



## RESEARCH ARTICLE

# Optimisation of topical antibacterial preparation from Malaysian kelulut honey by using xanthan gum as polymeric agent

Mohd-Aspar, M.A.<sup>1</sup>, Edros, R.<sup>1,2\*</sup>, Hamzah, N.A.<sup>3</sup>

<sup>1</sup>Centre of Excellence for Advanced Research in Fluid Flow, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 23600 Kuantan, Pahang, Malaysia

<sup>2</sup>Faculty of Chemical & Process Engineering Technology, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 23600 Kuantan, Pahang, Malaysia

<sup>3</sup>The Pathology and Laboratory Medicine Department, Sultan Ahmad Shah Medical Centre, 25200 Kuantan, Pahang, Malaysia

\*Corresponding author: rzahirah@ump.edu.my

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### ABSTRACT

The study aims to formulate and optimise topical antibacterial preparation by using Malaysian kelulut honey as the active ingredient and xanthan gum as the polymeric agent. Response surface methodology was used to optimise the preparation. The acidity, honey concentration and xanthan gum concentration were the independent variables. The zone of inhibitions on *S. aureus* ATCC6538 and *E. coli* ATCC8739 were the response variables. The optimal preparation was evaluated on its physicochemical properties, viscosity, antibacterial efficacy and stability. The antibacterial efficacy of the optimal preparation was compared to the commercially antibacterial gel (MediHoney™, Comvita). The optimal preparation was formulated at pH of 3.5, honey concentration of 90% (w/v) and xanthan gum concentration of 1.5% (w/v) with the inhibition zones measured on *S. aureus* ATCC6538 was 16.2 mm and *E. coli* ATCC8739 was 15.8 mm respectively. The factors of acidity and honey concentration have significantly influenced the inhibition zone on *S. aureus* ATCC6538 and *E. coli* ATCC8739. The utilisation of xanthan gum as the polymeric agent was fit for the preparation which showed by adequate physicochemical properties and retained of the antibacterial effects. This was supported by constant viscosity and efficacy of the preparation within the six months of stability study indicating stable and reliable preparation. Xanthan gum is a potential polymeric agent due to its effective use in preparing stable preparation with effective antibacterial properties.

**Keywords:** Antibacterial properties; optimisation; Malaysian kelulut; topical preparation; xanthan gum.

### INTRODUCTION

Honey has been known as an effective antibacterial agent to prevent bacterial infection (Khalil *et al.*, 2014) with the presence of both bacteriostatic and bactericidal effects. The potent antibacterial properties of honey was effective against laboratory standard bacteria such as ATCC (Chua & Ismail, 2015; Almasaudi *et al.*, 2017) and also those obtain from clinical isolation (Johnston *et al.*, 2018; Kateel *et al.*, 2018). There are numerous honey harvested from various countries including New Zealand (Weston *et al.*, 1999), Australia (Irish *et al.*, 2011), Italy (Grego *et al.*, 2016), Brazil (Sousa *et al.*, 2016), Saudi Arabia (Zakaria, 2015), and Taiwan (Liu *et al.*, 2013) have been studied on its used to prevent bacterial infection. Among the honey, the manuka honey that is native to New Zealand (Johnston *et al.*, 2018) and Australia (Carter *et al.*, 2016) is known to possess potent antibacterial properties.

In Malaysia, there are honey such as tualang, kelulut and acacia have been identified to possess beneficial

bioactive properties including antibacterial (Tan *et al.*, 2009; Syazana *et al.*, 2013; Zainol *et al.*, 2013; Ismail, 2016; Ranneh *et al.*, 2018). Among the Malaysian honey, kelulut was being studied and found to own potent antibacterial properties including bacteriostatic and bactericidal effects (Jalil *et al.*, 2017; Tuksitha *et al.*, 2018; Yaacob *et al.*, 2018). Interestingly, in our recent study, it was revealed that kelulut has a higher antibacterial properties as compared to manuka against the common pathogenic bacteria species such as *P. aeruginosa* (Mohd-aspar & Edros, 2019; Mohd *et al.*, 2020a, 2020b). Although Malaysian kelulut honey has been reported to possess potent antibacterial properties, until now, there are only limited studies that have reported on utilisation of kelulut honey as an alternative to prevent bacterial infection. Instead, the usage of Malaysian honey are limited to medicinal tonic for health consumption (Ismail, 2016). The development progress on such local honey is still unsatisfied thus demanding more efforts to discover more pharmacological properties and applications.

Based on the presence of antibacterial properties, honey has been successfully applied as a topical preparation in the treatment of wound infection (Mohd-aspar *et al.*, 2021). Other than antibacterial properties, the additional characteristics of honey such as non-irritant, non-toxic, self-sterile, nutritive, and easily applied (Boukraâ, 2014; Ismail, 2016) enhanced its potential to be effectively utilised as a topical antibacterial agent. Based on the tropical rainforest, the honey harvested in Malaysia contained high moisture content (exceeding 20%) which is less viscous and unsuitable to be directly applied without improvement on its rheological properties. The honey could not remain on the site of action as long as needed and maintain the concentration within the effective range to promote bacteriostatic and bactericidal effects (El-kased *et al.*, 2017; Zhu *et al.*, 2019).

Polysaccharides derived from natural gums such as xanthan gum is widely used as polymeric agent due to its biodegradability and non-toxic characteristics (Badwaik *et al.*, 2013; Benny *et al.*, 2014; Thombare *et al.*, 2016). Xanthan gum is a high molecular weight polysaccharide produced predominantly by *Xanthomonas campestris* in an aerobic condition from sugar cane, corn or their derivatives (Badwaik *et al.*, 2013). These natural polysaccharides give highly viscous solution even at 1% of the aqueous dispersion of the gum. Xanthan gum is used mainly as a thickener in food, cosmetic, and pharmaceutical industries (Garcia-Ochoa *et al.*, 2000; Bueno *et al.*, 2013; Benny *et al.*, 2014).

In the effort to implement the Malaysian honey as an antibacterial agent, the study aims to evaluate the potential development of a topical preparation from Malaysian kelulut honey with the employment of xanthan gum as the polymeric agent. The rheological properties of Malaysian honey were aimed to be improved by using the natural xanthan gum as thickening agent. In order to maximise the effect on preventing the growth of bacteria, the preparation was optimised through response surface method by considering the most effective pH, honey concentration and polymeric agent concentration used. The optimal preparation was evaluated on its physicochemical properties, antibacterial efficacy, and stability to affirm on the stability and adequacy of the formulated preparation. As for antibacterial efficacy, the commercially available antibacterial gel (MediHoney™) was used as a basis of comparison.

## MATERIALS AND METHODS

### Honey Samples

Kelulut samples were obtained from local apiarist and aseptically stored in the sterile bottles. The information of the collected honey were recorded in the form of certificate of analysis (CoA) that has been accredited by Malaysian Agriculture Research and Development Institute (MARDI). Additional assay by using the RapidRaw™ method, which was developed by the Malaysia Genome Institute (MGI) was included to verify the purity of honey. Honey that has not been processed contains more natural compounds to react with the reagents which resulted with formation of sediment. The honey without visible sediment are processed honey. The kelulut sample used in this study showed clear visible sediment indicating the purity of honey. This is in agreement with our negative control with the sole presence of sugar substance. The honey were stored in sterile glass bottles for experimental works and kept in the dark plastic container away from direct sunlight at room temperature.

### Materials and Methods

The xanthan gum was purchased from Sigma, US. Sodium benzoate and triethanolamine (TEA) were purchased from Bendosen, Malaysia.

### Bacteria

The study has employed eight standard strains and fourteen clinically isolated strains of common bacteria infecting wound. The eight standard strain bacteria were kindly supplied by the Department of Pathology & Laboratory Medicine, International Islamic University Malaysia Medical Centre (IIUMMC) and Central Laboratory Universiti Malaysia Pahang (UMP) and labelled as standard strains of American Type Culture Collection (ATCC, US). These includes three gram-positive bacteria of *Staphylococcus aureus* ATCC 6538, *Streptococcus pyogenes* ATCC 19615 and *Enterococcus faecalis* ATCC 29212, and five gram-negative bacteria of *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14028, *Proteus mirabilis* ATCC 12453, and *Klebsiella pneumonia* ATCC BAA 1144. The 14 clinically isolated bacteria were obtained primarily from Department of Pathology & Laboratory Medicine, International Islamic University Malaysia Medical Centre (IIUMMC). These include five gram-positive bacteria of *Staphylococcus aureus*, *Staphylococcus hominis*, *Staphylococcus haemolyticus*, *Streptococcus pyogenes*, and *Streptococcus agalactiae* and another nine gram-negative bacteria of *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella sp.*, *Proteus mirabilis*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Enterobacter cloacae*, and *Enterococcus aerogenes*. The bacteria were re-cultured in a nutrient or soy agar and incubated at 37°C for 24 hours in which by then is known as primary culture.

### Preparation of Working Bacteria

Working bacterial culture was prepared by inoculating a loop of primary culture into sterile screw-capped test tubes containing 10 ml of broth and incubated in a shaking incubator for 24 hours at a temperature of 37°C and rotational speed of 150 rpm. The prepared working bacteria cultures were adjusted to 0.5 McFarland standard which equivalent to  $1.5 \times 10^8$  CFU/mL. It was prepared based on optical density by diluting the working bacteria into fresh sterile broth and adjusted to the absorbance range of 0.08 to 0.13 (Franklin *et al.*, 2012). The absorbance of prepared cultures was measured by using UV-viscometer (Shimadzu, Japan) at the reference wavelength of 600nm.

### Preparation of the Topical Preparation

The preparation with kelulut honey was prepared by employing the cold mechanical method (El-kased *et al.*, 2017) in which the desired amount of natural polymer was dissolved in the sterile deionised water with continuous stirring for 1 h until the polymer was completely soaked in water. This step was followed by the addition of 0.02% (w/v) sodium benzoate (Bendosen, Malaysia) as a preservative in the preparation. Sodium benzoate is a commonly used as preservative agent especially in food products due to its non-toxic and safe additive (Shahmihammadi *et al.*, 2016), with the allowed amount of usage of 0.03% (w/w) by FDA (Pongsavee, 2015). The mixture was stirred continuously for 30 min. The desired amount of honey was added to the mixture with continuous stirring for another 30 min until the honey was dissolved. The final volume of each preparation was set to 100 mL by adding sterile deionised water. The preparation was kept in a sterile, wide-mouth glass

container that was covered with a lid and stored at 28°C for 24 h for a complete swelling.

#### Measurement of Inhibition Zone

Prior to investigating the potency to inhibit bacteria growth, a study was conducted to measure the zone of inhibition on the bacterial strains when exposed to the preparation. This was performed qualitatively by using the agar well diffusion assay as previously described (Sherlock et al., 2010; Moussa et al., 2012) to gain an understanding of the sensitivity of bacteria towards the preparation. In this assay, two types of agars were used, i.e., nutrient agar and soy agar depending on the bacteria. Soy agar was used to grow *E. faecalis* and *S. pyogenes*, while nutrient agar was used to grow *S. aureus*, *E. coli*, *P. aeruginosa*, *S. typhimurium*, *P. mirabilis* and *K. pneumonia*. The nutrient and soy agar were prepared by dissolving 23 g and 40 g of agar to 1 L of distilled water and later autoclaved at the pressure of 100 kPa and temperature of 121°C for 20 min. The agars were allowed to cool down slightly and was poured into 90 mm × 15 mm (Brandon™, Malaysia) petri dishes.

The working bacterial culture, which was adjusted to 0.5 McFarland bacteria concentration, was prepared as described previously. A volume of 100 µL of the adjusted 0.5 McFarland culture was spread onto the agar by using the spread plate technique. Upon inoculation, 6 mm diameter wells were cut on the agar surface. A plate was divided into four quadrants, where a single well was created in each quadrant to contain 80 µL of the preparation. Plates were incubated at 37°C for 24 h. The diameters of inhibition zones were measured by using a ruler in millimetres (mm) based on the diameter of circles formed around the tested well areas in which the bacterial colonies do not grow. This diameter is inclusive of the diameter of the 6 mm well that was used to occupy the tested preparation. The commercially available topical gel was used as a positive control. Based on the inhibition zones measured, the sensitivity of bacteria towards preparation was categorised as not sensitive, sensitive, very sensitive, and extremely sensitive as previously described (Moussa et al., 2012). The not sensitive was denoted by the diameter of inhibition zone of lower than 8 mm, sensitive for the diameter from 8 to 14 mm, very sensitive for the diameter from 15 to 19 mm, and extremely sensitive for the diameter of 20 mm and above.

#### Design of Response Surface Methodology

In RSM, the experimental domain is defined as the antibacterial properties of the preparation. Three independent variables were pH, honey concentrations (% w/v), and polymeric agent concentrations (% w/v), and designated as  $X_1$ ,  $X_2$ , and  $X_3$ , respectively. The inhibition zone (mm) on *S. aureus* ATCC 6538, and *E. coli* ATCC 8739 were collected as the response variables which were designated as  $Y_1$  and  $Y_2$ , respectively. The bacteria of *S. aureus* and *E. coli* were selected since these species were commonly isolated from an infected wound and have various mechanisms of resistance toward antibacterial agents, such as biofilm formation (Jefferson, 2004), active beta-lactamase production (Brudzynski & Sjaarda, 2014; Peacock & Paterson, 2015), and high pH tolerance (Molan, 1992; Cotter & Hill, 2003).

In each of the independent variables  $X_1$ ,  $X_2$ , and  $X_3$ , the optimum parameters were determined within the range that were set during the optimisation work. The lowest value was referred as low level and the highest value was referred as the high level respectively (Shahzad et al., 2012; Shekar et al., 2014). In this study, the low and high levels were set

between 3.5 and 6.5 for acidity ( $X_1$ ), 50% and 90% (w/v) for honey concentration ( $X_2$ ) and 1.0% and 2.0% (w/v) for the xanthan gum concentration ( $X_3$ ) respectively. The low and high levels of the independent variables were set according to the literature.

In this study, RSM by using the central composite design (CCD) was used to optimise the antibacterial properties of the preparation. According to the CCD, the total number of experimental combinations is based on Equation 1 below (Shekar et al., 2014; Anitha & Pandey, 2016):

$$2^k + 2k + n_0 \quad \text{Equation [1]}$$

Where  $k$  is the number of independent variables and  $n_0$  is the number of repetitions of the experiments at the central points. In this study, three independent variables were involved ( $k = 3$ ) with five replicates at the centre points ( $n_0 = 5$ ) leading to a total of nineteen runs.

The Design of Experts Software (DOE version, 7.1.3, STAT-EASE Inc., Minneapolis, USA) was used for Analysis of Variance (ANOVA), regression, and graphical analyses of the data obtained. The desirability function to get the optimum combinations of independent variables was fitted by the least square method using the software. The three-dimensional response graph and profile for predicted values and desirability level for independent variables were plotted by employing the same software. In ANOVA, the analysis included overall model significance, correlation coefficient ( $R$ ), and determination coefficient ( $R^2$ ) that measure the goodness of fit of the regression model.

#### Evaluation of the Optimal Preparation

The optimal preparation resulting from each optimisation process were evaluated in terms of physicochemical properties, antibacterial efficacy, and stability. This is essential in order to decide on adequate and reliable preparation.

#### Physicochemical Properties

The physicochemical properties of the optimal preparation was evaluated in term of physical appearance, colour, homogeneity, grittiness, lump formation, viscosity, and pH. The viscosity was measured by using Viscometer VL210001 (Fungilab, Spain), spindle number R5, at 100 rotations per min. The pH was measured by using the pH meter SevenCompact™ (Mettler Toledo, USA).

#### Centrifugation Test

The centrifugation test was performed by using a refrigerated centrifuge 5810R (Eppendorf, Germany) as previously described by (Dantas et al., 2016). It was done by adding 10 g of the preparation in a tapered test tube and was subjected to a cycle of 3000 rpm for 30 min at the room temperature of 25°C.

#### Antibacterial Efficacy

The antibacterial efficacy of the optimal preparation was evaluated by using two separate methods. The first method was the evaluation on the inhibition effect by using the measurement of inhibition zone and the second method was the evaluation on the bactericidal effect by using the tube dilution method. For both evaluations, the experiments were performed on 22 bacterial species, including eight standard strains and fourteen clinical strains as previously listed.

### Measurement of Inhibition Zone

The ability of the preparation to inhibit the growth of wound-associated bacteria was determined through the measurement of inhibition zone by using agar well diffusion assay as previously described (Measurement of inhibition zone). The diameters of each inhibition zone was measured in millimetres (mm), including the diameter of well created. Each test was carried out in triplicate, and the average values were calculated.

### Bactericidal Effect

The bactericidal effect of the optimal preparation was determined by using tube dilution method as previously described (Shagana & Geetha, 2017). An equal volume of 0.5 mL of the preparation was mixed with 0.5 mL of freshly prepared broth in screw cap tube (Jain et al., 2016; Shagana & Geetha, 2017). Then, a loopful of the test organism that has been adjusted to 0.5 McFarland was transferred into the tube (Dewanjee et al., 2008). A tube that contained 1 mL of freshly prepared broth and seeded with a loopful of test organism was used as a control. The prepared tubes were then incubated in the incubator shaker at 37°C at a rotational speed of 150 rpm for 24 h. After overnight incubation, a loopful suspension was suspended and inoculated onto freshly prepared TSA by using the streak plate method. Then, the plate was incubated for another 24 h in 37°C before being observed for any bacterial growth. A plate with no visible growth of bacteria was considered to possess bactericidal effect while the plate with the visible formation of bacterial colony was considered as the absence of a bactericidal effect.

### Stability Study

Evaluation of the stability of the optimal preparation was adapted from previous studies (Chen et al., 2016; Dantas et al., 2016; Majumdar et al., 2018) with slight modification. The preparation was kept in the glass containers and stored in long-term and accelerated conditions, which were at 25°C ± 2/60% ± 5 RH and 40°C ± 2/75% ± 5 RH, respectively for six months and evaluated at 0, 1, 2, 3, and 6 months. The storage conditions during the stability study were set according to the ICH guideline (World Health Organisation, 2018). The evaluations were observed based on colour, pH, homogeneity, viscosity, and antibacterial efficacy, which were conducted similar to the procedures described in the previous sections. In the measurement of the inhibition zone and bactericidal effect, three Gram-positive i.e., *S. aureus* ATCC 6538, *E. faecalis* ATCC and *S. pyogenes* and three Gram-negative i.e., *E. coli* ATCC 8739, *K. pneumonia* and *E. aerogenes* were considered.

## RESULTS AND DISCUSSION

### Response Surface Methodology (RSM) for the Preparation

The nature of kelulut honey is less viscous which unsuited to be applied topically as it could not remain on the site of action as long as needed and maintain the concentration within the effective range to promote bacteriostatic and bactericidal effects. Due to its incompatibility, kelulut honey has been prepared as a preparation. The natural polymers i.e., xanthan gum was used to improve the rheological properties of the honey to make it topically applicable. The optimisation through Response Surface Methodology (RSM) has been employed to optimise the preparation for prevention of bacterial infection. The optimal independent variable i.e., acidity ( $X_1$ ), concentration of honey ( $X_2$ ) and concentration of xanthan gum ( $X_3$ ) were determined with respond to the diameter of the inhibition zones on *S. aureus*

**Table 1.** Experimental design of the central composite design and response for the experimental runs

Run	Factor			Response of inhibition zone (mm)	
	$X_1$	$X_2$	$X_3$	<i>S. aureus</i> ( $Y_1$ )	<i>E. coli</i> ( $Y_2$ )
1	3.50	50.00	1.00	11.7 ± 0.58	11.3 ± 0.58
2	6.50	50.00	1.00	7.8 ± 0.00	8.0 ± 0.29
3	3.50	90.00	1.00	13.8 ± 0.29	13.7 ± 0.58
4	6.50	90.00	1.00	8.0 ± 0.00	8.3 ± 0.58
5	3.50	50.00	2.00	12.2 ± 0.29	10.7 ± 0.58
6	6.50	50.00	2.00	8.0 ± 0.00	8.3 ± 0.58
7	3.50	90.00	2.00	13.8 ± 0.29	12.3 ± 0.29
8	6.50	90.00	2.00	9.0 ± 1.00	8.3 ± 0.58
9	2.48	70.00	1.50	16.2 ± 0.58	15.8 ± 0.58
10	7.52	70.00	1.50	8.3 ± 0.58	8.3 ± 0.58
11	5.00	36.36	1.50	7.8 ± 0.00	7.8 ± 0.58
12	5.00	103.64	1.50	9.7 ± 0.58	9.5 ± 0.00
13	5.00	70.00	0.66	8.3 ± 0.58	8.5 ± 0.58
14	5.00	70.00	2.34	9.0 ± 0.00	9.2 ± 0.58
15	5.00	70.00	1.50	8.7 ± 0.76	8.7 ± 0.50
16	5.00	70.00	1.50	9.2 ± 0.29	9.2 ± 0.29
17	5.00	70.00	1.50	8.7 ± 0.76	9.0 ± 0.29
18	5.00	70.00	1.50	8.8 ± 0.76	9.0 ± 0.50
19	5.00	70.00	1.50	8.8 ± 0.29	9.2 ± 0.76

The symbol ± represents the standard deviation, which was calculated between the three biological replicates. Student's t-test shows significant differences for the data collected ( $P$ -value < 0.05).

ATCC 6538 ( $Y_1$ ) and *E. coli* ATCC 8739 ( $Y_2$ ). The results for optimisation of preparation by using xanthan gum were described below.

### Analysis of Variance (ANOVA) and Model Fitting

A total of 19 experimental runs were conducted which combined different levels of independent variables (Shekar et al., 2014; Anitha & Pandey, 2016). The observed responses from each run are tabulated in Table 1.

Based on the findings, the diameter of inhibition zones measured were found to range from 7.8 ± 0.00 mm to 16.2 ± 0.58 mm on *S. aureus* and 7.8 ± 0.58 mm to 15.8 ± 0.58 mm on *E. coli*, respectively. The largest zones of inhibition of 16.2 ± 0.58 mm and 15.8 ± 0.58 mm were measured on *S. aureus* and *E. coli*, respectively in run number 9, while the smallest zones of inhibition of 7.8 ± 0.00 mm and 7.8 ± 0.58 mm were measured on *S. aureus* and *E. coli*, respectively in run number 11.

A second-order polynomial model was developed by using RSM for the prediction of optimum responses in a function of independent variables and their interactions (Ammer et al., 2016). It was performed by applying multiple regression analysis on the experimental data. Based on the generated model, the role of each independent variable ( $X_1$ ,  $X_2$ , and  $X_3$ ) and their interaction can be explained and the optimum zone of inhibition that corresponds to the optimum levels of pH, honey concentration, and xanthan gum concentration can be estimated (Madiha et al., 2017). The second-order polynomial model generated and their estimated regression coefficients are shown in the following equation:

$$Y_1 = 8.82 - 2.34X_1 + 0.59X_2 + 0.21X_3 - 0.31X_1X_2 + 0.088X_1X_3 + 0.038X_2X_3 + 1.33X_1^2 + 0.094X_2^2 + 0.059X_3^2 \quad \text{Equation [2]}$$

$$Y_2 = 9.01 - 2.03X_1 + 0.52X_2 - 0.038X_3 - 0.46X_1X_2 + 0.29X_1X_3 - 0.14X_2X_3 + 1.12X_1^2 - 0.086X_2^2 - 0.015X_3^2 \quad \text{Equation [3]}$$

Where  $Y_1$  and  $Y_2$  are the responses, i.e., the inhibition zones on *S. aureus* and *E. coli*, respectively.  $X_1$ ,  $X_2$ , and  $X_3$  are the coded values of the independent variables, i.e., pH, honey concentration, and xanthan gum concentration, respectively.

In general, both Equation 2 and Equation 3 consist of three linear terms ( $X_1$ ,  $X_2$ , and  $X_3$ ), three quadratic terms ( $X_1^2$ ,  $X_2^2$ , and  $X_3^2$ ), and three combinations of two factorial terms ( $X_1X_2$ ,  $X_1X_3$ , and  $X_2X_3$ ) to explain the role of independent variables ( $X_1$ ,  $X_2$ , and  $X_3$ ) that affect response variables ( $Y_1$  and  $Y_2$ ). The positive coefficients indicate a linear effect which is directly proportional to the zone of inhibition while the negative coefficients show a negative effect that is inversely proportional to the zone of inhibition (Wang et al., 2011; Shahzad et al., 2012).

From both equations, the effect of pH ( $X_1$ ) is more pronounced compared to honey concentration ( $X_2$ ) and xanthan gum concentration ( $X_3$ ) since the coefficient of  $X_1$  is 2.34, which is 4-fold and 11-fold higher than the coefficient of  $X_2$  and  $X_3$  with values of 0.59 and 0.21, respectively for Equation 2. This also applies to Equation 3 with a value of 2.03 for  $X_1$ , which is 4-fold and 53-fold higher compared to  $X_2$  and  $X_3$  with values of 0.52 and 0.038, respectively.

The significant impact of each term in the second-order polynomial equation was evaluated through ANOVA and the results are tabulated in Table 2. The degree of significance for every term in the equation including linear ( $X_1$ ,  $X_2$ ,  $X_3$ ), quadratic ( $X_1^2$ ,  $X_2^2$ ,  $X_3^2$ ), and combination ( $X_1X_2$ ,  $X_1X_3$  and  $X_2X_3$ ) were analysed at 95% confident interval ( $P$ -value < 0.05) (Ammer et al., 2016; Madiha et al., 2017).

Among the linear terms, the main effect of pH ( $X_1$ ) and honey concentration ( $X_2$ ) on inhibition zones of both *S. aureus* and *E. coli* were highly significant as proven by their respective  $P$ -values with  $P_{X_1} < 0.0001$  and  $P_{X_2} = 0.0005$  for *S. aureus*, and  $P_{X_1} < 0.0001$  and  $P_{X_2} = 0.0007$  for *E. coli*, respectively. In contrast, xanthan gum concentration ( $X_3$ ) has an insignificant effect on inhibition zones for both *S. aureus* and *E. coli* with  $P_{X_3} = 0.0894$  and  $P_{X_3} = 0.7205$ , respectively. For quadratic terms, only  $X_1^2$  was significant at 5% level with  $P_{X_1^2} < 0.0001$  for both *S. aureus* and *E. coli*. Meanwhile,  $X_2^2$  and  $X_3^2$  were insignificant with  $P_{X_2^2} = 0.4171$  and  $P_{X_3^2} = 0.6081$  for *S. aureus*, and  $P_{X_2^2} = 0.4305$  and  $P_{X_3^2} = 0.8891$  for *E. coli*, respectively. For combined variables, none of the terms had a significant effect on the zones of inhibition. The results suggested that pH and honey concentration have a direct relationship with inhibition zones since small variations in their values will considerably

alter the zones of inhibition for both *S. aureus* and *E. coli*. This is consistent with a study conducted previously in which pH and honey concentration have a strong influence on antibacterial properties of honey (Johnston et al., 2018; Kateel et al., 2018).

The precision of the quadratic regression model is also supported by the ANOVA results (Wang et al., 2011; Anitha & Pandey, 2016). From Table 2, the quadratic regression model was highly significant with  $P$ -value < 0.0001 for both *S. aureus* and *E. coli*. This indicates that the quadratic regression model as expressed in Equation 2 and Equation 3 provides a suitable model to describe the response of the experiment pertaining to the zone of inhibition (Shahzad et al., 2012). In addition, the model also showed a statistically insignificant lack of fit with  $P$ -value of 0.0507 and 0.0620 for *S. aureus* and *E. coli* respectively, indicating the adequate fit of the models (Shahzad et al., 2012; Shekar et al., 2014; Khuri, 2017).

The model's goodness of fit based on RSM can be further checked by the determination coefficient ( $R^2$ ) and adjusted  $R^2$  (Shahzad et al., 2012; Shekar et al., 2014). The  $R^2$  provides a measure of variability in the observed response values that can be explained by the experimental factors and their interactions. The  $R^2$  value is always between 0 and 1, and the closer the  $R^2$  value to 1, the stronger the model to predict the response (Mulye et al., 2014; Madiha et al., 2017). An  $R^2$  value higher than 0.9 indicates a strong correlation (Shahzad et al., 2012). In this study, the coefficient of determination  $R^2$  were 0.9860 and 0.9839 (Table 2), implying that the zones of inhibition are attributed to the given range of pH, honey concentration, and xanthan gum concentration. The  $R^2$  also indicates that 98% of the total variation was explained by the model while only 2% was not explained which is due to external factors. The values of the adjusted determination coefficient (adjusted  $R^2 = 0.9719$  and 0.9678) were also high, indicating good accuracy and ability of the polynomial model to analyse the response trend (Wang et al., 2011; Ammer et al., 2016). Therefore, it is concluded that the model is adequate to predict the response within the range of variation employed.

**Response Surface Analysis**

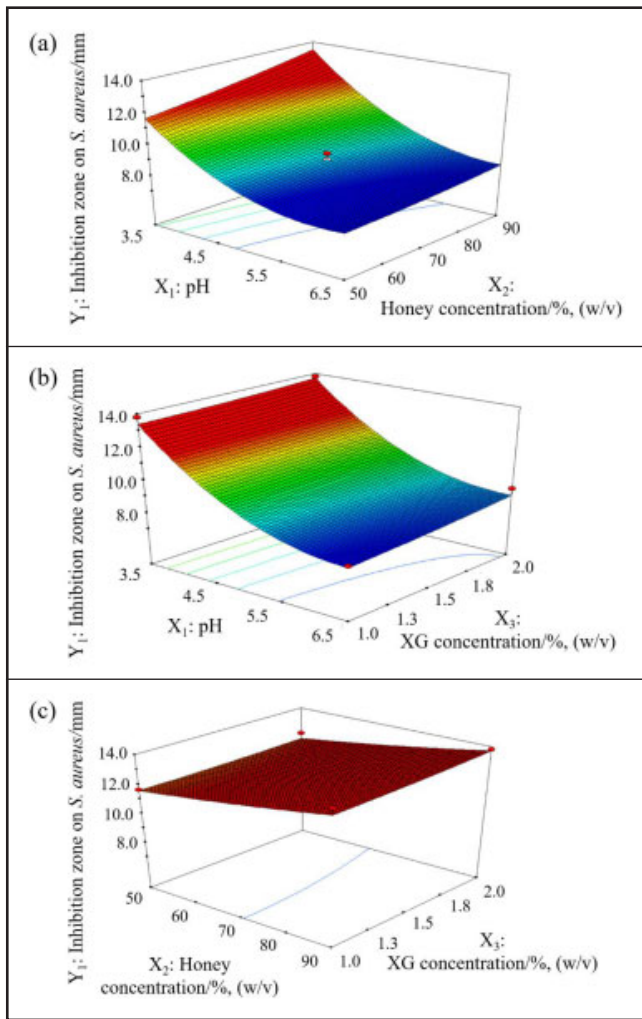
The interaction between independent variables;  $X_1X_2$ ,  $X_1X_3$ , and  $X_2X_3$  as indicated in Table 2 can be visualised by using 3D response surface and 2D contour plots as shown in Figure 1 for *S. aureus* ATCC 6538 and Figure 2 for *E. coli* ATCC 8739. These plots are important to illustrate the effects of independent variables, and their interactions on the response variables.

Figure 1 (a) and Figure 2 (a) show the 3D plots and their corresponding contour plots, showing the effect of pH ( $X_1$ ) and honey concentration ( $X_2$ ) on inhibition zones of *S. aureus* and *E. coli*, while the concentration of xanthan gum ( $X_3$ ) was fixed at its middle level which was 1.5% (w/v). For both *S. aureus* and *E. coli*, at any pH level between 3.5 and 6.5, honey concentration showed an inverse correlation with the zone of inhibition. As the preparations were prepared at lower pH, the zones of inhibition were increased. In contrast, the honey concentration showed a direct correlation to the zone of inhibition, regardless of which pH level was employed within pH 3.5 and 6.5. As the concentration of honey increased, the zone of inhibition also increased. The findings indicate acidity has no significant interaction with honey concentration which has proven by the unaffected response of pH with regards to the concentration of honey and vice versa. The analysis of Figure 1 (a) and Figure 2 (a) reveals that the optimal pH was at the lowest pH which was at 3.5 and the honey concentration was at the concentration of 90% (w/v).

**Table 2.** Analysis of variance (ANOVA) of the quadratic model and coefficient of determination ( $R^2$ ) of the quadratic model

Factors	Inhibition zone on <i>S. aureus</i>		Inhibition zone on <i>E. coli</i>	
	$P$ -value	Model term	$P$ -value	Model term
$X_1$	*<0.0001	Significant	<0.0001	Significant
$X_2$	*0.0005	Significant	0.0007	Significant
$X_3$	0.0894	Not Significant	0.7205	Not Significant
$X_1X_2$	0.0590	Not Significant	0.0077	Not Significant
$X_1X_3$	0.5601	Not Significant	0.0628	Not Significant
$X_2X_3$	0.8012	Not Significant	0.3365	Not Significant
$X_1^2$	*<0.0001	Significant	<0.0001	Significant
$X_2^2$	0.4171	Not Significant	0.4305	Not Significant
$X_3^2$	0.6081	Not Significant	0.8891	Not Significant
Model	*<0.0001	Significant	<0.0001	Significant
Lack of fit	0.0507	Not significant	0.0620	Not significant
$R^2$	0.9860		0.9839	
Adjusted $R^2$	0.9860		0.9678	

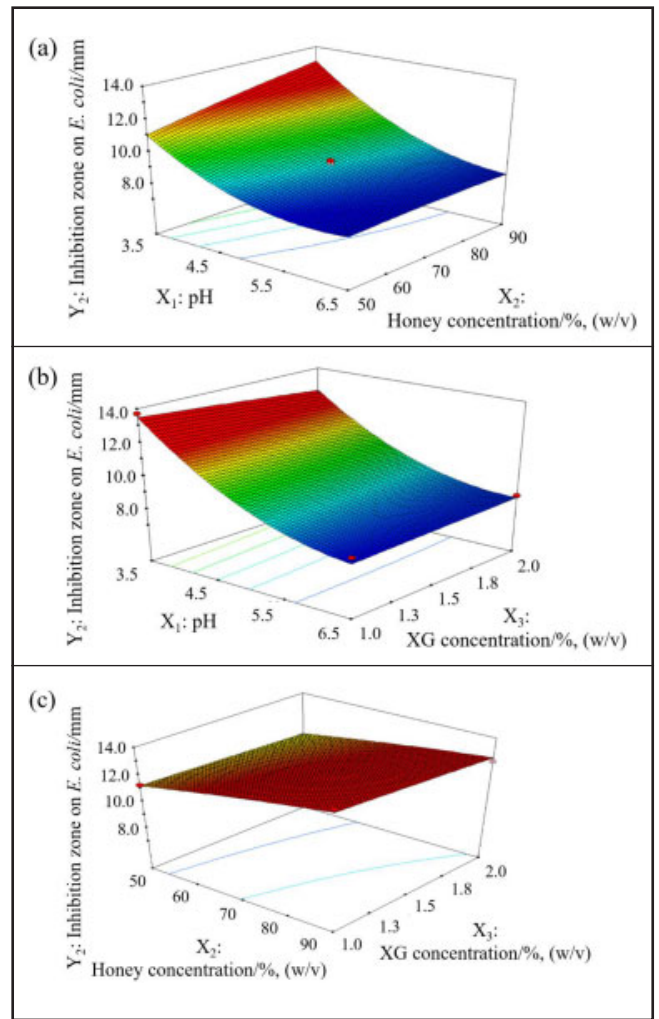
Significant at 5% level ( $P$ -value < 0.05).



**Figure 1.** Response surface plot showing the effect of pH ( $X_1$ ), honey concentration ( $X_2$ ), and xanthan gum concentration ( $X_3$ ) on inhibition zones against *S. aureus* ATCC 6538 ( $Y_1$ ). (a)  $X_1X_2$ , (b)  $X_1X_3$ , and (c)  $X_2X_3$ .

Figure 1 (b) and Figure 2 (b) depict the 3D plots and their corresponding contour plots showing the effects of pH ( $X_1$ ) and xanthan gum concentration ( $X_3$ ) on the zones of inhibition of *S. aureus* and *E. coli*, while the honey concentration was fixed at its middle level which was 70% (w/v). As the preparation was prepared with xanthan gum concentrations between 1.0% and 2.0% (w/v), the zones of inhibition remained unchanged, regardless of the variation in pH level. Similarly, as the pH of the preparation was increased from 3.5 to 6.5, the zones of inhibition decreased without being affected by the concentration of xanthan gum. According to Figure 1 (b) and Figure 2 (b), it can be observed that there was a lack of interaction between pH and xanthan gum concentration. The interaction between pH ( $X_1$ ) and xanthan gum concentration ( $X_3$ ) does not give a mutual impact on inhibition zones. No optimal concentration of xanthan gum was observed across the tested range between 1.0% and 2.0% (w/v).

Figure 1 (c) and Figure 2 (c) present the 3D plots and their corresponding contour plots showing the effect of honey concentration ( $X_2$ ) and xanthan gum concentration ( $X_3$ ) on the inhibition zones of *S. aureus* and *E. coli*, while the pH was fixed at its middle level which was 5.0. As the preparation was formulated with xanthan gum concentrations between 1.0% and 2.0% (w/v), the zones of inhibition remained



**Figure 2.** Response surface plot showing the effect of pH ( $X_1$ ), honey concentration ( $X_2$ ), and xanthan gum concentration ( $X_3$ ) on inhibition zones against *E. coli* ATCC 8739 ( $Y_2$ ). (a)  $X_1X_2$ , (b)  $X_1X_3$ , and (c)  $X_2X_3$ .

unchanged. However, as the preparation was formulated with concentrations of honey from 50% to 90% (w/v), the zones of inhibition increased in size. The changes in xanthan gum concentration and honey concentration did not show mutual interaction towards the inhibition zones, indicating the lack of interaction between concentrations of honey ( $X_2$ ) with the concentration of xanthan gum ( $X_3$ ).

The 3D plots and their respective contour plots are useful to analyse the effect of independent variables towards the response variables and enabled the identification of the optimal response. Based on the results obtained from Figure 1 and Figure 2, the optimal preparation was observed with pH 3.5 and honey concentration of 90% (w/v) while the concentration of xanthan gum was chosen at the middle of level which was at 1.5% (w/v). This is due to the consistent inhibition zone with less than 5% of variation across the tested range between 1.0% and 2.0% (w/v).

#### Verification of the Optimal Responses

Based on the second-order polynomial models and surface response, the levels of independent variables to produce optimal responses were predicted to be at pH of 3.5, honey concentration of 90% (w/v) and concentration of xanthan gum of 1.5% (w/v). According to the RSM analysis, the optimal inhibition zones were estimated to be 13.5 mm for *S. aureus*

**Table 3.** Summary of the predicted and observed responses for the optimal preparation

Factor	Optimal value			
pH	3.5			
Honey concentration (% w/v)	90			
Xanthan gum concentration (% w/v)	1.5			
Response	Predicted	Observed	Residual	Prediction error (%)
Inhibition zone on <i>S. aureus</i> (mm)	13.5	13.7	±0.2	1.5
Inhibition zone on <i>E. coli</i> (mm)	13.1	13.9	±0.8	6.1

The symbol ± represents the standard deviation, which was calculated between three biological replicates.

and 13.1 mm for *E. coli* respectively. An experiment was carried out to verify the results and the outcomes are tabulated in Table 3.

Based on the conducted experiment, the inhibition zones measured were 13.7 mm for *S. aureus* and 13.9 mm for *E. coli* respectively. The results were in close agreement with the predicted responses with residual percentages of 1.5% and 6.1% for *S. aureus* and *E. coli*, respectively. The results confirmed the validity of the predicted models, as the residual percentages were less than 10% (Shahzad *et al.*, 2012; Madiha *et al.*, 2017).

The RSM is an effective tool for optimising preparation which allows the user to predict the optimal response through the generation of mathematical equations and three-dimensional response surfaces. In preparing a topical preparation, RSM has been used to optimise the percentage of drug release (Mulye *et al.*, 2014), permeability (Shahzad *et al.*, 2012), and spreadability (Mulye *et al.*, 2014). The response to be optimised is selected depending on the main goal of which the preparation has been developed. In the present study, the RSM has been successfully utilised to optimise a preparation that can be topically used to prevent bacterial infection on wound sites. The response variables which were the inhibition zone on *S. aureus* and *E. coli*, have been precisely interpreted in form of mathematical equations and three-dimensional response surfaces to assist on the prediction of optimal responses based on the considered independent variables.

In optimising the topical preparation, pH was one the considered independent variables. The range of pH between 3.5 and 7.5 was used as it is a suitable range to promote wound healing (Serra *et al.*, 2015). As the preparation was set with the lower pH i.e., pH 3.5, the inhibition zone measured was noted to increase. This result was found to support the effectiveness of an acidity in preventing bacterial growth (El-kased *et al.*, 2017). The reason for larger inhibition zones in acidic pH compared to neutral pH may due to the unsuitable of bacterial growth condition which required pH between 6.6 to 7.0 (Jones *et al.*, 2015) and also the antimicrobial properties of compounds such as flavonoids and phenolic acids that are available in kelulut honey which reported to increase at lower pH (Sanchez-Maldonado *et al.*, 2011).

Other than pH, honey concentration was also the considered independent variable in which it was optimised between the concentration of 50% and 90% (w/v). As the honey concentration was increased, the diameter of the inhibition zone also increased significantly. This can be explained by the presence of antibacterial compounds such as phenolic acids and flavonoids that increased as the honey concentration increased (Bakar *et al.*, 2017; Tuksitha *et al.*, 2018). In addition, as the honey concentration increased,

the degree of sugar content that naturally present in kelulut honey will also increase that can causes dehydration to bacteria (Dluya, 2016). These findings were similar to the previous study that found higher inhibition zone with respond to increments of honey concentration used in honey-based preparation (El-kased *et al.*, 2017).

An ideal topical preparation requires sufficient polymeric cross-link structures to sustain an optimum effect. The concentration of polymer used directly impacts the strength of polymeric cross-links (Wong *et al.*, 2015). The utilisation of xanthan gum at concentrations between 1% and 2% were reported to provide sufficient cross-linking strength (Cunha *et al.*, 2005; Bueno *et al.*, 2013; Mulye *et al.*, 2014). Based on the optimisation through RSM, a variation on the concentration of xanthan gum between 1% and 2% resulted with no significant effect towards the inhibition zone on both *S. aureus* and *E. coli* and no optimum concentration observed during the optimisation process. The insignificant effect may be due to the use of a narrow range of polymeric agent concentration between 1% and 2%. Although the range was reported to significantly affect the rheological properties of a preparation (Mulye *et al.*, 2014), however, it does not significantly affect the zone of inhibition.

As the optimal preparation was finalised at a pH of 3.5, honey concentration of 90% (w/v) and xanthan gum concentration of 1.5% (w/v), further evaluations on physico-chemical properties, antibacterial efficacy, and stability were necessary to ascertain the effective use of the xanthan gum as the polymeric agent in conveying kelulut honey as a topical preparation. Therefore, the description of the outcomes of further evaluations are described in the following sections.

#### Evaluations of the Optimal Preparation

Based on the optimal preparation obtained from RSM, the physicochemical properties, antibacterial efficacy, and stability of the optimal preparation was evaluated. The evaluations on physicochemical properties include both physical and chemical characteristics of the preparation, which described the compatibility and reliability of the preparation. Meanwhile, antibacterial efficacy revealed the antibacterial properties the preparation which determined through measurement of inhibition zone and formation of the bacterial colony to specify the bacteriostatic and bactericidal effects. Finally, the stability study was performed to observe the consistency and durability of the preparation upon storage. Within the six months of storage period, the uniformed characteristics with minimal variation on both physical and chemical properties indicate an ideal topical preparation (Hemendrasinh & Dhruvi, 2015; Dantas *et al.*, 2016).

**Physicochemical Properties**

The physicochemical properties of the optimal preparation were evaluated in terms of physical appearance, homogeneity, colour, grittiness, lump formation, pH, viscosity, and centrifugation test. The results are tabulated in Table 4.

The preparation resulted in opaque, homogeneous, and dark brown colour. No grittiness and formation of lump were observed. The pH of the preparation was recorded at  $3.52 \pm 0.10$ . The pH recorded can be considered suitable for topical preparation, as the pH ranging between 2.8 and 7.4 was acceptable for therapeutic effect with non-irritant (Panther & Jacob, 2015; Dantas *et al.*, 2016;) since the normal pH range of human skin is between 4 to 6 (Schmid-wendtner & Korting, 2006).

The viscosity of the preparation was measured at  $3047 \pm 130.1$  cps. The viscosity was within the range that is sufficient for good spreadability and clarity, as the viscosity that suitable for topical preparation was recorded between 512 and 15000 cps (Singh *et al.*, 2013; Pande *et al.*, 2014; Chen *et al.*, 2016).

The centrifugation test was also conducted to evaluate the gravitational effect on the preparation. This is essential to analyse on adequate quality and stability of the preparation (Dimeski *et al.*, 2011; Iradhati & Jufri, 2017). Based on the results obtained, no noticeable instability was observed on the preparation upon spinning at 3000 rpm for 30 min at 25°C. The preparation remained intact without phase separation indicating adequate and stable formulation (data not shown).

**Antibacterial Efficacy**

The antibacterial efficacy of the optimal preparation was evaluated through the measurement of inhibition zone and formation of a bacterial colony. These two evaluations were performed to investigate the sensitivity of bacteria towards the preparation and to examine the ability of the preparation to kill the bacteria. In both evaluations, the preparation was tested on 22 bacterial species associated with wound infection, which include eight standard laboratory (ATCC) and fourteen clinically-isolated bacteria from the wound sites. The commercially available topical antibacterial preparation (MediHoney™) was used as the basis of comparison.

**Measurement of Inhibition Zone**

The results for the inhibition zones measured are shown in Figure 3 (a) for Gram-positive and Figure 3 (b) for Gram-negative bacteria. The sensitivity of bacteria towards the preparation was decided according to the range that was previously used.

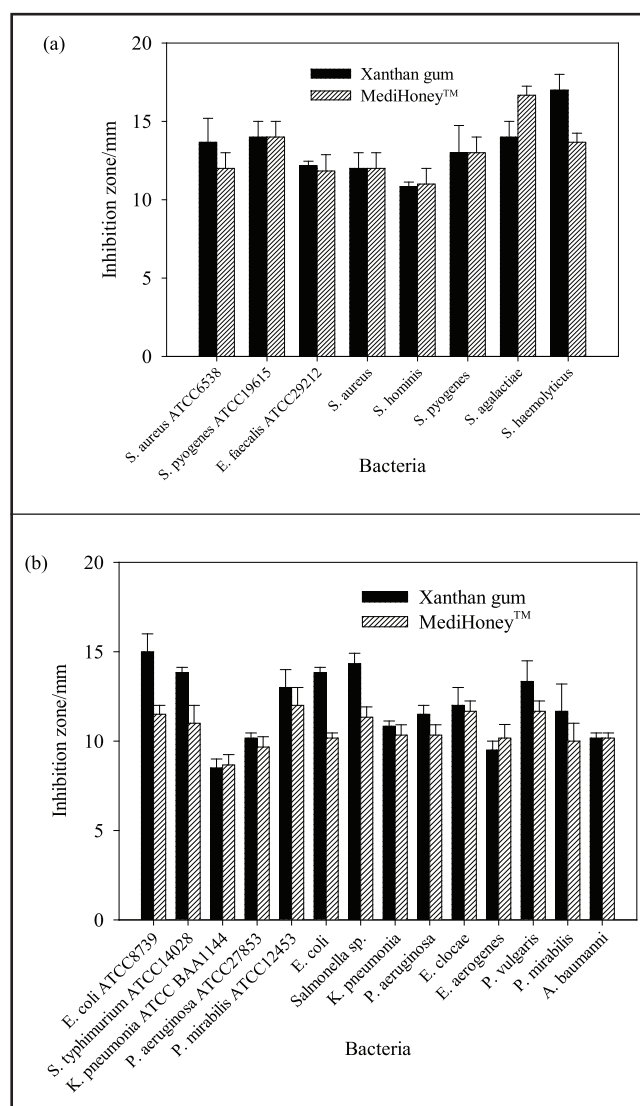
The range of inhibition zone measured was between  $8.5 \pm 0.50$  mm and  $17.0 \pm 1.00$  mm which indicate that the bacteria responded between sensitive to very sensitive towards the preparation. In the commercially available preparation by using manuka, the inhibition zone was measured in the range between  $8.7 \pm 0.58$  mm and  $16.7 \pm 0.58$  mm. The smallest inhibition zone was measured on *K. pneumonia* while the largest was on *S. agalactiae*, respectively. Based on the range of inhibition zone measured, the tested bacteria were responded between sensitive to very sensitive towards the manuka preparation.

In comparing between the optimal preparation and commercially gel, for Gram-positive bacteria (Figure 3 (a)), the optimal preparation showed the highest inhibition zone on three bacteria, including *S. aureus* ATCC 6538, *E. faecalis* ATCC 29212, and *S. haemolyticus* with inhibition zones of  $13.7 \pm 1.00$  mm,  $12.2 \pm 0.29$  mm, and  $17.0 \pm 1.00$  mm, respectively. These were 1.1-, 1.1-, and 1.2-fold higher as compared to the

**Table 4.** Physicochemical properties of the optimal preparation

Formulation	Xanthan gum
Physical appearance	Opaque
Homogeneity	Homogeneous
Colour	Dark brown
Grittiness	No
Lump formation	No
pH	$3.52 \pm 0.10$
Viscosity at 100rpm (cps)	$3047 \pm 130.1$
Centrifugation Test	No phase separation observed

The symbol  $\pm$  represents the standard deviation which was calculated between three biological replicates.



**Figure 3.** The inhibition zone measured by the preparation tested against the standard laboratory and clinical isolated bacteria of (a) Gram-positive and (b) Gram-negative.

commercially gel. As for Gram-negative bacteria [Figure 3 (b)], out of 14 species, the optimal preparation demonstrated the largest inhibition zone against ten bacteria with an exception on *K. pneumonia* ATCC BAA 1144, *E. cloacae*, *E. aerogenes*, and *A. baumannii*. Based on the results obtained, the optimal preparation was able to be used as a topical



antibacterial agent which proved by the manifestation of inhibition zone on the tested bacteria.

**Bactericidal Effect**

In this section, an attempt was made to investigate on the ability of the preparation to kill the bacteria through formation of bacterial colony. The results were tabulated in Table 5 (a) for Gram-positive and Table 5 (b) for Gram-negative respectively.

In the 22 tested bacteria, no formation of bacterial colony was observed on the surface of agar for the optimal preparation after 24 hours of incubation, indicating the presence of bactericidal effect (Dewanjee et al., 2008; Shagana & Geetha, 2017). The presence of bactericidal effect for the optimal preparation was observed on the tested Gram-positive including *S. aureus*, *E. faecalis* ATCC 29212, *S. hominis*, and *S. haemolyticus*) and Gram-negative including *E. coli*, *P. aeruginosa*, *Salmonella* sp. and *K. pneumonia*) bacteria, respectively. Similar results demonstrated by the preparation of manuka, except on *E. faecalis* ATCC 29212 which formation of bacterial colonies were observed. In the control sample, which the bacteria was cultured in broth alone, the formation of bacteria colonies were observed for the 22 bacteria indicating the absence of a bactericidal effect.

In many types of acute and chronic wounds, *S. aureus* and *P. aeruginosa* are usually isolated from the infected wounds (Serra et al., 2015; Negut et al., 2018). These bacteria often cause biofilm development and chronic infections which may suppress immune activities and promote the development of antibiotic-resistant strains (Serra et al., 2015). Similar to *S. aureus* and *P. aeruginosa*, other wound-associated bacteria such as *E. coli*, *S. pyogenes*, *E. faecalis*, and *P. mirabilis* can also develop a biofilm, antimicrobial inactivating enzymes, and other resistance mechanisms to eliminate the antibacterial action (Lu et al., 2014; Kim et al., 2018). In this study, 22 wound associate bacteria which include standard laboratory and clinical strains that isolated from infected wounds have been tested and were found to be susceptible to the preparation. This was proven by the formation of inhibition zone and the presence of bactericidal effect for the optimal preparation against the tested bacteria. The results indicate that the xanthan gum was effective in conveying the kelulut honey as a topical preparation without compromising on its antibacterial properties.

**Stability Study**

The stability study was conducted according to the ICH guideline for storage condition, which was performed by keeping the preparation long-term (25°C ± 2 /60% ± 5 RH) and accelerated storage (40°C ± 2/75% ± 5 RH) conditions for six months (World Health Organisation, 2018). The physico-chemical properties i.e., colour, homogeneity, pH, and viscosity, and antibacterial efficacy i.e., inhibition zone and

formation of bacterial colony, of the optimal preparation was determined at 0, 1, 2, 3, and 6 months.

The results for the physicochemical properties of the preparation are shown in Table 6 and Figure 4 respectively. The colour, homogeneity, and pH level after six months of storage remained unchanged. The pH was measured to be in the range between 3.50 ± 0.10 and 3.58 ± 0.10 with the difference between the lowest and highest pH levels was 2%. Based on the results obtained, the physicochemical properties of the optimal preparation were remained unchanged. The ability to maintain the fundamental

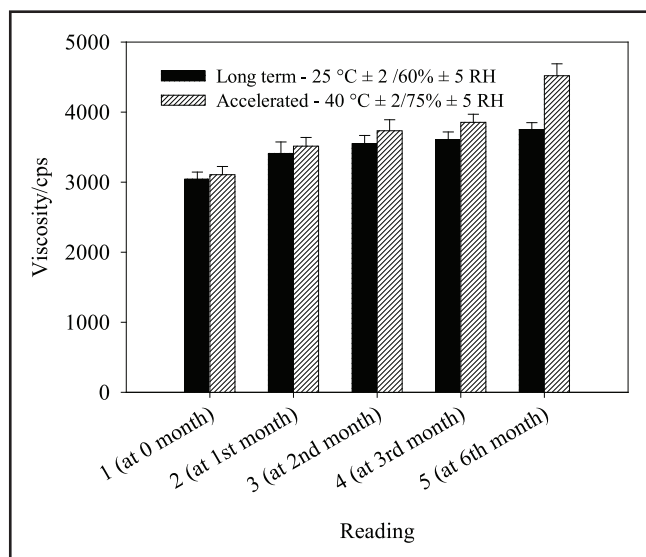
**Table 5.** The results on formation of bacterial colony for (a) Gram-positive and (b) Gram-negative bacteria

(a)			
	Formation of bacteria colony		
	Xanthan gum	Comvita	Control (broth only)
Standard strain			
<i>S. aureus</i> ATCC6538	No	No	Yes
<i>S. pyogenes</i> ATCC19615	No	No	Yes
<i>E. faecalis</i> ATCC29212	No	Yes	Yes
Clinical isolated strain			
<i>S. aureus</i>	No	No	Yes
<i>S. hominis</i>	No	No	Yes
<i>S. pyogenes</i>	No	No	Yes
<i>S. agalactiae</i>	No	No	Yes
<i>S. haemolyticus</i>	No	No	Yes
(b)			
	Formation of bacteria colony		
	Xanthan gum	Comvita	Control (broth only)
Standard strain			
<i>E. coli</i> ATCC 8739	No	No	Yes
<i>S. typhimurium</i> ATCC14028	No	No	Yes
<i>K. pneumonia</i> ATCCBAA1144	No	No	Yes
<i>P. aeruginosa</i> ATCC27853	No	No	Yes
<i>P. mirabilis</i> ATCC12453	No	No	Yes
Clinical isolated strain			
<i>E. coli</i>	No	No	Yes
<i>Salmonella</i> sp.	No	No	Yes
<i>K. pneumonia</i>	No	No	Yes
<i>P. aeruginosa</i>	No	No	Yes
<i>E. cloacae</i>	No	No	Yes
<i>E. aerogenes</i>	No	No	Yes
<i>P. vulgaris</i>	No	No	Yes
<i>P. mirabilis</i>	No	No	Yes
<i>A. baumannii</i>	No	No	Yes

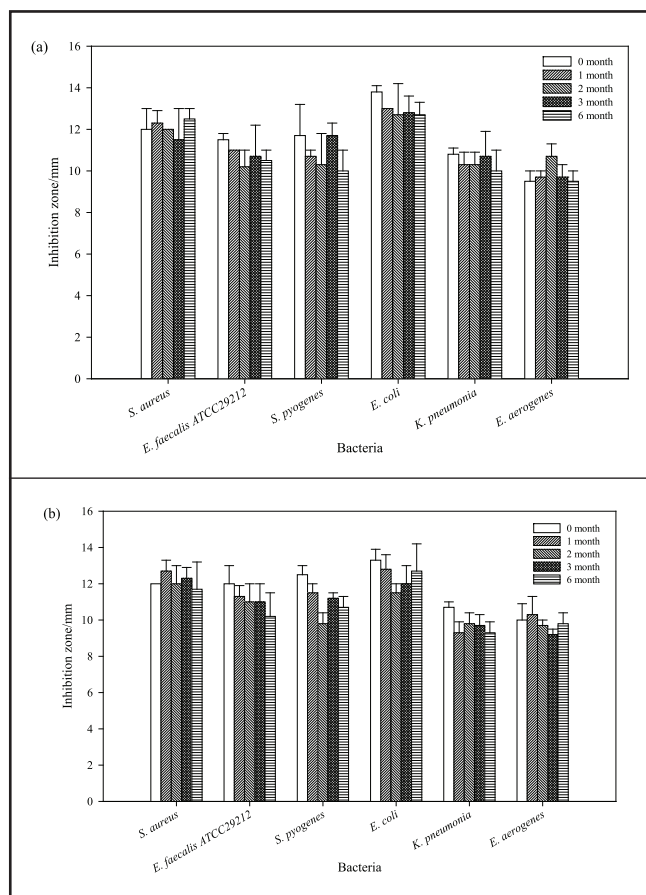
**Table 6.** The results for colour, homogeneity, and pH during six months stability test for the optimal preparation

	(Long term) 25°C ± 2 /60% ± 5 RH					(Accelerated) 40°C ± 2/75% ± 5 RH				
	Months					Months				
	0	1	2	3	6	0	1	2	3	6
Colour	Dark brown					Dark brown				
Homogeneity	Homogeneous					Homogeneous				
pH	3.52±0.05	3.50±0.05	3.51±0.06	3.57±0.04	3.56±0.03	3.57±0.07	3.52±0.07	3.53±0.05	3.58±0.04	3.56±0.04

The symbol ± represents the standard deviation which was calculated between three biological replicates.



**Figure 4.** Viscosity of the preparation during the six-month of stability study after been stored at long term -25°C ± 2 /60% ± 5 RH and accelerated -40°C ± 2 /75% ± 5 RH storing conditions.



Error bars symbolise the errors calculated from three biological replicates. One sample t-test represent significant differences between the mean of the sampled population and the hypothesised population mean ( $P$ -value < 0.05).

**Figure 5.** Measurement of inhibition zone on six bacteria for the optimal preparation after stored in (a) long-term and -25°C ± 2 /60% ± 5 RH, and (b) accelerated -40°C ± 2 /75% ± 5 RH storage conditions.

physicochemical properties indicate acceptable and reliable preparation (Chen *et al.*, 2016).

As for viscosity, the preparation showed a directly proportional relationship with time. The lowest viscosity of 3043 ± 100.7 cps for the long term and 3107 ± 116.8 cps for the accelerated storage were recorded at 0 month, respectively. The highest viscosity of 3750 ± 100.0 cps for the long term and 4520 ± 170.6 cps for the accelerated storage were recorded after the six months of storage, respectively. The variation in viscosity between 0 month and sixth month was 23% for the long term and 46% for the accelerated storage, respectively.

**Antibacterial Efficacy**

The antibacterial efficacy of the preparation within the six months of stability study was evaluated through the measurement of inhibition zone and formation of bacterial colony. In both evaluations, six wound-infecting bacteria, which include three Gram-positive (*S. aureus*, *E. faecalis*, and *S. pyogenes*) and three Gram-negative (*E. coli*, *K. pneumoniae* and *E. aerogenes*) were used. The measurement of the inhibition zone on Gram-positive and Gram-negative bacteria are shown in Figure 5 (a) and Figure 5 (b), respectively followed by the results of the formation of the bacterial colony.

The zone of inhibition showed a variation of 10.7% within the six-month stability study. The inhibition zones measured were ranged from 9.2 ± 0.30 to 13.8 ± 0.30 mm. As for the formation of bacterial colony, the preparation remained valid to prevent the formation of the bacterial colony within the six months of the stability study (data not shown). Based on the outcomes obtained, the efficacy of the preparation remained effective without deficiency indicating a stable and reliable preparation that can sustain its antibacterial properties over time (Irish *et al.*, 2011).

The synergistic interaction is considered positive if the therapeutic effect that initially present in the preparation remained without interruption. The results indicated that the preparation which prepared in this study are capable to retain the antibacterial properties which proven by the identical inhibition zone and bactericidal effect. The porous structure facilitates uniformed dispersion of honey across the polymeric structure of the preparation may explain the uninterrupted antibacterial properties (Sharma *et al.*, 2015). In addition, the interactions between the charges of the polymeric agent i.e., xanthan gum which loaded with honey and the charges on the surface of the bacterial membrane formed a contact (Kaith *et al.*, 2015) that allow the compounds such as phenolic acids and hydrogen peroxide to promote cell disruption and lysis to kill the bacteria (Henriques *et al.*, 2010, 2011). A similar finding with the positive synergistic effect of honey in preparation that led to more than 95% of drug release was demonstrated by the previous study (El-kased *et al.*, 2017).

**CONCLUSION**

The optimal preparation was finalised at a pH of 3.5, the honey concentration of 90% (w/v), and xanthan gum concentration of 1.5% (w/v). The utilisation of xanthan gum as polymeric agent was fit for preparing kelulut as a topical preparation. Based on the optimal preparation, the preparation demonstrated reliable physicochemical properties, and most importantly, the potent antibacterial properties, i.e., bactericidal effect, were retained after the honey was converted to a topical preparation. This was supported by constant viscosity and efficacy of the

preparation within six months of stability study indicating stable and reliable preparation.

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#### Conflict of Interests

The authors declare that they have no conflict of interests.

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