showing *in vitro* bactericidal effect of *Trigona* honey on *E. coli.*

Table 6. Log reduction (LR) for *E. coli* after 24 hours of exposure to 30% of *Trigona* honey.

Time (hours)	Log_{10}CFU/ml (A)	Log_{10}CFU/ml+ honey (B)	LR = log_{10}(A)-log_{10}(B)	*p*-value
0	7.3	7.3	0	Initial
3	7.4	6.3	1.1	0.021
6	7.5	5.4	2.1	0.015
9	7.8	4.8	3.0	0.011
12	7.9	3.2	4.5	0.014
15	7.9	2.4	5.4	0.011
18	8.2	0.8	7.4	0.007
21	8.5	0.4	8.1	0.002
24	8.6	0.0	8.0	0.001

1–3 log for *S. aureus*, and >3 log for *P. aeruginosa* after treated with 20% of honey [55]. Four major antibacterial properties of honey including acidity, non-hydrogen peroxide activity, high osmotic effect, and the presence of phytochemical components [49]. The high osmotic effect of honey due to the high contents of sugar in honey also plays a role in reducing biofilm mass [7]. Besides the high osmotic effect of Trigona honey, the acidity of honey is assumed to play a role in reducing biofilm mass as well. The acidity of Trigona honey which is within the range of pH 3.2 to 4.5, creates an unfavorable environment for bacterial growth whereas their optimum pH for growth is about pH 7.2 to 7.4 [7].

Conclusion

This study has provided evidence to show that *Trigona* honey can significantly inhibit the growth of *E. coli* cell culture *in vitro*. Further 30% honey dilution is found to be the most appropriate concentration for getting significant results in inhibiting the growth. Therefore, it can be concluded that *Trigona* honey in its diluted form can be effectively applied in controlling *E. coli* infections. However, further investigation is needed to understand the mechanism of this inhibition, which was not the scope of this study. Further study with a scanning electron microscope followed by quantification of active compounds would help to understand the mechanism of inhibition.

Acknowledgments

This important piece of research would have not been possible without the generous sport of faculty of health sciences at University Sultan Zainal Abidin, Terengganu, Malaysia.

References

[1] Mandal MD, Mandal S. Honey: its medicinal property and antibacterial activity. Asian Pac J Trop Biomed 2011; 1(2):154–60.

[2] Dunford C, Cooper R, Molan P, White R. The use of honey in wound management. Nurs Stand 2000; 15(11):63–8.

[3] Kalidasan G, Saranraj P, Ragul V, Sivasakthi S. Antibacterial activity of natural and commercial

honey—a comparative study. Adv Biol Res 2017; 11(6):365–72.
[4] Eteraf-Oskouei T, Najafi M. Traditional and modern uses of natural honey in human diseases: a review. IJBMS 2013; 16(6):731.
[5] Getaneh A, Belyhun Y, Moges F, Anagaw B, Destaw B, Unakal C, et al. In vitro assessment of the antimicrobial effect of Ethiopian multi-flora honey on methicillin-resistant *Staphylococcus aureus*. Int J Curr Res Rev 2013; 5(11):64.
[6] Kuropatnicki AK, Kłósek M, Kucharzewski M. Honey as medicine: historical perspectives. J Apic Res 2018; 57(1):113–8.
[7] Fatima IJ, Mohd Hilmi AB, Salwani I, Lavaniya M. Physicochemical characteristics of Malaysian stingless bee honey from *Trigona* species. Int Med J Malaysia 2018; 17:187–91.
[8] Abeshu MA, Geleta B. Medicinal uses of honey. Biol Med, 2016; 8(2):1.
[9] Soliman M, Islam MA, Paul S, Ali Y, Khalil MI, Alam N, et al. Physicochemical properties, minerals, trace elements, and heavy metals in honey of different origins: a comprehensive review. Comp Rev Food Sci Food Saf 2016; 15(1):219–33.
[10] Pan SY, Litscher G, Gao SH, Zhou SF, Yu ZL, Chen HQ, et al. Historical perspective of traditional indigenous medical practices: the current renaissance and conservation of herbal resources. J Evid Based *Complement* Altern Med 2014; 2014:20.
[11] Maddocks SE, Jenkins RE. Honey: a sweet solution to the growing problem of antimicrobial resistance? Future Microbiol 2013; 8(11):1419–29.
[12] Maddocks SE, Lopez MS, Rowlands RS, Cooper RA. Manuka honey inhibits the development of *Streptococcus pyogenes* biofilms and causes reduced expression of two fibronectin binding proteins. Microbiology 2012; 158(3):781–90.
[13] Roberts AE, Maddocks SE, Cooper RA. Manuka honey is bactericidal against *Pseudomonas aeruginosa* and results in differential expression of oprF and algD. Microbiology 2012; 158(12):3005–13.
[14] Roberts AE, Maddocks SE, Cooper RA. Manuka honey reduces the motility of *Pseudomonas aeruginosa* by suppression of flagella-associated genes. J Antimicrob Chemother 2014; 70(3):716–25.
[15] Biluca FC, Braghini F, Gonzaga LV, Costa AC, Fett R. Physicochemical profiles, minerals and bioactive compounds of stingless bee honey (Meliponinae). J. Food Compos Anal 2016; 50:61–9.
[16] Rao PV, Krishnan KT, Salleh N, Gan SH. Biological and therapeutic effects of honey produced by honey bees and stingless bees: a comparative review. Rev Bras Farmacogn 2016; 26(5):657–64.
[17] Molan PC. The antibacterial activity of honey: 1. The nature of the antibacterial activity. Bee World 1992; 73(1):5–28.
[18] Brudzynski K. Effect of hydrogen peroxide on antibacterial activities of Canadian honeys. Can J microbiol 2006; 52(12):1228–37.
[19] Jaganathan SK, Mandal M. Antiproliferative effects of honey and of its polyphenols: a review. BioMed Res Int 2009; 2009:13.
[20] KekSP, Chin NL, Yusof YA, Tan SW, Chua LS. Total phenolic contents and colour intensity of Malaysian honeys from the *Apis* spp. and *Trigona* spp. bees. Agric Agric Sci Proc 2014; 2:150–5.
[21] Limsong JE, Benjavongkulchai E, Kuvatanasuchati J. Inhibitory effect of some herbal extracts on adherence of Streptococcus mutans. J Ethnopharmacol 2004; 92(2–3):281–9.
[22] Jibril FI, Hilmi ABM, Manivannan L. Isolation and characterization of polyphenols in natural honey for the treatment of human diseases. Bull Nat Res Centre 2019; 43(1):4.
[23] Ng WJ, Chan YJ, Lau ZK, Lye PY, Ee KY. Antioxidant properties and inhibitory effects of trigona honey against *Staphylococcus aureus* planktonic and biofilm cultures. Int J 2017; 12(37):28–33.
[24] ELMetwally A. Factors affecting the physical and chemical characteristics of Egyptian beehoney. Fac Agric Cairo Univ, Giza, Egypt, 2015.
[25] El Sohaimy S, Masry S, Shehata M. Physicochemical characteristics of honey from different origins. An Agric Sci 2015; 60(2):279–87.
[26] Buba F, Gidado A, Shugaba A. Analysis of biochemical composition of honey samples from North-East Nigeria. Biochem Anal Biochem 2013; 2(3):139.
[27] Da Silva PM, Gauche C, Gonzaga LV, Costa ACO, Fett R. Honey: chemical composition, stability and authenticity. Food Chem 2016; 196:309–23.
[28] Jull AB, Cullum N, Dumville JC, Westby MJ, Deshpande S, Walker N. Honey as a topical treatment for wounds. Cochrane Database Syst. Rev 2015; (3).
[29] AlqurashiA, Masoud E, Alamin M. Antibacterial activity of Saudi honey against Gram negative bacteria. J Microbiol Antimicrob 2013; 5(1):1–5.
[30] Mahendran S Kumarasamy D. Antimicrobial activity of some honey samples against pathogenic bacteria. Int Let Nat Sci 2015; 7.
[31] Zainol MI, Yusoff KM, Yusof MYM. Antibacterial activity of selected Malaysian honey. BMC Complement Altern Med 2013; 13(1):129.
[32] Al-Waili N, Salom K, Al-Ghamdi AA. Honey for wound healing, ulcers, and burns; data supporting its use in clinical practice. Sci World J 2011; 11:766–87.
[33] Kwakman PH Zaat SA. Antibacterial components of honey. IUBMB Life. 2012; 64(1):48–55.
[34] Noori AL, Al-Ghamdi A, Ansari MJ, Al-Attal Y, Salom K. Synergistic effects of honey and propolis toward drug multi-resistant *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* isolates in

single and polymicrobial cultures. Int J Med Sci 2012; 9(9):793.
[35] Ansari MJ, Al-Ghamdi A, Usmani S, Al-Waili NS, Sharma D, Nuru A, et al. Effect of jujube honey on *Candida albicans* growth and biofilm formation. Arc Med Res 2013; 44(5):352–60.
[36] NooriAL, Al Ghamdi A, Ansari MJ, Al-Attal Y, Al-Mubarak A, Salom K. Differences in composition of honey samples and their impact on the antimicrobial activities against drug multiresistant bacteria and pathogenic fungi. Arc Med Res 2013; 44(4):307–16.
[37] Rossi C, Chaves-López C, Serio A, Goffredo E, Goga BTC, Paparella A. Influence of incubation conditions on biofilm formation by Pseudomonas fluorescens isolated from dairy products and dairy manufacturing plants. Ital J Food Saf 2016; 5(3):5793.
[38] Nishio EK, Ribeiro JM, Oliveira AG, Andrade CGTJ, Proni EA, Kobayashi RKT, et al. Antibacterial synergic effect of honey from two stingless bees: Scaptotrigona bipunctata Lepeletier, 1836, and S. postica Latreille, 1807. Sci Rep, 2016; 6:21641.
[39] Bouacha M, Ayed H, Grara N. Honey bee as alternative medicine to treat eleven multidrug-resistant bacteria causing urinary tract infection during pregnancy. Sci Pharm 2018; 86(2):14.
[40] Nyenje ME, Green E, Ndip RN. Evaluation of the effect of different growth media and temperature on the suitability of biofilm formation by Enterobacter cloacae strains isolated from food samples in South Africa. Molecules 2013; 18(8):9582–93.
[41] Nyenje ME, Green E, Ndip RN. Biofilm formation and adherence characteristics of Listeria ivanovii strains isolated from ready-to-eat foods in Alice, South Africa. Sci World J 2012; 2012:7.
[42] Shehu A, Ismail S, Rohin MAK, Harun A, Aziz AA, Haque M. Antifungal properties of Malaysian Tualang honey and stingless bee propolis against *Candida albicans* and *Cryptococcus neoformans*. J Appl Pharm Sci 2016; 6(2):044–50.
[43] Nassar HM, Li M, Gregory RL. Effect of honey on Streptococcus mutans growth and biofilm formation. Appl Environ Microbiol 2011; AEM. 05538-11.
[44] Chauhan A, Pandey V, Chacko KM, Khandal RK. Antibacterial activity of raw and processed honey. Electronic J Biol 2010; 5(3):58–66.
[45] Moussa A, Noureddine D, Abdelmelek M, Saad A. Antibacterial activity of various honey types of Algeria against Pathogenic Gram–Negative Bacilli: *Escherichia coli* and *Pseudomonas aeruginosa*. *Asian* Pac J Trop Dis 2012; 2(3):211–4.
[46] Wasfi R, Elkhatib WF, Khairalla AS. Effects of selected Egyptian honeys on the cellular ultrastructure and the gene expression profile of *Escherichia coli*. PLoS One 2016; 11(3):e0150984.
[47] Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 2008; 3(2):163.
[48] Kim S, Hong I, Woo S, Jang H, Pak S, Han S. Isolation of abscisic acid from Korean acacia honey with anti-Helicobacter pylori activity. Pharm Mag 2017; 13(Suppl 2):S170.
[49] Bogdanov S. Nature and origin of the antibacterial substances in honey. LWT-Food Sci Technol 1997; 30(7):748–53.
[50] Singleton P. Bacteria in biology. Biotechnology and medicine. John Wiley & Sons, Hoboken, NJ, 2004.
[51] Klepser ME, Ernst EJ, Lewis RE, Ernst ME, Pfaller MA. Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. Antimicrob Agents Chemother 1998; 42(5):1207–12.
[52] Nzeako B, Hamdi J. Antimicrobial potential of honey on some microbial isolates. *Sultan Qaboos Univ* Med J 2000; 2(2):75–9.
[53] Cooper RA, Wigley P, Burton NF. Susceptibility of multiresistant strains of Burkholderia cepacia to honey. Lett Appl Micribiol 2000; 31(1) 20–4.
[54] Cooper RA, Halas E, Molan PC. The efficacy of honey in inhibiting strains of *Pseudomonas aeruginosa* from infected burns. J Burn Care Rehabil 2002; 23(6):366–70.
[55] Boorn KL, Khor YY, Sweetman E, Tan F, Heard TA, Hammer KA. Antimicrobial activity of honey from the stingless bee *Trigona carbonaria* determined by agar diffusion, agar dilution, broth microdilution and time-kill methodology. J Appl Microbiol 2010; 108(5):1534–43.
[56] Andualem B. Synergistic antimicrobial effect of Tenegn honey (Trigona iridipennis) garlic against standard and clinical pathogenic bacterial isolates. Int J Microbiol Res 2013; 4(1):16–22.
[57] Mandal MD, Mandal S. Honey: its medicinal property and antibacterial activity. Asian Pac J Trop Biomed 2011; 1(2):154.
[58] Abbas HA. Comparative antibacterial and antibiofilm activities of manuka honey and Egyptian clover honey. Asian J Appl Sci 2014; 2(2).
[59] ShenoyVP, Ballal M, Shivananda PG, Bairy I. Honey as an antimicrobial agent against *Pseudomonas aeruginosa* isolated from infected wounds. J Glob Infect Dis 2012; 4(2):102.
[60] Lye PY. Interactive effect of trigona honey and ampicillin on *Staphylococcus aureus* isolates of infected wound. UTAR, 2015.