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Evaluating the Wound Healing Activity of Fabricated Stingless Bee Honey Hydrogels in an Animal Model

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Abstract

Introduction: Honey is a well-established treatment for wound healing and had been used for ages. However, there are limitations when it is directly applied to the wound such as inconsistent dosage and pain during dressing changes. The incorporation of honey into hydrogel could overcome these problems. The study is aimed at developing a suitable honey-based hydrogel formulation for wound healing application by using systematic experimental design from response surface methods (RSM). **Methods:** The hydrogel's base was made from polyvinyl alcohol (PVA), polyethylene glycol (PEG), glycerol and agar. A two-level factorial design was selected to screen the factors followed by centred composite design for optimization. The characterizations of the optimized formulation were observed in term of hydrophilicity and rheological. The optimized formulation was further assessed in an in vivo wound healing study in New Zealand albino rabbits. **Results:** PEG and agar concentration was found to be the most important process variable based on the screening result. The optimised hydrogel has a good hydrophilicity ability and rheological property. In in vivo healing study, the healing in the honey incorporated hydrogel treated group was significantly faster than the no treatment group, as demonstrated in wound closure percentage and histological assessment. From the results, the wound in the honey hydrogel treatment group has entered the remodelling phase compared with the control group that was still in the proliferation phase. **Conclusion:** Based on all of these results, stingless bee honey incorporated hydrogel has a promising application as an efficient wound dressing.

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Introduction

Wound healing remains one of the major concerns among healthcare practitioners and scientists to this day. Poor wound healing not only causes trauma to the patients, but the process itself becomes time-consuming and can place a significant strain on healthcare resources. For example, in the United Kingdom the National Health Services (NHS) estimated that the cost for managing wound cases was approximately £8.3 billion per annum, where 81% of the total annual NHS cost was covered in the community(Guest et al., 2020).This number may rise in the future, since the prevalence of wounds is increasing due to the ageing population, in addition with other comorbid factors that could hinder the healing processes such as cardiac disease, diabetes, and obesity (Versey et al., 2021). Therefore, a careful wound healing management therapy should be considered seriously to reduce the burden and improve the outcome of wound healing cases.

Over the years, there have been a lot of methods and inventions introduced to overcome wound healing problems. Since the beginning of the 20th century advanced technologies in polymerisation research have contributed refreshing materials for wound healing products. A few studies indicated that a moist environment is the ideal condition to improve wound healing process (Farahani & Shafiee, 2021; Gao et al., 2021; Liang et al., 2021; Tottoli et al., 2020). Therefore, a dressing that can imbibe sufficient water content was developed, and it is now known as hydrogel. Hydrogel also has a promising attribute in drug delivery systems due to their high-water content that can imitate similar environment as the human body tissue and provides good biocompatibility to encapsulate hydrophilic drugs (Asadi et al., 2021).

Numerous types of honeys have been identified and one of them is stingless bee honey that are widely used in Latin America, the mainland of Australia, Eastern and Southern Asia, and Africa (Engel et al., 2017). The therapeutic effect of honey that contribute to the wound healing process is mainly due to the broad-spectrum antibacterial activity (Domingos et al., 2021; Dżugan et al., 2020; Wasihun & Kasa, 2016). This antibacterial property is contributed by the nature of honey, which contain high osmolarity,(Al-Masaudi et al., 2020) acidity (Bouhlali et al., 2019) and strong non-peroxide activity (Guttentag et al., 2021). Furthermore, with these characteristics, stingless bee honey is able to protect or confront against pathogenic microbial colonization at the injury site, which, if not handled properly, may lead to chronic wound formation (Rao et al., 2016).

Stingless bee honey also possesses numerous good phytochemical components such as phenolic acids, flavonoids, glucose oxidase and catalase enzymes (Ávila et al., 2018; Sousa et al., 2016).) These compounds are highly associated with the antioxidant activities from honey. Besides antibacterial activity, antioxidant activity could also enhance the healing process by protecting the wound site against the detrimental effects from oxidative stress (Abd Jalil et al., 2017).

Direct application of honey is inefficient, as tissue rapidly absorbs the fluid, leading to inconsistent dosing and the therapeutic concentration is not constantly maintained. Besides, when it is combined with traditional dressings materials, such as gauze or bandage, it would cause discomfort to the patient due to the frequent dressing change that might be painful during the dressing replacement (Resch et al., 2021). These predicaments can be solved by incorporating the honey within a hydrogel formulation.

The incorporation of honey with hydrogel could not only provide an ideal moist environment on the wound but also good fluid absorption that promotes permeation of the nutrient content of the honey (Ahmad et al., 2021; Gull et al., 2019). Furthermore, due to its jelly-like structure that is similar to the granulation tissue, it would reduce the pain and provide a soothing effect (Phaechamud et al., 2015). Hydrogel also can allow a sustained delivery of the bioactive substances to the wound over time.

The conventional optimization method for hydrogel development involves changing one factor at a time while keeping the other factors constant. The disadvantage of this method is it may take a long period and can be costly (Kaith et al., 2014).

Therefore, response surface method (RSM) can be used as an alternative method, whereby various factors can be designed simultaneously, and they can be related with the dependent response by using a design matrix that eventually produces an optimal formulation (Karkare et al., 2022).

In this study, stingless bee honey incorporated into a hydrogel made from synthetic polymer (PVA-PEG) and natural polymer (agar) was developed by using response surface methodology. A few optimized formulations developed were further characterized to observe the robustness as a wound healing agent. Then, the in vivo assessment was conducted on the most effective formulation to support the healing activity. Previously, there are plenty of other combination between hydrogel and different types of honey such as Medihoney® using manuka honey(Woodward, 2019), Malaysia Gelam honey (Mohd Zohdi et al., 2012), Egyptian Arabic honey (El-Kased et al., 2017), Korean Chestnut honey (Park et al., 2017), and Indonesian Euphoria longana sp. Honey (Kosimaningrum et al., 2020). However, this study is the first combination of hydrogel and stingless bee honey.

Materials and methods

Materials

The materials and chemicals used throughout this study were Muller-Hinton agar (MHA) (Merck, Germany), Muller-Hinton broth (MHB) (Merck, Germany), ethanol (R&M Chemicals, UK), polyethylene glycol 400 (PEG 400) (Merck, Germany), polyvinyl alcohol (PVA) with molecular weight of 195,000 and 99% hydrolyzed (Sigma-Aldrich, USA), protein-free agar (1st Base, Singapore), distilled water (Brandon, Malaysia), phosphate-buffered saline (PBS) tablets (Sigma-Aldrich, USA), stingless bee honey (Bris Trigona, Malaysia), Ketamin (Ilium, Australia), Xylazine (Ilium, Australia), normal saline solution (Opticare, Malaysia), 37% formaldehyde (Merck, Germany), 100% ethyl alcohol (Fisher Scientific, UK), toluene (Fisher Scientific, UK), xylene (Fisher Scientific, UK), Mallory's trichrome staining kit (DiaPath, Italy), haematoxylin (Surgipath® Leica Microsystem, USA) and eosin (Surgipath® Leica Microsystem, USA).

Stingless bee honey hydrogel preparation

The methods in preparing and characterizing the hydrogel were adapted from the study conducted by Hwang et al., (2010) for the number of freeze and thawed cycle, Kamoun et al., (2015) for the use of PVA- PEG excipient and Mohd Zohdi et al., (2012)for the use of agar as excipient and honey as main active ingredient of the hydrogel formulation. Ten percent PVA was mixed with 6% PEG (w/v) together with 1% protein-free agar solution (w/v), 1% glycerol (w/v), and 40% honey (w/v). To prepare the PVA aqueous solution, 100 g of PVA was dissolved in 600 mL distilled water for 1 h until the solution was clear. Later, 10 g of agar was dissolved in 330 mL distilled water and was heated for about 1 h until the solution was clear. The solution was stirred continuously before 60 g of PEG was added to create an agar solution. Then, 1 g of glycerol was dissolved in the mixture. After that, the mixture of the PVA aqueous solution and the agar solution was submerged in an ultrasonic water bath at 37 °C for 1.5 h for degassing purpose. When the temperature reached 40 °C, 40 g of honey was added. To prepare a control hydrogel, a similar mixture was prepared without honey. They were kept in the freezer at -20 °C for 18 h and then thawed at 25 °C for 6 h to complete a cycle (F–T cycle). They were grouped into first, second, and third cycles. Fig. 1 summarize the preparation of hydrogel.

Experimental design and model for PVA-agar-honey development

The swelling ratio of the prepared hydrogel could be influenced by many factors such as the number of cycles, temperature during the freezing cycle or percentage of PEG, agar and glycerol. It would be time consuming to test each of these factors one at a time. Therefore, the introduction of factorial design may provide a better solution to screen the effects of each factor. In addition, the usage of this design could also study both antagonistic and synergistic factor interactions (Anderson & Whitcomb, 2017).

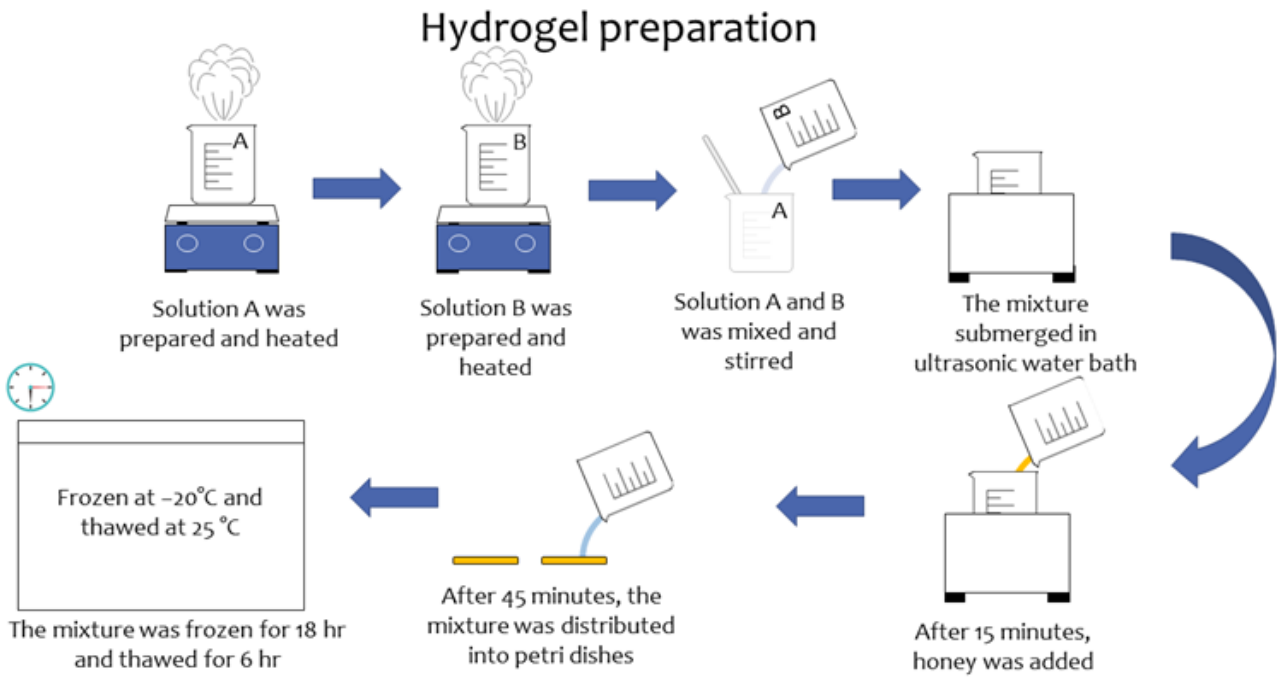

Fig. 1: Summary of hydrogel preparation. Solution A consist of PVA aqueous solution while solution B consist of agar and PEG solution.

Table 1: List of factors with their maximum (+1) and minimum (−1) points.

Factors	Factor significance	Minimum Level (−1)	Maximum Level (+1)
X1	PEG % (w/v)	3	5
X2	Agar % (w/v)	0	1
X3	Glycerol % (w/v)	0	1
X4	Temperature (°C)	−80	−20
X5	Cycles	1	3

Table 2: List of factors with their maximum (+1), central (0), and minimum (−1) points.

Factors	Factor significance	Minimum Level (−1)	Central Level (0)	Maximum Level (+1)
X1	PEG % (w/v)	4.5	5.0	5.5
X2	Agar % (w/v)	0	0.5	1

For two-level factorial design, five variables were arranged at their maximum (+1) and minimum (−1) points to improve the swelling percentage of the hydrogel formulation. Table 1 shows the list of formulations that was generated by the software. Significant variables that maximized the swelling percentage were selected by using Pareto charts. Sequential ANOVA modelling was used to fit the empirical data while the significance of the model was evaluated based on p-values. Then, based on the screening result, the two most significant process variables (PEG and agar percentage) were optimized using central composite design (CCD). Table 2 shows a list of factors with their maximum (+1), central (0), and minimum (−1) points.

Response tests

From the formulation list, a swelling ratio test was conducted as a response test to study the influence of the selected factors toward the formulation.

Determination of swelling ratio

The hydrogel samples (2 × 2 cm) were dried at 60 °C in the oven for 12 h (Wa). Then, they were soaked in pH 7.4 PBS and put inside an oven at 37 °C (Ws) for 24 h. The swelling ratio (SR) was calculated using Eq. (1) below.

$$\text{Swelling Ratio \%} = \frac{W_s}{W_a} \times 100 \quad (1)$$

where Wa is the weight of hydrogel samples dried for 12 h at 60 °C and Ws is the weight of hydrogel samples soaked in PBS at 37 °C.

Characterization of optimum formulation

Based on the results from the optimized formulations, the composition of the hydrogel was sorted as shown in Table 3. The formulations were characterized in terms of swelling and rheological properties.

Rheological Properties

This test was conducted using a Rheometer (HAAKE MARS, Thermal analysis, Germany) and analysed using the RheoWin version 3.61.0000 software. The instrument uses cone and plate geometries and the base plate temperature was maintained at 32 ± 0.05 °C using a universal temperature controller. The spindle is PP35 Ti with a diameter of 35 mm. The linear viscoelastic region (LVR) was measured through oscillation stress sweep test. The shear rates for the preformed stress sweep test were between 0.01 to 1000 Pa.

Table 3: Hydrogel composition for each formulation

Ingredient	F1	F2	F3	F4
PVA % (w/v)	10	10	10	10
PEG % (v/v)	6	6	6	6
No. of F–T cycle	3	3	3	3
Honey conc. % (w/v)	Distilled water	40	40	40
Agar conc. % (w/v)	0.25	0.25	0.25	0.5
Glycerol % (w/v)	1	1	Distilled water	1

In vivo healing efficacy test on the animals

Animal care and handling was carried out as described by Azis et al., (2017). Males New Zealand White Albino rabbits weighing from 1.7 to 2.5 kg were used for the study. The rabbits were placed individually in aluminium cages throughout the study in a holding room with the temperature at 23±2°C, relative humidity of 45% to 55% and 12-hours light/dark cycles. The acclimatization period for the animals were seven days. Animal study was approved by Institutional Animal Care and Use Committee (IACUC), International Islamic University Malaysia with approval number IIUM/IACUC- 2019 (7).

Wound induction

The hair on the dorsal thoracic region of the rabbit was shaved at the beginning of the test. Then, rabbits were anesthetised with intramuscular injection of Ketamine: Xylazine (0.4 mL: 0.1 mL). The shaved area was cleaned and disinfected with 70% ethanol. By using a biopsy punch, a 4 mm diameter excision wound was inflicted. (Azis et al., 2017) The wounding day was considered as Day 0. The wounds were categorized as no treatment, blank hydrogel treatment and honey incorporated hydrogel treatment. The wounds were treated once daily until they completely heal. The arrangement of the dressings was shifted randomly in Animal 1 to Animal 4 to avoid bias in term of location of the wounds or environmental effect that could influence the assessment of healing process.

Measurement of wound area

At the Day 0, Day 3, Day 6 and Day 9, the image of the wounds was captured using the videoscope probe of the Dermal Lab (Dermalab Series Skinlab Combo, Denmark) and their size were measured.

The relative wound size reduction was calculated according to Eq. (2).

$$Relative\ wound\ size\ reduction\ (\%) = \frac{(A_\circ - A_t)}{A_\circ} \times 100 \quad (2)$$

where Ao is wound size at the initial time and At is the wound size at the predetermined time.

Histopathological studies

After nine days of treatment, the rabbits were sacrifice by carbon dioxide overdose (Leary, Underwood & Anthony, 2013). The wound tissues were extracted and fixed immediately in 10% formalin. The tissues were dehydrated by using ethanol and toluene solution before embedded in paraffin wax. The paraffin-embedded tissues were sliced by using a microtome (RM 2135, Leica, Germany) for staining either by Haemotoxyllin & Eosin or Mallory's Trichrome. The thickness of the epidermis and dermis layer of the regenerated wound on the histology slide was measured using Leica Application Suite software (version 4.0) together with Image-J software (version 1.50i) at 10 different random locations

Statistical Analysis

Statistical evaluation was carried out using the IBM SPSS statistics version 20 and Microsoft Office Excel 2016. Significant differences between the treated groups and the control were determined by one-way ANOVA using Kruskal-Wallis test, with a significance level of $p < 0.05$.

Results and discussion

Fig. 2. Stingless bee honey-based hydrogel.

Fig. 2 displayed the hydrogel that been prepared. The PVA types were carefully selected based on several literature related to hydrogel formulations and after a few trials PVA with relatively lower molecular weights (145,000–195,000 MW and 146,000–186,000 MW) were omitted from this study since they were unable to produce a stable hydrogel with good characteristics. The concentration of PVA was capped to 10% due to the risk of irritation to the skin and eye (Rowe et al., 2009). A relatively high molecular weight, PEG 400, was selected due to its flexible chain structure, leading to a better hydrophilic portion to the hydrogel chain (Ghobadi Jola et al., 2018). The PEG plays an important role in the fabrication of the hydrogel where it acts as a plasticiser to assist the mobility of the molecular chain and reduces the hydrogel stiffness (Laboulfie et al., 2013). On the other hand, the concentration of PEG was considered based on the results from the Design of Experiment (DOE) software in which the higher concentration of PEG was predicted to produce better hydrogel characteristics.

Recently, the combination of natural and biopolymers with synthetic polymers as a product had gained increasing attention. This due to its better characteristics in terms of the increased number of polymer chains, chemical derivation convenience, and better biocompatibility (Kamoun et al., 2017). Agar was an example of a biopolymer selected to enhance the cross-linking between the PVA–PEG and eventually improve the mechanical structure of the hydrogel. The hydrogel with agar incorporated displayed better strength and flexibility. In addition, agar as a polysaccharide polymer, has a good response towards blood, mucosa, and tissues. Hence, it can be a good drug carrier and may improve drug release as well (Das & Pal, 2015; Koneru et al., 2020).

Screening of the factors for hydrogel synthesis

Table 4 indicated three factors that could influence the swelling ratio response which are PEG concentration (X1), agar concentration (X2), and number of cycles (X5). The main effects of these factors also are presented by the Pareto chart provided in Fig. 3. The bar lengths in this chart are proportionate to the 95% confidence level of the estimated effects to the absolute value, in which the orange bars denote positive influence and the blue bars denote negative influence. According to the Pareto chart, the number of cycles (X5) displayed the most significant and positive effect on the swelling ratio in which an increase in the number of cycles will increase the swelling ratio value. PEG concentration (X1) also gave a positive and significant effect on the response, while agar

concentration (X2) contributed significantly to a

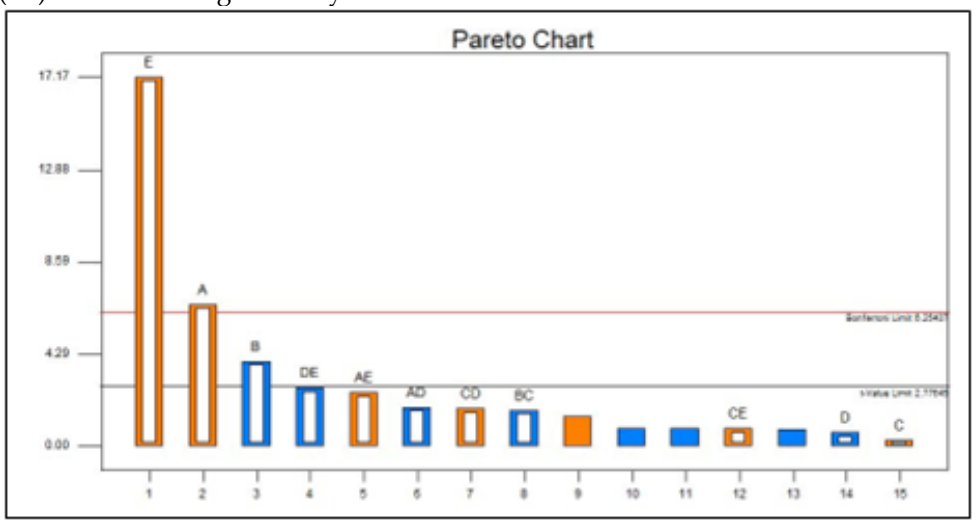

Fig. 3 Pareto chart showing significant factors that could influence the swelling ratio of the formulation where A) PEG, B) agar, C) glycerol, D) temperature, and E) number of cycles.

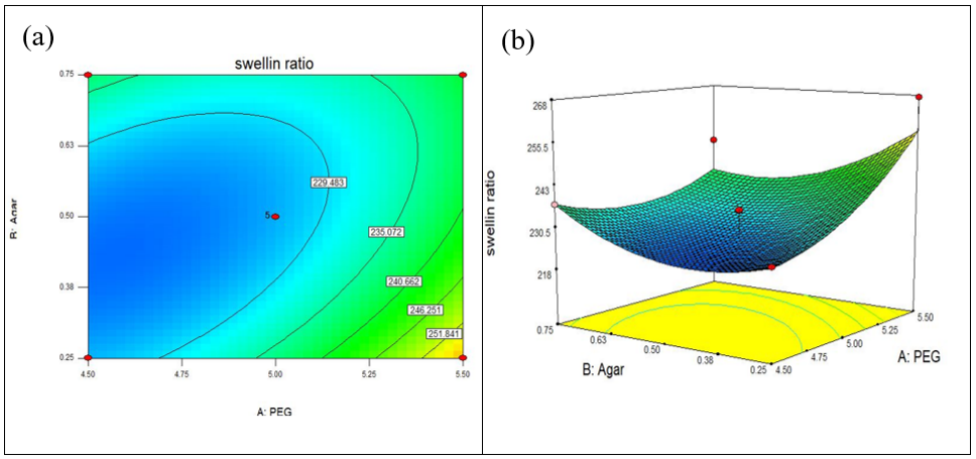

Fig. 4 Contour plot (a) and response surface (b) of swelling ratio as function of PEG and agar concentrations. The blue colour (darkened region) represents the area where the prediction is unreliable while brighter region (green and yellow), represent the area where the prediction is reliable due to enough information collected.

Table 4. Analysis of variance (ANOVA) of the factors.

Source	Mean Square	*F* value	*p*-value Prob > *F*
Model	2248.51	34.40	0.0019*
A-PEG	2860.31	43.76	0.0027*
B-Agar	1021.14	15.62	0.0168*
C-Glycerol	5.23	0.080	0.7913
D-Temperature	26.69	0.41	0.5576
E-Cycles	19273.41	294.84	< 0.0001*
AD	219.61	3.36	0.1407
AE	410.40	6.28	0.0664
BC	187.36	2.87	0.1657
CD	199.89	3.06	0.1553
CE	44.14	0.68	0.4574
DE	485.47	7.43	0.0527

*p < 0.05, significant value

negative effect on the response. The other factors were below the t-value limit line are considered as not significantly influencing the swelling ratio response.

Central composite design (CCD)

To determine the optimum value for PEG and agar concentrations for the swelling ratio response, an experiment was designed according to a face-centred CCD with the two variables following RSM. Each variable was varied at 5 levels, which are -α, -1, 0, + 1, + α, resulting in 13 experimental runs.

The contour plot and their corresponding three-dimensional (3D) response surface for swelling ratio percentage against PEG and agar are shown in Fig. 4. The swelling ratio percentage ranging from

maximum (267.51%) and minimum (218.75%).

Table 5 summarizes the ANOVA and p-value that were used to estimate the coefficients of the model in assessing the significance and interaction strength of each parameter. From the ANOVA analysis, the confidence level is greater than 95% and the p-value of the model is 0.0139, demonstrating that the model is suitable for this experiment. The R2 and adjusted-R2 are 0.8251 and 0.7002, respectively, which displayed in Table 6 indicated that the estimated model fits the experimental data satisfactorily. Since R2 for these response variables is close to 100%, which is 83%, it showed that the regression models explain the mechanism well and contains a good correlation between the predicted and observed values(Diwan et al., 2021).

Table 5. ANOVA for response surface quadratic model.

Source	Mean Square	*F* Value	*p*-value Prob > *F*
Model	537.50	6.61	0.0139*
A-PEG	800.05	9.83	0.0165*
B-Agar	40.15	0.49	0.5050
AB	188.07	2.31	0.1722
A^2	776.22	9.54	0.0176*
B^2	1325.21	16.29	0.0050
Residual	81.36		
Lack of Fit	96.25	1.37	0.3720
Pure Error	70.19		
Correction total			

*p < 0.05, significant value

Table 6. The value of fitting the model parameter.

Parameter	Value
Standard deviation	9.02
Mean	239.55
CV percent	3.77
R^2	0.8251
Adjusted-R^2	0.7002
Predicted-R^2	0.1218
Adequate precision	6.464

Characterization of hydrogel formulation

Swelling ratio

Formulation 2 with 40% (w/v) honey that contained glycerol indicates a significantly ($p<0.05$) higher swelling ability than others, while the control formulation without honey displays the lowest swelling ability as been shown in Fig. 5. The swelling ratio determines the capacity of the formulation to absorb fluids such as the wound exudate when applied as a wound dressing. A high value of swelling ratio indicates a better absorption capacity. As the number of different components in the formulation increases the swelling ratio decreases since the porosity of the hydrogel may be higher. This reduction is likely due to enhanced intermolecular interactions and a denser cross-linked polymeric network, which can limit the free volume available for water absorption. However, the addition of PEG 400 could promote the absorption capacity of the formulation and it is postulated the presence of agar can hinder its swelling ability. Pal et al., (2007) suggested that a swelling capacity of 260% for a hydrogel membrane causes it to be super absorbent.

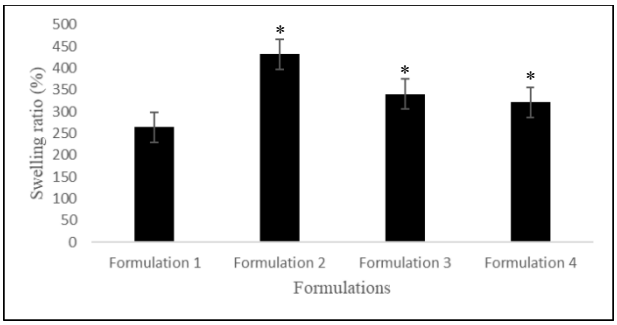

Fig 5. Effect of the formulations on the swelling ratio. The asterisks (*) indicated significant differences ($p< 0.05$) compared to the control group (Formulation 1). Results are expressed as mean ± standard error mean (SEM) (n = 3).

Rheological Properties

Fig. 6a, 6b, 6c, and 6d display the rheological properties for the Formulation 1,2,3 and 4respectively. Storage modulus, represented by G′, is the maximum stored energy available to pull back when shear stress is applied. Loss modulus, represented with G″, is the deformation energy that dissipates when shear stress is applied (Engleder et al., 2014).

The control formulation (Formulation 1) has a poor rheological property due to its low critical strain point, which was approximately 85 ± 5 Pa. The critical strain point is the point at which the network structure of the gel is disrupted. Formulations 2 and 3, with the highest concentration of honey, showed the highest value of critical strain point, which were approximately 900 ± 50 Pa. Formulation 3 that did not contain glycerol, on the other hand, showed a better and clearer LVR line. Therefore, Formulation 3 possess the best rheological properties among the formulations.

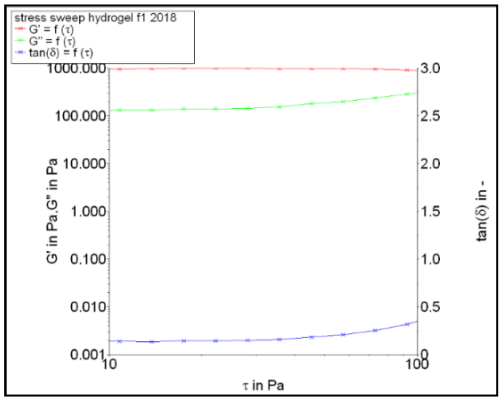

Fig. 6a. Formulation 1

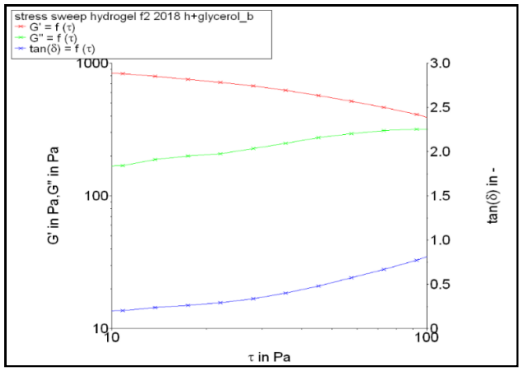

Fig. 6b: Formulation 2

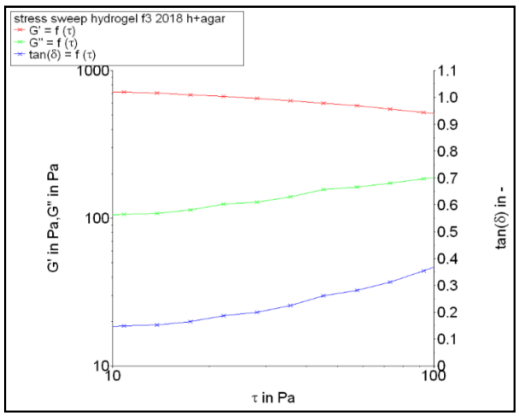

Fig. 6c: Formulation 3

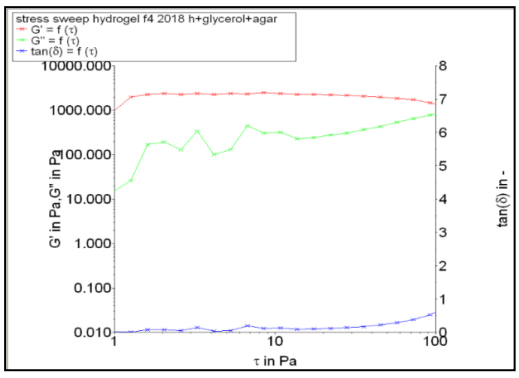

Fig. 6d: Formulation 4

The rheological properties of these hydrogel were studied in terms of liner viscoelasticity range (LVR) by using stress sweep test. The LVR indicates the range of shear stress that would cause the network between the polymer to still be interconnected. Whereas, beyond the LVR range, the cross-linking between the polymer network is broken, thus reducing its elasticity (Weiss, 2014). From the rheological testing results, the values of G′ in all formulations were relatively higher than the value of G″. This indicates that these formulations possess viscoelastic behaviour and are stable upon storage due to their higher elasticity compared to their viscosity.

In vivo wound healing test

Wound contraction is determined by the reduction of the unhealed area, where a higher contraction indicates a better rate of healing. Fig. 7 displays the percentage of contraction over time for the three different group of rabbits, whereby the rate of healing varied among the groups. Formulation 3 was selected for in vivo experiments since it possessed a lot of good physicochemical properties compared with other formulation.

On day 3 the percentage of wound contraction in the honey hydrogel group was significantly higher ($p<0.05$) than in the no treatment group (18.8 ± 3.3% vs. 11.3 ± 2.0%) and this trend continued in day 6 (66.1 ± 2.3% vs. 36.0 ± 5.1%) and day 9 (98.6 ± 2.3% vs. 88.8 ± 3.2%). It is important to point out that on day 6, the percentage of wound closure in the honey hydrogel group was also significantly higher than the blank hydrogel (66.1 ± 2.3% vs. 40.7 ± 0.7%).

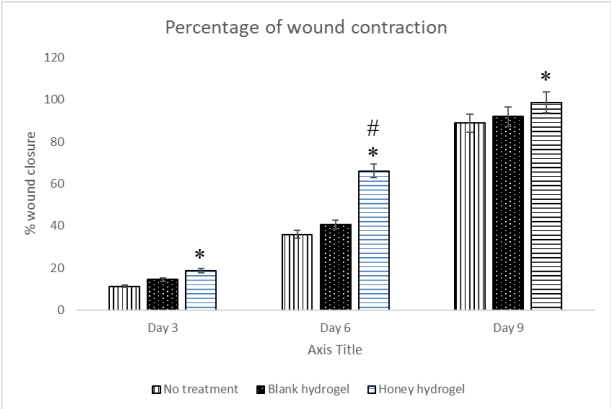

Fig 7 Percentage of wound closure for different groups form day 0 to day 9. The asterisks (*) indicated significant differences ($p< 0.05$) compared to the no treatment group. The hashtag (#) indicated a significant difference compared to the blank hydrogel group ($p<0.05$). Results are expressed as mean ± standard error mean (SEM) (n = 4).

Histological analysis

Histopathological study is used to observe the pathological changes at a microscopic level. The wound tissues were stained with H&E as well as

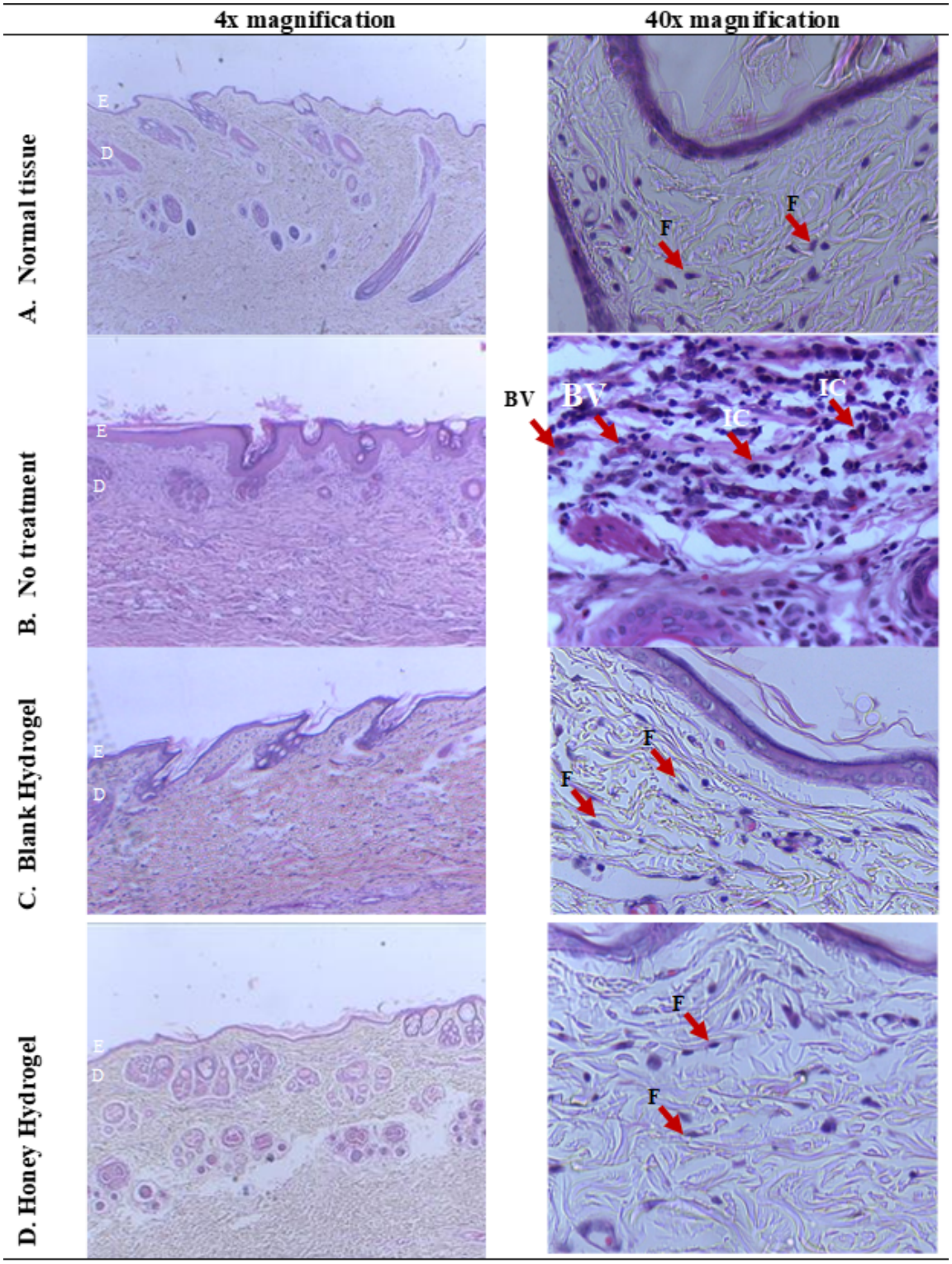

Fig 8. Histological observation of the skin at day 9 under 4x and 40x magnification for H&E. E = epidermis layer; D = dermis layer; BV = blood vessel, IC = inflammatory cells, F = fibroblast. Scale: 10325.9 pixels/cm.

Mallory trichrome. In addition, normal skin tissue was also stained with H&E and Mallory trichrome for baseline comparison with the wounded tissue. In normal tissue histology (Fig 8), it can be observed that there is a thin epidermis and the presence of many skin appendages such as hair follicles and sebaceous glands.

In the no treatment group, there were a lot of blood vessels and inflammatory cells seen when compared to the honey hydrogel group. Clumps of multinucleated cells, which are the distinguishing

feature of inflammatory cells, can be observed with H&E staining (Fig. 8B). On the other hand, in the stingless bee honey hydrogel group (Fig. 8D) the presence of inflammatory cells was much lower, and the arrangement of the fibroblasts, which are single nucleated cells, were much more organised. The higher number of fibroblasts represents an ongoing active healing process at the wound site as they play an important role in restoring damaged tissue. Therefore, it can be postulated that on day 9 the wounds in the no treatment group were still in the transition between the inflammation and proliferative phases while for the other two groups they have progressed to the following phase, which is the remodelling phase.

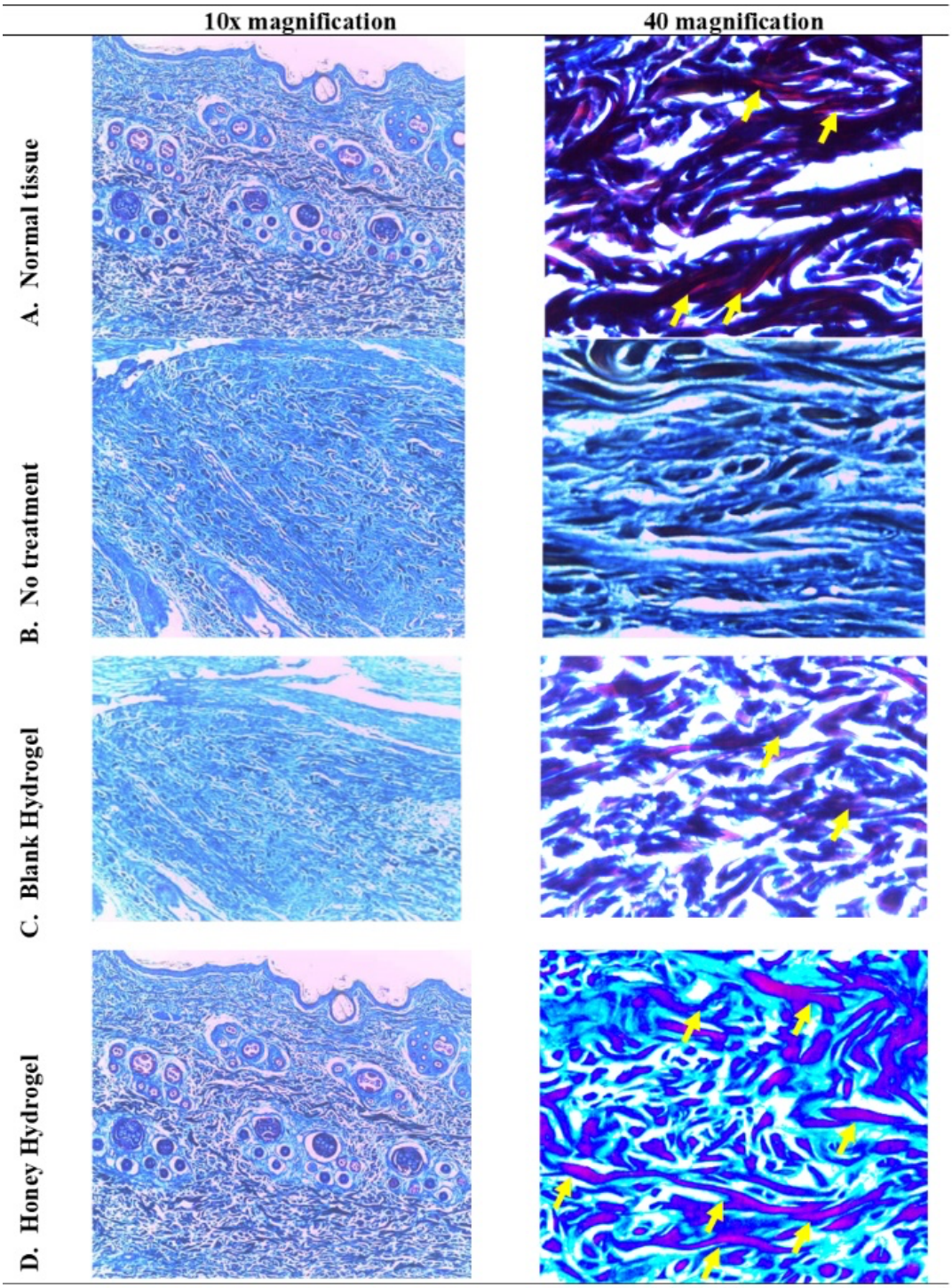

Fig 9. Histological observation of the skin at day 9 under 10x and 40x magnification with Mallory trichrome staining. Yellow arrows (➡) demonstrated the presence of collagen fibres. Scale: 10325.9 pixels/cm.

In the remodelling phase, the collagen fibres, fibroblast and keratinocytes will be elevated, leading to a well-organised structure that eventually resembles normal tissue. There is a balance between the synthesis and degradation of collagen and other proteins at the wound site to improve the wound structure. In the end, the type 1 collagen present during the healing phases will be replaced with mature type 3 collagen that resembles normal tissue (Singh et al., 2017).

In Fig. 9, using Mallory trichrome staining, the histological arrangement of collagen fibres can be observed in all tissues, except in the no treatment group (Fig. 9B). The collagen fibres are much more well-structured and in greater amount in the stingless bee honey hydrogel group, thus, it can be postulated that the structure of the tissue in this group would be stronger when compared to the other groups due to the role of collagen that supports the tissue structure (Maçin, 2021).

Fig. 10a shows the dermis thickness of the no treatment group is significantly higher than the normal group. Besides fibroblast, collagen deposition also leads to dermal thickness. In the proliferative stage, the collagen and fibroblast were disorganised in contrast with remodelling phase, where the cross-linkage between collagen fibres rearrange the structure of the dermis (Lee et al., 2017). In contrast, the thickness of honey hydrogel group closely resembles the normal tissue. That can be an indication of collagen fibres in this group is more organised than the others.

In Fig. 10b, the epidermal thickness layer of skin in the no treatment group was significantly ($p<0.05$) higher than the normal tissue. The main reason for the increased epidermal thickness is the changes in cytokine concentration that affect the ability of epithelial cells to proliferate more rather than differentiate, in order to overcome the loss of epidermal structure after a wound insult (Jacków et al., 2016). Eventually, in the remodelling phase, the thinning of epidermis would occur by the cornification of epithelial cells to reinstate the structure as before (Sullivan & Myers, 2021). Since the thickness of epidermis in honey hydrogel group is significantly ($p<0.05$) thinner than the no treatment and closely mirrors normal tissue, this can support the fact that the wound treated with honey hydrogel was at the more advance remodelling phase as compared to the no treatment group.

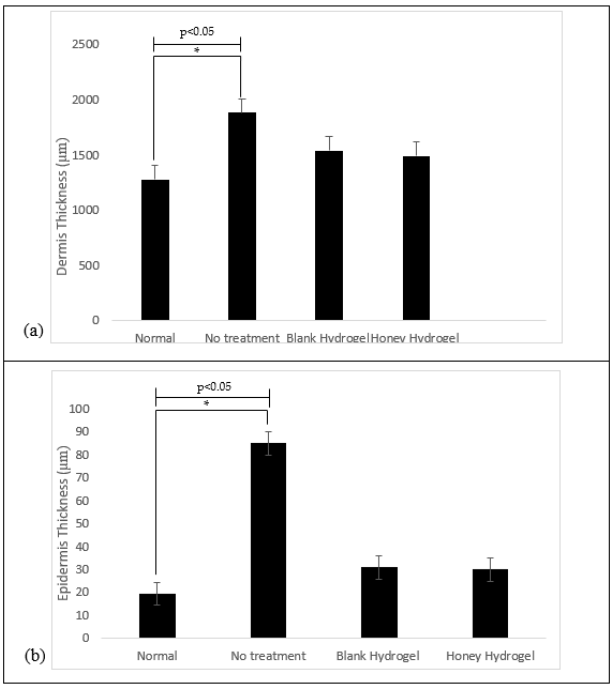

Fig 10a. Thickness of (a) dermis and (b) epidermis layers. The asterisks (*) indicated significant differences ($p< 0.05$) compared to normal tissues.

In wound contraction percentage, stingless bee honey-based hydrogel displayed a significant ($p<0.05$) increase in healing than the no treatment group. The result was consistent with the histological observations of the wound (Fig. 8 and Fig. 9) together with the dermal (Fig. 10a) and epidermal (Fig. 10b) thickness measurements, where the stingless bee honey-based hydrogel features were similar with the normal tissue. These findings were proven by gross observation via the videoscope images generated by Dermal Lab (Dermalab Series Skinlab Combo, Denmark) as seen in Fig.11.It can be observed that at day 9, the wound is completely healed for the stingless bee honey hydrogel treatment group when compared to the others. Furthermore, the closure of the wound in stingless bee honey hydrogel treatment is much better compared to the others from day 3 onwards. The stingless bee honey incorporated hydrogel displayed a significant improvement in the rate of wound healing, primarily due to the therapeutic effects of honey in the formulation. Honey stimulates immune cells, promoting wound debridement and accelerating healing. (Masad et al., 2021). In addition, the presence of honey positively influences the fibroblast proliferation, granulation tissue formation and collagen synthesis (Majtan, 2014) which could mediate the process of wound contraction. After the wound insult, growth factors (TGF- β and PDGF) stimulates the proliferation of fibroblast which subsequently results in the

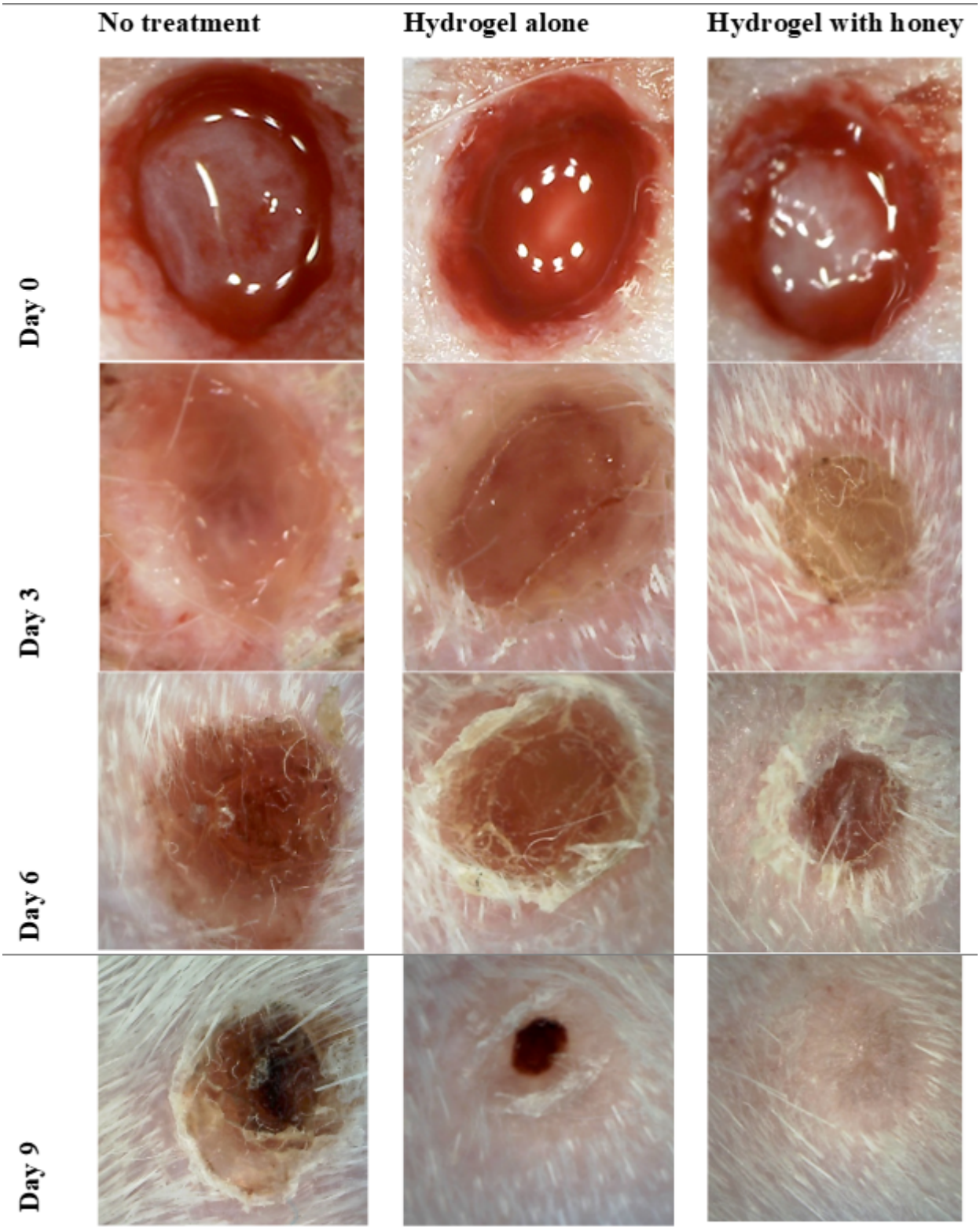

Fig 11 Gross wound observation from day 0 to day 9 Scale: 1834.72 pixels/cm.

formation of granulation tissue and an abundance of collagen. Collagen play an important role in providing strength to the matrix for contraction of the wound to occur. The action of actin and myosin in the matrix will draw the edge of the wound closer and thus decrease the wound size (Maçin, 2021). Presence of hydrogel also plays an essential part in promoting wound healing. As previously mentioned, hydrogel could provide a moist condition at the wound site, which could reduce dermal necrosis and promote wound reepithelialisation (Ahmad et al., 2021; Gull et al., 2019). Reepithelialisation of wound also occurs during the wound contraction later. Contraction of the wound must occur for the number of epithelial cells to be reduced significantly in the wound area and for epithelialisation to follow. Keratinocytes will then migrate from the edges of the wound and proliferate to reconstruct the epidermis structure. The epithelialisation period is essential to the fate of the wound, where a longer period will result in a scar formation at the later phase over many weeks (Loh et al., 2018).

In the epidermal and dermis layers, the blank hydrogel and honey did not display any significant difference, but in term of wound contraction, there were significant differences ($p<0.05$) at day 6. This indicated that blank hydrogel only promotes the thinning of the skin structure while honey hydrogel promotes thinning of the skin and the arrangement of the collagen fibres to assist in the wound contraction.

Honey could promote the wound contraction by stimulating fibroblast and re-epithelization that eventually promotes proliferation of keratinocytes to the skin surfaces(Oryan et al., 2016). The hydrogen peroxide possessed by honey can stimulate the fibroblast proliferation which contributes to the arrangement of the cells and collagen during the proliferation phase of the healing process (Al-Jadi et al., 2014). In addition, the glucose content and minerals in honey could promote epithelial migration across the wound surface and keratinocyte proliferation (Al-Masaudi et al., 2020). .

Conclusion

The experimental design by using RSM was successful in developing stingless bee honey-based hydrogel. After performing the characterization tests, Formulation 3 displayed good swelling ability, rheological property and antimicrobial activity. For wound healing assessment, stingless bee honey-based hydrogel demonstrated promising healing properties as proven by using gross appearance and wound contraction percentage where it was significantly ($p<0.05$) better than the untreated group. This result was further supported qualitatively via histological observations and quantitatively via epidermal and dermal thickness measurements. The result suggested that stingless bee honey-based hydrogel was able to promote full thickness wound healing by promoting wound contraction, re-epithelisation as well as collagen formation. In addition, since blank hydrogel consisted of PVA-PEG-agar composition that was able to imbibe water content, it has the ability to maintain a moist condition and provide an occlusive effect to the wound. Therefore, it implies that the introduction of stingless bee honey hydrogel could provide a synergistic effect with the PVA-PEG hydrogel to enhance the rate of wound healing where honey hydrogel promotes thinning of the skin structure which immediately followed by better wound contraction.

Authors contributions

Conceptualization, M.A.A.J and H.H.; Experimental studies M.A.A.J., M.L.M.I, U.A and H.H.; Data and statistical analysis, M.A.A.J and M.L.M.I; Manuscript preparation, M.A.A.J and U.A; Manuscript editing, M.A.A.J, U.A., K.A.H and H.H; Manuscript review, K.A.H, H.H and M.A.B. All authors have read and agreed to the published version of the manuscript.

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Ethical approval statement (if applicable)

The animal study protocol was approved by the

Institutional Animal Care and Use Committee (IACUC), International Islamic University Malaysia with approval number IIUM/IACUC-2019 (7).

Conflict of interest

The authors declare that there are no conflicts of interest to disclose

Declaration of generative AI and AI-assisted technologies in the writing process

A few artificial intelligent tools (e.g ChatGPT, Gemini and DeepSeek) were used to assist in improving the readability and language in certain parts of this work. The authors have reviewed and edited the content as necessary and take full responsibility for the final content of the publication.

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