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Research Article

In Vitro Investigation on the Effectiveness of *Trigona* Honey against Biofilm Formation by *Escherichia coli*

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Abstract

Aim: This study was designed to assess the effectiveness of *Trigona* honey in blocking the biofilm formation by *E. coli*.

Methods: Biofilms were cultivated in microtiter plates with varying concentrations (10, 20, 30, 40 and 50%) of honey for specific incubation time (24, 48 and 72 hours). The extent of biofilm biomass was estimated by staining with crystal violet.

Results: It was found that the mean values of biofilms were significantly different ($F = 425.42$, $P < 0.001$) within groups based upon time of incubation. The mean biofilm values were significantly different among groups ($F = 121.82$, $P = 0.029$). It was also observed that a difference of 10% between the treatments was found to be non- significant. Overall it was found that 30% *Trigona* honey application significantly reduce the formation of biofilms during first 24 hours of incubation.

Conclusion: This study has shown that *Trigona* honey has significantly inhibited biofilm formation by *E. coli in vitro*. Hence *Trigona* honey can be used as an alternative to antibiotic in retarding biofilm formation by *E. coli*. However further research and development is required to explore the active compounds that are responsible for attributing this inhibitory effect.

Keywords: *Trigona Honey; E. coli; Biofilm; Inhibitory Effects*

Introduction

Biofilm formation is one of the defensive mechanisms of bacteria wherein bacteria aggregate in the form of a complex structure in order to withstand harsh conditions [1]. The major features that distinguish biofilm forming bacteria from their planktonic counterparts are their surface attachment ability, high population density, extracellular polymeric substances (EPS) and a wide range of physical, metabolic and chemical heterogeneities [2,3]. It is now recognized that biofilm formation is an important aspect of many diseases including native valve endocarditis, osteomyelitis, dental caries, middle ear infections, medical device-related infections, ocular implant infections and chronic lung infections in cystic fibrosis patients [4]. Biofilms can tolerate antimicrobial agents at concentrations of 10 - 1000 times higher than that needed to kill genetically equivalent planktonic bacteria [5]. Moreover, biofilms are extraordinarily resistant to phagocytosis making biofilms extremely difficult to eradicate from living hosts [6]. Biofilm formation has therefore called for concerted efforts from microbiologists and life scientists to investigate this phenomenon in order to develop better strategies to prevent inhibit and destroy biofilm formation.

Honey has been used in curing ailments and preventing the onset of ailments since prehistoric times [7]. In recent times of modern clinical practices [8], cost and difficulty of chronic wound care has asked for better and cost effective remedies [9,10]. Moreover, the side

effects of antibiotics along with acquired antibiotic resistance pose a serious concern for health care management systems. Honey in general reported to have more than 100 distinctive compounds that are functionally very important in creating a healthy body functions [11]. Major antibacterial factor, in most honey types, is hydrogen peroxide [12]. However, *Trigona* honey is considered to have this activity due to phenolic compounds [7,13-15], which act as strong antioxidants [15-17]. These compounds have been argued to have anti-adhesive properties that could be exploited to limit the biofilm formation [18]. In this study, effectiveness of *Trigona* honey against biofilm formation in *E. coli* was investigated. *E. coli* being attributed to a greater genomic variability coupled with diverse environmental niches presents serious challenges in devising anti biofilm formation [19,20] remedies [21,22]. Biofilm formation of pathogenic strains of *E. coli* is considered to be a virulence factor in a host with compromised immune system [21,23].

Materials and Methods

Honey samples

Trigona honey was purchased from Kuala Terengganu, Malaysia in October 2018. The sample was kept in dark at room temperature. All chemicals used were of analytical grade [24].

Bacterial growth assays

One of the most common genera associated with catheter-associated infections and food safety dangers of *E. coli* strain (ATCC 25922) was used in this study. *E. coli* was grown in Brain Heart Infusion (BHI) from the available in-house agar slopes. The inoculated broth was incubated at 37°C for 24 hours without shaking. Thus 0.1% concentrations of *E. coli* were prepared in BHI broth [29].

Biofilm assays

The supernatant medium containing planktonic bacteria was gently aspirated to clear flat- bottomed 96-well plates for measurement of planktonic bacteria using plate reader absorbance at 570 nm. Each well of the experimental plates was rinsed three times with 100 mL of sterile distilled water without disturbing the adherent biofilm. The plate was air-dried for 5 minutes. Then 150 µL of 0.1% crystal violet was added for each well for 15 minutes [27]. The crystal violet was removed by rinsing with 200 mL of distilled water for three times and left to air dry. About 200 mL of 95% ethanol per well was applied and the plates were incubated at room temperature for 15 minutes. The contents of each well were thoroughly mixed and 125 mL of the crystal violet/ethanol solution was transferred to clear flat-bottomed 96-well plate. The extent of biofilm was determined by measuring absorbance using the micro plate reader at 570 nm [9,11,26,28].

Assessing the effect of *Trigona* honey

A sterile solution of 50% (w/v) honey was prepared by weighing 10g sterile honey into a sterile test tube containing 10 mL of sterile BHI. These stock solutions were used to prepare a range of honey dilutions (Table 1) to determine the effect on bacterial growth and biofilm formation [9,26].

Serial Number	50% stock honey (g)	Sterile MHB (mL)	Final honey concentration (% w/v)
1	0.4	1.6	10
2	0.8	1.2	20
3	1.2	0.8	30
4	1.6	0.4	40

Table 1: *Trigona honey dilutions preparation.*

Dilutions of honey (50 µl) were added to each well of 150 µl bacterial cultures while a positive control was prepared without honey. The treated plates were incubated at 37°C for 24, 48 and 72 hours with honey. Cultures were washed three times with distilled water to remove planktonic cells. These were then air dried, and subsequently dyed with crystal violet 200 µl of 0.1%. Then, it was washed with distilled water followed by 200 µl of 95% ethanol while a positive control was retained with untreated bacteria. The extent of growth was determined by measuring absorbance at 570 nm wavelength in microplate reader [9,26,28].

Statistical analysis

Data was analyzed using Statistical Package for Social Science version 21. The repeated measures analysis of variance (ANOVA) was used to compare biofilm results based on percentage of honey based on incubation time. The level of significance was set at 0.05 with two-tailed fashion. The differences of mean values with groups (time effect) were analyzed by using pair wise comparisons within groups based on time. The assumption of compound symmetry was applied with Mauchly's test of sphericity. The differences of mean values among groups regardless of time (treatment effect) were analyzed by applying separate ANOVA followed by post-hoc multiple comparison. The difference of mean values among groups with regard to time (time- treatment interaction) were analyzed by using mixed repeated measures ANOVA. The assumptions of normality and homogeneity of variance were applied to check the fit of the model.

Results and Discussion

Between group analysis (Treatment effect regardless of time)

The mean biofilm values were significantly different among groups (F = 121.82, P = 0.029). Post-hoc multiple comparison was performed and there were significant differences among groups except 50% vs 40%, 40% vs 30%, 30% vs 20%, 30% vs 10% and 20% vs 10% (Table 2). Repeated Measures ANOVA between group analysis was applied followed by post-hoc multiple comparisons using Bonferroni method F-stat (df) = 121.82 (5), P-value = 0.029.

Comparison	Mean difference (95% CI)	P-value
50% vs 40%	-0.03 (-0.08, 0.01)	0.139
50% vs 30%	-0.07 (-0.11, -0.03)	0.004
50% vs 20%	-0.08 (-0.12, -0.04)	0.001
50% vs 10%	-0.10 (-0.14, -0.06)	0.001
50% vs control	-0.20 (-0.25, -0.16)	< 0.001
40% vs 30%	-0.04 (-0.08, 0.01)	0.117
40% vs 20%	-0.05 (-0.09, -0.01)	0.024
40% vs 10%	-0.06 (-0.11, -0.02)	0.006
40% vs control	-0.17 (-0.21, -0.13)	< 0.001
30% vs 20%	-0.01 (-0.06, 0.03)	> 0.95
30% vs 10%	-0.03 (-0.07, 0.01)	0.262
30% vs control	-0.14 (-0.18, -0.09)	< 0.001
20% vs 10%	-0.02 (-0.06, 0.03)	>0.95
20% vs control	-0.12 (-0.16, -0.08)	< 0.001
10% vs control	-0.11 (-0.15, -0.06)	< 0.001

Table 2: *Overall mean difference of biofilm among groups (Treatment effect).*

Within-between analysis (Time-treatment interaction)

As shown in table 3, there were significant differences of mean biofilm values in Day 1 (50% vs 30%, 50% vs 20%, 50% vs 10%, 50% vs control, 40% vs 20%, 40% vs 10%, 40% vs control, 30% vs 10%, 30% vs control and 20% vs control), Day 2 (50% vs 20%, 50% vs 10%, 50% vs control, 40% vs 10%, 40% vs control, 30% vs control and 20% vs control) and Day3 (50% vs 30%, 50% vs 20%, 50% vs 10%, 50% vs control, 40% vs control, 30% vs control, 20% vs control and 10% vs control).

	Comparison	Mean difference (95% CI)	P-value
Day 1	50% vs 40%	-0.01 (-0.04, 0.02)	> 0.95
	50% vs 30%	-0.03 (-0.06, -0.01)	0.018
	50% vs 20%	-0.05 (-0.07, -0.02)	0.03
	50% vs 10%	-0.07 (-0.10, -0.05)	< 0.001
	50% vs control	-0.09 (-0.12, -0.07)	< 0.001
	40% vs 30%	-0.02 (-0.05, 0.003)	0.088
	40% vs 20%	-0.04 (-0.07, -0.01)	0.009
	40% vs 10%	-0.06 (-0.09, -0.04)	0.001
	40% vs control	-0.09 (-0.11, -0.06)	< 0.001
	30% vs 20%	-0.01 (-0.04, 0.01)	0.809
	30% vs 10%	-0.04 (-0.07, -0.01)	0.007
	30% vs control	-0.06 (-0.09, -0.03)	0.001
	20% vs 10%	-0.03 (-0.05, 0.001)	0.065
	20% vs control	-0.05 (-0.07, -0.02)	0.003
	10% vs control	-0.02 (-0.01, 0.05)	0.161
Day 2	50% vs 40%	-0.01 (-0.07, 0.04)	> 0.95
	50% vs 30%	-0.03 (-0.09, 0.02)	0.376
	50% vs 20%	-0.05 (-0.11, -0.002)	0.041
	50% vs 10%	-0.08 (-0.13, -0.03)	0.005
	50% vs control	-0.12 (-0.18, -0.08)	< 0.001
	40% vs 30%	-0.02 (-0.07, 0.03)	> 0.95
	40% vs 20%	-0.04 (-0.09, 0.01)	0.153
	40% vs 10%	-0.07 (-0.12, -0.12)	0.012
	40% vs control	-0.11 (-0.17, -0.06)	0.001
	30% vs 20%	-0.02 (-0.07, 0.03)	> 0.95
	30% vs 10%	-0.05 (-0.10, 0.002)	0.063
	30% vs control	-0.09 (-0.15, -0.04)	0.002
	20% vs 10%	-0.03 (-0.08, 0.02)	0.657
	20% vs control	-0.07 (-0.13, -0.02)	0.009
	10% vs control	-0.05 (-0.10, 0.01)	0.098

Day 3	50% vs 40%	-0.08 (-0.21, 0.05)	0.467
	50% vs 30%	-0.14 (-0.27, -0.01)	0.037
	50% vs 20%	-0.15 (-0.28, -0.01)	0.031
	50% vs 10%	-0.14 (-0.27, -0.01)	0.039
	50% vs control	-0.39 (-0.52, -0.26)	< 0.001
	40% vs 30%	-0.06 (-0.19, 0.07)	> 0.95
	40% vs 20%	-0.07 (-0.20, 0.07)	0.821
	40% vs 10%	-0.06 (-0.19, 0.07)	> 0.95
	40% vs control	-0.31 (-0.45, -0.18)	< 0.001
	30% vs 20%	-0.01 (-0.14, 0.13)	> 0.95
	30% vs 10%	0.002 (-0.13, 0.13)	> 0.95
	30% vs control	-0.25 (-0.38, -0.12)	0.002
	20% vs 10%	0.01 (-0.13, 0.14)	> 0.95
	20% vs control	-0.25 (-0.38, -0.11)	0.002
	10% vs control	-0.25 (-0.39, -0.12)	0.002

***Table 3**: Comparison of mean biofilm among different groups based on time (Time-treatment interaction).*

Figure 1 showed the profile plot for the adjusted mean (estimated marginal means) of biofilm for Day 1, 2 and 3. The mean of biofilm for each treatment showed the incremental effect for each time. It can be inferred here that the effect of *Trigona* honey is time and concentration dependent with a cut off value of 30% honey dilution; being the baseline for having a significant effect on biofilm formation during 24 hours of incubation at 37°C. It was also observed that a difference of 10% between the treatments were non-significant. These findings are in line with earlier study of [26], which suggested a decline in inhibitory activity on biofilm formation when honey dilutions are applied. Moreover, honey is reported to maintain a moist wound condition to allow its gluconic acid and peroxides to act on bacteria especially in biofilm form [8,25]. Also, a higher viscosity of honey helps to provide a protective barrier to prevent the infection. This study has shown that *Trigona* honey has significant inhibitory effects on *E. coli* biofilm formation based on time. Therefore, it can be concluded that *Trigona* honey can be developed into an alternative to antibiotics for treating microbial infections. Minimum inhibitory concentration recommended based upon this study was found to be ≥ 30% honey concentration.

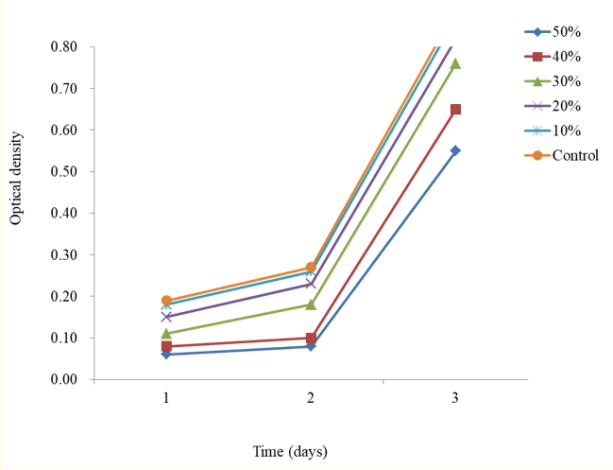

***Figure 1:** The mean values of biofilm formation (OD values) for Day 1, 2 and 3.*

Conclusion

This study has shown that *Trigona* honey has significantly inhibited biofilm formation by *E. coli in vitro*. Hence *Trigona* honey can be used as an alternative to antibiotic in retarding biofilm formation by *E. coli*. However further research and development is required to explore the active compounds that are responsible for attributing this inhibitory effect.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding to this publication.

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