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RESEARCH ARTICLE

Susceptibility of Stingless Bee, Giant Bee and Asian Bee Honeys Incorporated Cellulose Hydrogels in Treating Wound Infection

Rathosivan Gopal[a], Alex Zhen Kai Lo[a], Noor Masuriani Masrol[a], Chian-Hui Lai[b,*], Norhidayu Muhamad Zain[c], Syafiqah Saidin[a,d,*]

[a] School of Biomedical Engineering & Health Sciences, Faculty of Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia; [b] Graduate Institute of Biomedical Engineering, National Chung Hsing University, Taichung, Taiwan; [c] Academy of Islamic Civilization, Faculty of Social Sciences and Humanities, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia; [d] IJN-UTM Cardiovascular Engineering Centre, Institute of Human Centered Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

Abstract Wound healing and wound management are among challenging clinical problems, despite the advancement in medical technology and research. Honey is one of the natural products, synthesized by honey bees that exhibits great antibacterial and medicinal properties. Incorporation of honey into modern dressing materials such as cellulose hydrogel is beneficial to anticipate cell proliferation while preventing infection in a wound region. This study reports the fabrication of honey cellulose hydrogels for reliable alternative treatment of wound infection. The cellulose hydrogels were incorporated with three types of mainland Southeast Asia honeys of stingless bee, giant bee and Asian bee, independently. Each hydrogel was subjected to ATR-FTIR analysis for the determination of chemical composition. The antibacterial properties of honey hydrogels were evaluated through zone inhibition and colony count tests against Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus). The cytocompatibility of the honey hydrogels was then evaluated through MTT assay and cell scratch assay with human skin fibroblast cells. The composition of honey and cellulose hydrogel were verified with the appearances of fingerprint bandwidth and identical peaks of both compounds. The giant bee honey hydrogels produced the highest bacterial retardation through both antibacterial tests. The stingless bee honey hydrogels projected susceptibility towards E. coli while the Asian bee honey hydrogels projected susceptibility towards S. aureus. Among these three variations of honey hydrogels, the in-vitro cytocompatibility analyses testified the greatest cell viability and cell migration on the stingless bee honey hydrogels compared to the Asian bee honey hydrogels, giant bee honey hydrogels and control hydrogels. The findings support the potential of honey hydrogels as a reliable alternative treatment for wound infection.

Keywords: Honey, Hydrogel, Antibacterial, Wound healing

***For correspondence:**
syafiqahsaidin@biomedical.utm.my

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Introduction

Wound healing is a commencement series of physiological responses when the human body experiences injury [1]. Infected wounds require more time and attention to be healed compared to normal

wounds due to interruptions in the healing process [1]. Localization of aggressive bacteria in wound disrupts cell migration, thus prolonging wound healing [2]. Among four-overlapping processes of wound healing (hemostasis, inflammation, proliferation and tissue remodeling), the inflammation stage is crucial for a wound to fight against bacteria and foreign body [3]. However, failure in this stage leads to tissue destruction and wound repair inhibition [3]. Along with the emergence of advance technology in medical research, several commercially available wound dressings have been introduced in recent years that contained antibacterial agents [4]. However, these dressings possess several drawbacks, such as cytotoxic effects from the incorporation of synthetic antibacterial agents, especially over an extended treatment period that could results in delayed wound healing [4,5]. The incorporation of natural products or natural drugs is a reliable alternative to overcome the complication of synthetic antibacterial agents [5].

Honey is a natural product, synthesized by honey bees from flower nectars [6]. Honey has been used as medicines and for palliative cancer treatments due to its nutrient and therapeutic values [7]. The properties of honey depend on several factors such as the source of honey bee food, climate, honey bee species, the presence of impurities and geographical region [6,7]. Honey is composed of flavonoids, phenolic acids, methylglyoxal and bee peptide that exhibits medicinal values such as antibacterial, antioxidant, anti-fungicidal, anti-inflammatory, antiviral and hepatoprotective [2,6]. There are several types of honey available in the mainland Southeast Asia region such as stingless bee, giant bee and Asian bee honeys.

Stingless bee honey known as *Kelulut* honey is produced by *Trigona spp.* [8]. It possesses antibacterial properties and excellent antioxidant capacities against various bacterial species, especially Gram-positive bacteria [9]. While, giant bee honey known as *Tualang* honey is produced by rock bees or giant bees called *Apis dorsata* [7]. Giant bee honey has high phenolic acids as well as flavonoids [10] and has been recognized with antibacterial effect as it can treat wound infection caused by some Gram-negative bacteria [10,11]. Asian bee honey is another type of commercialized honey, known as *Apis cerana* honey which share similar properties as the Western honey bee, *Apis mellifera* [12,13]. Asian bee honey is capable in retarding bacterial growth of *Eschericia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Salmonella typhimurium* (*S. typhimurium*) [14].

Hydrogels are one of the biomaterial-based dressings, broadly used in tissue engineering which exhibit an outstanding physiological property due to its high water content, good fluid absorbance and moisture maintainance to encourage cell development [4,15]. Specifically, cellulose-based hydrogels are nature-derived and sustainable asset with excellence biocompatibility, biodegradability, low-cost, ingest wound fluids, give sufficient gaseous exchange at wound region and possess mucoadhesive properties [15,16]. The incorporation of honey into cellulose-based hydrogels can be a very good shield in attaching and covering a wound region as well as preventing bacterial infection [17]. Therefore, in this study, cellulose-based hydrogels were fabricated with the incorporation of three types of mainland Southeast Asia honeys which were stingless bee, giant bee and Asian bee honeys, independently. The honey hydrogels were characterized with an attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). The antibacterial properties of the honey hydrogels were evaluated through antibacterial analyses of zone inhibition and colony count. While MTT assay and cell scratch assay were used as a platform to verify the cytocompatibility of the honey hydrogels through cell viability and cell migration measurements, respectively.

Materials and methods

Materials

Sodium carboxymethyl cellulose (SCMC) with M_w of 90,000 g/mol, hydroxypropyl methyl cellulose (HPMC) with M_w of 10,000 g/mol, polyethylene glycol (PEG) 400 and analytical grade ethanol were purchased from Sigma Aldrich (Missouri, USA). Three types of mainland Southeast Asia honeys, stingless bee, giant bee and Asian bee honeys at 22% concentration, were purchased from Bahtera

Yubalam Enterprise, Malaysia.

Fabrication of honey hydrogels

A casting-solvent evaporation technique was used to fabricate honey hydrogels. The hydrogel composition was standardized at 15% SCMC, 10% HPMC, 5% PEG 400, 30% respective honey and 40% distilled (DI) water [18]. The hydrogels without the incorporation of honey were set as a control. The fabrication of honey hydrogels was conducted by dissolving SCMC in ethanol at a stirring constant of 250 rpm. Moderately, PEG 400 and HPMC were added into the SCMC solution to prevent clumping formation. In a separate beaker, stingless bee honey was dissolved in DI water. The dissolved honey was incorporated into the hydrogel mixture and allowed to stir for 30 min at 200 rpm. Finally, the honey hydrogel was transferred into a small petri dish and solidified in an oven for 48 h at 40°C. The stingless bee honey hydrogel was refrigerated until further use. The fabrication process was repeated by replacing the stingless bee honey with giant bee honey and Asian bee honey, respectively, to produce giant bee honey hydrogels and Asian bee honey hydrogels.

ATR-FTIR analysis

For the measurement of hydrogel composition, an approximately 10 mm × 10 mm of each hydrogel was cut and subjected under ATR-FTIR (FT-IR Spectrometer Spectrum Two (UATR Two), PerkinElmer, United States). The wavenumber reading was recorded from 500 to 4000 cm^{-1}. The resolution was set at 8 cm^{-1} with 32 scans using a diamond crystal with background subtraction.

Antibacterial analysis

The antibacterial properties of the hydrogels were evaluated against Gram-negative *Eschericia coli* (*E. coli*, ATCC 11229) and Gram-positive *Staphylococcus aureus* (*S. aureus*, ATCC 6538) through two antibacterial analyses of disc diffusion and colony count. Prior to the tests, a single colony of bacteria was transferred from nutrient agar plate into 100 mL of Luria-Bertani (LB) broth by using a sterile inoculation loop to prepare the suspension of bacterial growth. The bacterial culture was incubated in a shaking incubator (SI-50D, Lab-Tech Scientific, Malaysia) at 200 rpm for 16 h. An amount of 10 mL bacterial suspension was then transferred into 100 mL of fresh LB for another hour incubation. The bacterial concentration was adjusted at 1×10^6 CFU/mL using UV-vis spectrophotometer (GENESYS 10S, Thermo Scientific, USA) at an optical density (OD) of 0.6.

Zone inhibition test

The control and honey hydrogels were further solidified in an oven for 48 h to obtain a dried hydrogel form. The dried hydrogels were punched into small disc shape with a diameter of 1.5 cm and a thickness of 0.5 cm. The punched hydrogels were firmly placed on nutrient agar plates which have been smeared with the 1×10^6 CFU/mL bacterial suspension. The hydrogels were pushed slowly and slightly within the nutrient agars to ensure tight contact between the punched hydrogels and the nutrient agar surfaces. All nutrient agar plates were incubated for 24 h in an incubator at 37°C. The lengths of zone of inhibition were measured using a ruler from one edge of the zone to the other edge. The average and standard deviation (SD) values were then calculated from three consecutive measurements.

Colony count test

The punched dried hydrogels were immersed in 5 mL of the 1×10^6 CFU/mL bacterial suspension. The suspensions were placed in a shaking incubator at 200 rpm for 3 h [19]. The incubated suspensions were then diluted to eight dilution series. An amount of 10 µL of each dilution series was dropped on nutrient agars for 24 h incubation at 37°C. This bacterial enumeration method is known as a drop plate method. After 24 h incubation, the bacterial colonies formed on the agars were counted for further calculation of bacterial reduction percentage as presented in Equation 1.

$$Bacterial\ reduction\ (\%) = \frac{CFU_{control} - CFU_{experiment}}{CFU_{control}} \times 100 \quad (1)$$

In-vitro cytocompatibility analysis

The indirect cytocompatibility analyses were conducted according to ASTM F813 standard protocol [20] with human skin fibroblast cells (HSF 1184, ECACC, UK). A complete medium which comprised of Minimum Essential Medium (MEM), fetal bovine serum (FBS) and penicillin/streptomycin was prepared with the ratio of 100:10:1. Human skin fibroblast cells were cultured in the complete medium by incubating the cells in a humidified incubator at 37ºC with 5% CO_2 supplement and 95% humidity. The cells were detached using TrypLE Express enzyme at 80% confluency. Then, the cells were seeded in cell culture plates and were further incubated to reach 100% confluency. The cell concentration was finally adjusted at 1×10^5 cells/mL for the MTT assay and the cell scratch assay. An extraction medium was also prepared at 0.2% w/v by dissolving the hydrogels in the new complete medium without cells.

MTT assay

An amount of 1 mL of sterile phosphate buffer saline (PBS) was added to 5 mg of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) in a vial to prepare 12 mM MTT solution. The MTT solution was stored at 4ºC without UV light exposure [21]. In 96-well plate, the 1×10^5 cells/mL cells were seeded and incubated for 24 h. The cells media were discarded and the cells were treated with the extraction media by pipetting 200 µL of extraction medium into each well. Further incubation for 24 h was performed to assess cell viability. The extraction media were then removed while 200 µL of MTT solution was added to each well. The plate was further incubated for 4 h to allow the MTT reaction.

After 4 h incubation, the MTT solution was replaced with 200 µL of dimethylsulfoxide (DMSO) to dissolve the crystalised formazan. The well plate was swayed and absorbance values of the purple formazan products (indicating the viable cells) were recorded at 540 nm wavelength using a spectrophotometer (Thermo Scientific, Multiskan FC 51119000, Taiwan). The cell viabilities were measured in triplicate to obtain reliable data. Equation 2 was used to express the results of cell viability.

$$Cell\ viability\ (\%) = \frac{OD_{test}}{OD_{positive\ control}} \times 100 \quad (2)$$

Cell scratch assay

A small linear scratch on the confluent monolayer culture of 1×10^5 cells/mL was created by scraping the monolayer with a sterilized 200 µL pipette tip. The cell media were replaced with the corresponding extraction media and incubated for 24 h at 37ºC with 5% CO_2 supplement. Each well was then observed under a bright-field inverted fluorescence microscope (Carl Zeiss Axio Vert A1, USA) and the cell images were captured at 5× magnification. The initial lengths of the scratched gap and the lengths after 24 hours incubation was measured to evaluate the capability of cell migration. An image analysis software called ImageJ software (version 1.8.0_172, NIH, USA) was utilized to measure the gap lengths. Equation 3 was used to express the cell migration results as a percentage of gap closure.

$$Gap\ closure\ (\%) = \frac{Length_{initial} - Length_{final}}{Length_{initial}} \times 100 \quad (3)$$

Statistical analysis

A statistical analysis was performed on the *in-vitro* cytocompatibility data using Statistical Package for the Social Sciences (SPSS) software (version 25.0, IBM Corp., USA). The combination of One-way Analysis of Variance (ANOVA) and Dunnett's post-hoc test was utilized, appropriately, to determine the significance difference between means ($p<0.05$) of respective honey hydrogels and the control hydrogel.

Results and discussion

Chemical functionalities

Figure 1 shows the ATR-FTIR spectra in a transmittance mode for the control and the honey hydrogels. The characteristic of water band (O–H stretch) in the hydrogels can be seen at 3200 - 3500 cm^{-1} vibrations. The peaks between 2850 and 3000 cm^{-1} represented C–H stretching vibrations of the cellulose composition. The characteristic of cellulose in the hydrogels can also be observed at the

transmittance band of 1585 - 1620 cm^{-1} which indicated C–C aromatic [22,23]. On the other hand, C=O and C=C stretching bonds were appeared in the region bands between 1630 - 1730 cm^{-1} which associated to the cellulose hydrogels as well as phenolic molecules of the honey [24]. The vibrations of 1000 - 1200 cm^{-1} and 1200 - 1350 cm^{-1} annotated the compositions of C–O and C–H, respectively. Both chemical bonds were derived from the cellulose hydrogels, which also share the similar spectral region with flavanol and phenol in the honey [24,25]. Some authors have defined several important spectral peaks to characterize the honey [26,27]. However, it is difficult to establish a clear characteristic of the honey in the hydrogels due to the mixture of cellulose compound, PEG 400 and honey. Nevertheless, different color intensities of yellowish-brown hydrogels also testified the presence of honey in the hydrogels.

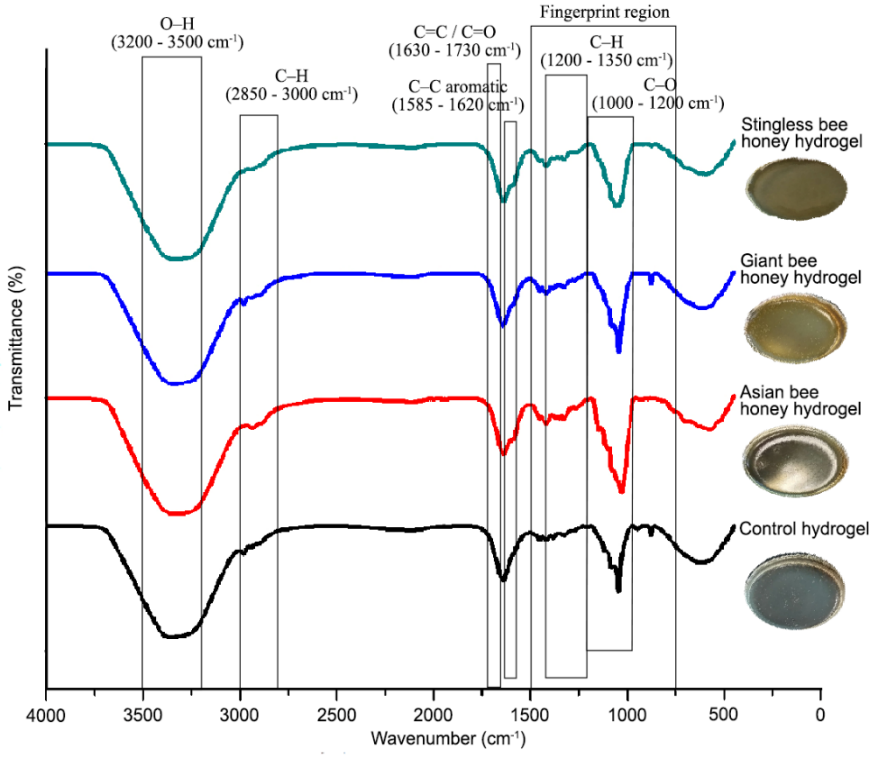

Figure 1. ATR-FTIR spectra of control, Asian bee honey, giant bee honey and stingless bee honey hydrogels.

Antibacterial analysis

Zone inhibition measurement

The migration of antibacterial agent from the hydrogels onto the nutrient agars that can inhibit bacterial growth or kill the bacteria was testified through the zone inhibition test. The zone inhibitions displayed by the honey hydrogels were indicated by clear and translucent circular zones compared to the color of nutrient agars which appeared in light-yellowish with the presence of observable colonies. The honey hydrogels showed distinct inhibition zones compared to the control hydrogels where the control hydrogels did not demonstrate any antibacterial activity (Table 1). The giant bee honey hydrogels exhibited the greatest inhibition zones for both bacteria. For *E. coli*, the stingless bee honey hydrogels showed slightly higher inhibition zones compared to the Asian bee honey hydrogels. While for *S. aureus*, the Asian bee honey hydrogels exhibited noticeable greater inhibition zones than the stingless bee honey hydrogels.

Zone inhibition test is a pre-antibacterial test to confirm the antibacterial properties of honey hydrogels and to verify the release of antibacterial substances [28]. However, this test has a limitation where it does not necessarily prove the capability of honey hydrogels in killing bacteria [29]. Moreover, it does not

accurately reflect the antibacterial effectiveness since the solubility and diffusion rate of honey hydrogels into the agar media can affect the inhibitory zones [30]. Therefore, a colony count measurement was conducted to validate the antibacterial data.

Table 1. Distances of inhibition zone produced by honey hydrogels on *E. coli* and *S. aureus*.

Hydrogel	Average distances of inhibition zone ± SD (mm)	
	E. coli	*S. aureus*
Asian bee honey hydrogel	11.23 ± 0.25	13.77 ± 0.06
Giant bee honey hydrogel	15.43 ± 0.23	15.47 ± 0.40
Stingless bee honey hydrogel	11.77 ± 0.50	12.60 ± 0.60
Control hydrogel	0.00 ± 0.00	0.00 ± 0.00

Colony count measurement

Different types of honey hydrogels have contributed to different bacterial growth trend on the agar plates as plotted in Figure 2. The giant bee honey hydrogels produced the highest bacterial reduction percentage against *E. coli* followed by the stingless bee honey hydrogels and the Asian bee honey hydrogels. It is recorded that the giant bee honey hydrogels were capable to provide 1.67 ratio higher antibacterial effects than the stingless bee honey hydrogels and 7.6 ratio higher antibacterial effects than the Asian bee honey hydrogels in retarding *E. coli*. The colony count measurements on *S. aureus* also showed that the giant bee honey hydrogels were capable to kill the bacteria the most, with the bacterial reduction percentages of 1.25 and 1.43 ratios higher than the Asian bee honey hydrogels and the stingless bee honey hydrogels, respectively. These results are accordance to the data of zone inhibition for both bacteria, thus validating the trend of antibacterial susceptibility for all honey hydrogels.

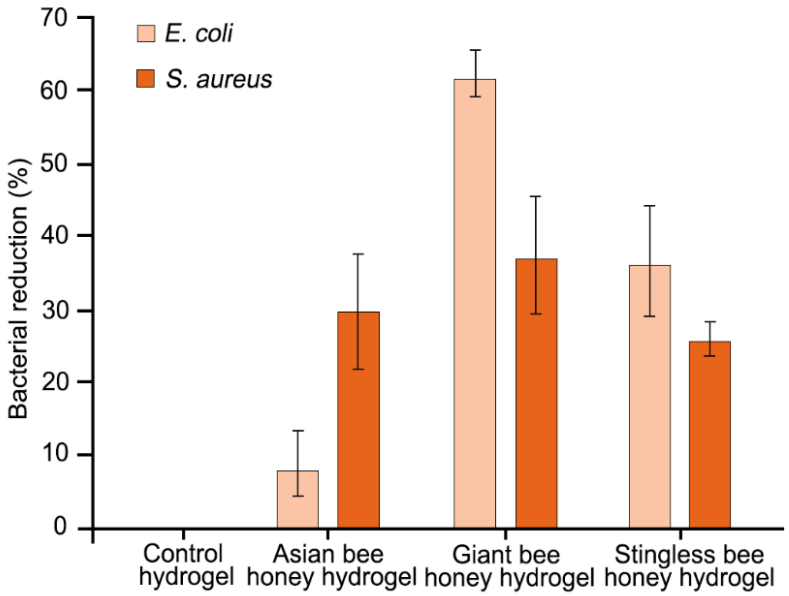

Figure 2. Bacterial reduction percentages produced by control, Asian bee honey, giant bee honey and stingless bee honey hydrogels.

Cytocompatibility analysis

Cell viability measurement

The MTT assay results showed approximately no cytotoxic effect on the HSF cells as shown in Figure 3 with cell viability percentages greater than 90% for all hydrogels. The highest cell viability was observed on the stingless bee honey hydrogels, followed by the Asian bee honey hydrogels and the giant bee honey hydrogels. The control hydrogels without the incorporation of honey were recorded at the minimum viability which only differed significantly ($p<0.05$) with the stingless bee honey hydrogels. The data for the honey hydrogels were intersected with each other which conclude that all honey hydrogels were not toxic to cells.

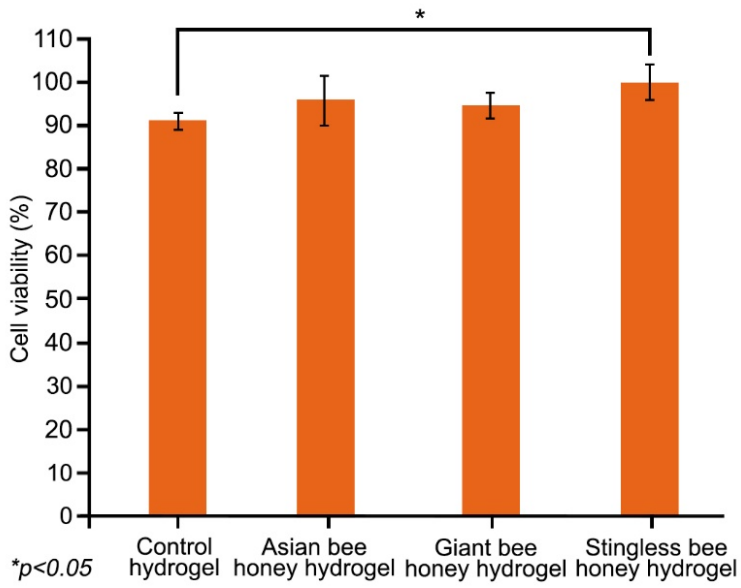

Figure 3. Cell viability data of control, Asian bee honey, giant bee honey and stingless bee honey hydrogels. The error bars represent the standard deviation of measurement for cells in triplicates for four hydrogel samples.

Cell migration measurement

The images of cell migration into the denuded areas after 24 h incubation were visualized in Figure 4. The Dunnett's test revealed that all cells treated with the honey hydrogels showed significant gap closure percentages when compared with the control hydrogel at $p<0.05$. The stingless bee honey hydrogels (11.08±0.88% gap closure) signified the greatest cell migration with 1.83 ratio more than the control hydrogels, followed by the Asian bee honey hydrogels (10.29±1.30% gap closure) with 1.70 ratio more than the control hydrogels. The giant bee honey hydrogels (8.55±1.24% gap closure) projected the least cell migration of 1.41 ratio higher than the control hydrogels.

The major component of honey is most sensitive to an infrared absorption region of 750 - 1500 cm^{-1}, known as the fingerprint region [26]. Each compound in honey has its own unique pattern of peaks at the fingerprint region that contained signals from numerous band vibrational modes [26,27]. This is the most appropriate region to quantify sugars of sucrose, glucose and fructose in various types of honey [26]. The sugars, in combination with honey acidity and phytochemicals are classified as non-peroxide contributors for the antibacterial effects of honey [31]. Flavonoids and phenols are the phytochemical components in honey which involved in its antibacterial effects [32].

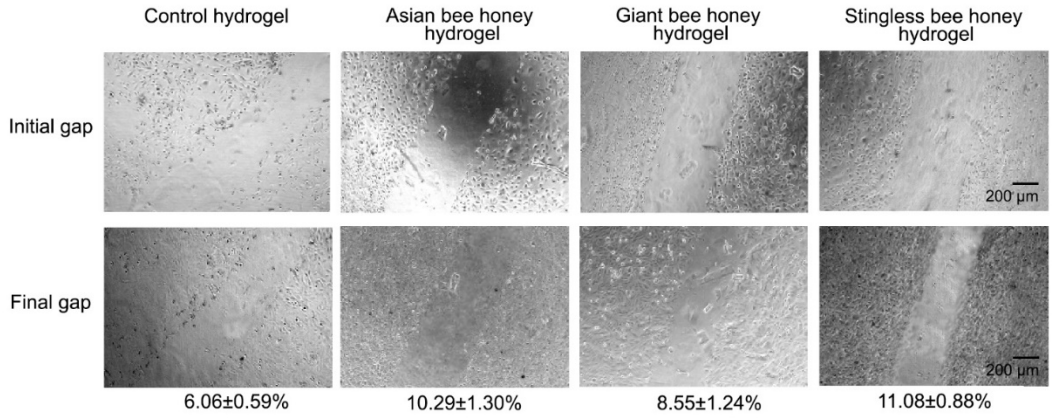

Figure 4. Cell migration images on control, Asian bee honey, giant bee honey and stingless bee honey hydrogels.

Giant bee honey is composed of high acidic compounds with pH values of 3.55 - 4 and contained high antibacterial agents of phenolic acids and flavonoids compared to stingless bee honey and Asian bee honey [33,34]. These properties cause the giant bee honey hydrogels to retard *E. coli* and *S. aureus* the most, in this study. The antibacterial efficacy of stingless bee honey is also mediated by non-peroxide action [35] that supported by its notable acidic pH of 3.29 - 6.02 [36,37]. While for Asian bee honey, its acidic pH is only reported from 4.03 to 4.32 [34,38]. According to the research by Ivanišová et al. [39], the total flavonoids of Asian bee honey is higher than stingless bee honey. Therefore, the lower acidity and greater flavonoids of Asian bee honey compared to stingless bee honey cause the Asian bee honey hydrogels to be more susceptible on Gram-positive *S. aureus*. Gram-positive bacteria are constructed of an extra peptidoglycan layer compared to Gram-negative bacteria which require greater acidity of antibacterial agents to dissolute the Gram-positive bacterial membrane [40]. Therefore, stingless bee honey has capability to dissolute and penetrate the membrane of Gram-negative *E. coli* more than Gram-positive *S. aureus* to deliver its antibacterial agent.

On the other hand, diluting honey in a certain extent will activate an enzyme called glucose oxidase that hydrolyses glucose in the honey to produce hydrogen peroxide (H_2O_2) [41]. The hydrogen peroxide and gluconic acid produced from the dissolution of sugar are another two key factors responsible for honey antibacterial effects [37,41]. It was noted by Irish et al. [42] that the efficacy of H_2O_2 radicals based honey is compromised by temperature which may depend on time, storage, composition and source of nectar.

The unique characteristic of honey that accelerates wound healing and cell epithelization is based on the reduction of oedema, exudate and inflammation [43]. The main element that contributes to the glory of honey is the therapeutic values of phenolic compound [8]. Phenolic compounds found in this natural product are correlated with the activity of antioxidant [41], antibacterial [34], anti-inflammatory [6] and anti-ageing [44]. Kek et al. [45] also proved that, there is a strong and positive correlation between the color intensity of honey and the total phenolic compound where darker color of the honey is an indicator for the higher phenolic content.

Conclusions

Asian bee, giant bee and stingless bee honeys are commonly known in the mainland Southeast Asia region for their therapeutic values. In this study, those honeys were incorporated in cellulose hydrogels for a reliable treatment of wound infection. The presence of honey in the cellulose hydrogels were clarified with the ATR-FTIR analyses. Both *E. coli* and *S. aureus* were most susceptible with the giant bee honey hydrogels. The stingless bee honey hydrogels were more prominent in retarding *E. coli* while the Asian bee honey hydrogels were more prominent in retarding *S. aureus*. Among those three honey

hydrogels, the stingless bee honey hydrogels assisted the highest cell viability and cell migration.

Data availability

Data are available on request.

Conflicts of interest

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

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