ORIGINAL ARTICLE

Polyhydroxyalkanoate and Stingless Bee 'kelulut' Propolis Improve Skin Wound Healing in Streptozotocin-induced Diabetic Rats

Samar Abdelrazeg[1,2], Mohamed Salih[1,2], Hasmah Hussin[1], Eshaifol Azam Omar[1], Manoj Lakshmanan[1,3], Kumar Sudesh[3], Bakiah Shaharuddin[1]

[1] Advanced Medical and Dental Institute, Universiti Sains Malaysia, sains@bertam, 13200 Kepala Batas, Pulau Pinang, Malaysia
[2] Stem Cells Department, National University Biomedical Research Institute, National University Sudan, 1111 Khartoum, Sudan
[3] School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia

ABSTRACT

Introduction: Chronic skin wounds in patients with serious medical conditions can significantly impact their clinical, social, and economic wellbeing. Diabetic wounds often become chronic due to infection and poor circulation. Currently, there is a lack of treatment options on effective wound management in these complex conditions. This study evaluates wound dressing patches made from polyhydroxyalkanoate (PHA) and stingless bee propolis in experimental diabetic rats. **Materials and methods:** PHA and propolis were evaluated *in vitro* as biocompatible wound dressing materials by studying the human dermal fibroblast (HDF) viability, cellular migration, gene expression and PHA haemocompatibility. PHA and propolis were evaluated in vivo to treat burn wounds in Streptozotocin (STZ)-induced diabetic rats. **Results:** The HDF scratch migration assay was significantly enhanced in culture media containing 10 µg/mL propolis at three time points ($P < 0.05$). PHA displayed good haemocompatibility (3.3%) and a high absorption capacity (1200%), while its water contact angle indicated slight hydrophobicity. Scanning electron microscopy (SEM) analysis showed that the PHA+propolis and propolis-only groups had an average pore size of 150 µm. The VEGF and b-FGF gene expressions in HDF cultured on various materials were higher in the PHA+propolis and propolis-only groups, though differences were not statistically significant ($P > 0.05$). In vivo, PHA+propolis patches exhibited significant wound contraction and healing via scab formation in burn wounds. **Conclusion:** The combination of PHA and propolis demonstrated promising results in treating experimental wounds in diabetic rats, suggesting it could be a low-cost, safe, and effective option for managing complex skin wounds.
Malaysian Journal of Medicine and Health Sciences (2025) 21(5): 203-213. doi:10.47836/mjmhs.21.5.24

Keywords: Propolis, *kelulut*, PHA, STZ-induced diabetic rats, Human dermal fibroblast

Corresponding Author:
Bakiah Shaharuddin, PhD
Email: bakiah@usm.my
Tel : +604-5622016

INTRODUCTION

Recovery from skin wounds is challenged by multiple factors including associated comorbidities such as diabetes, connective tissue diseases and inflammatory vascular conditions. In these patients, it is commonly aggravated by secondary bacterial contamination, which may present as skin ulcers, delayed healing and other complications. Diabetic foot ulcer affects around 9.1 to 26.1 million person worldwide (1).

Treatment modalities have improved over the years with deeper understanding of the cellular and molecular aspects of wound patho-physiology. Innovative and technological breakthroughs in the field of regenerative medicine have chartered significant improvement in the management of wounds. Advances in biomedical wound strategies include the development of dressing materials from natural polymers like chitosan, cellulose, alginates, silk and others. These biomaterials have biological characteristics inherent to the human body such as biodegradability, biocompatibility and regenerative capacity (2). However, low mechanical properties and rapid degradation rate limit its applications.

Conversely, synthetic polymers have demonstrated lower permeability, absorption, and adherence (3) , making them useful for superficial dressings. Yet, synthetic polymers that are biodegradable, biocompatible, and non-immunogenic have shown notable advantages in biomedical applications, particularly in wound dressings, due to their versatility in design and fabrication (4). This versatility supports the wound dressings that meet

the specific needs of complex wound environments, combining durability with biocompatibility.

Chronic wounds in diabetic patients often present with high level of exudates containing inflammatory mediators which is a media for bacterial colonization (4). In the case of diabetic patients, good control of hyperglycaemia and keeping other medical conditions in optimum control are crucial for wound healing. Keeping the wound free of infections is major hurdle in diabetic wound care (5). Hyperglycaemia causes microvascular complications, local ischaemia, reducing blood flow to the wound site and compromising the delivery of oxygen and essential nutrients necessary for tissue repair. Elevated glucose levels can impair the immune system, by reducing neutrophils and macrophages functions, impairment of migration of the fibroblasts and also defect in the production of growth factors that will help in wound healing mechanism (6).

Further incorporation of bioactive agents or biological substrates with polymers is another strategy to promote excellent wound healing and tissue regeneration. Honey and poly(vinyl alcohol) (PVA), electrospun as nanofiber scaffolds were fabricated to deliver an anti-inflammatory drug Dexamethasone sodium phosphate in a wound dressing showed excellent anti-microbial effect and promote wound healing (7).

Polyhydroxyalkanoates (PHA) are polyesters produced by many types of bacteria e.g. *Alcaligenes latus, Bacillus subtilis, Cupriavidus necator*, and others (8). Besides being non-toxic, once PHA has degraded inside the body, degradation products are absorbable by the metabolic pathways (9). The versatility and different properties of PHA is due to the different types of monomers that allow usage for different purposes. A hybrid wound dressing from 3 hydroxybutyric-4 hydroxybutyric acids [P(3HB/4HB)] and bacterial cellulose showed to be more effective than the commercial material (10). Another study tested the use of electrospun P(3HB))/ P(3HO-co-3HD coated with silver nanoparticles as a wound dressing materials, and showed promising biocompatibility and immunomodulatory, making it a good wound dressing option (12).

Propolis is a natural sticky resinous substance produced by bees to seal and protect bee hives from invasion of insects (12) and to maintain a low level of microorganisms (13). Diverse bioactive properties of propolis have been observed such as anti-microbial (14), anti-oxidant (15), anti-tumor (16) and anti-inflammatory effects. It also possesses strong wound healing properties (17). Indo-Malayan stingless bee or 'kelulut' is one of the bee species found in Malaysia which produces propolis that contains potent biological active molecules to expedite wound healing (18). To date, there is scant literature on the effects of "kelulut" bee propolis on wound healing. The aim of this paper is to evaluate whether employing a combination of PHA and "kelulut" bee propolis as dressing materials could be a novel therapeutic strategy in experimental diabetic rats' skin wounds. In the long term, we hope to translate this into an innovative solution for chronic wound management in the diabetic population.

MATERIALS AND METHODS

Research approval and ethics

This project was approved by the Regenerative Medicine Cluster, Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia (USM). All ethical rules for research conduct were observed. Animal ethics approval was granted by USM Institutional Animal Care and Use Committee (reference number: USM/IACUC/2018/(111) (902).

Human dermal fibroblasts (HDF) cell cultures and maintenance

Primary HDF was obtained and were purchased from the National University of Malaysia Tissue Engineering Center, cultured in DMEM:F12 at 37°C, 5% CO2 in a tissue culture incubator (ESCO, Barnsley, UK). The medium was changed every other day. The cultures were expanded when the cells reached 70-80% confluence (19). Cell passages of P2 – until P5 were used throughout this study.

Propolis collection and extraction

Raw Stingless Bees propolis from *Trigona thoracica* sp. was collected from Syamille Agrofarm, Taiping, Perak, Malaysia, an established commercial 'kelulut' beekeeper. The total weight of propolis was 365.5 grams, and 30 g was removed as a sample for extraction. The sample was frozen at -20°C overnight (Thermofisher, USA), then transformed into powder form using a grinder (Mill power tech, Thailand). Subsequently, the ethanolic extract of propolis was collected after rotary evaporation using Rotavap (BUCHI, United States) and freeze drying.

Propolis EC50 and PHA biocompatibility

To determine the concentration of propolis that gives 50% maximal response (EC50), the dose curve graph of HDF viability was plotted. HDFs were seeded in 6-well plate with a cell density of 100,000 cells per well. Two controls were used; C1 (no treatment added) and C2 (treated with DMSO 0.1%). Triplicate samples were prepared for each propolis concentrations; (10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, 50 µg/mL, 60 µg/mL, 70 µg/mL, 80 µg/mL, 90 µg/mL and100 µg/mL). After reaching 70% confluence, propolis was added to the cells and incubated for 24 hours.

For PHA biocompatibility, the cast films were placed into a polystyrene 24-well plate in triplicates, washed twice with PBS buffer and incubated in DPBS buffer for 30 min in a sterile hood under UV light (Esco life sciences, Singapore). In a 10% serum-supplemented culture medium, the cast films were incubated at 37oC

for 24, 48 and 72 hours. Each cast film was seeded with 75 000 cells in 400 µL of medium for 24 hours incubation, 50 000 cells/well for 48 hours incubation, 25 000 cells/well for 72 hours incubation. HDF cell viability for propolis effective concentration (EC50) and PHA biocompatibility was assessed by Presto Blue assay using the fluorescence spectrum of microplate reader Fluostar omega (BMG Labtech, Germany).

Scratch migration assay of HDF in propolis containing media

To study the effect of propolis on HDF cell migration, HDF were seeded in a 6-well plate with the seeding density of 150Ч103 until the cells were 80% confluent. Using a 10 µl pipette tip, a straight line (scratch) was made across the culture by keeping the pipette tip at an angle of 30°. Three wells were identified as control containing DMEM high glucose medium with 0.1 % DMSO only, and three wells as 'treatment' group containing DMEM high glucose + 10 µg/ml propolis. Phase contrast images were taken using an inverted microscope (Olympus, Tokyo, Japan) at 0, 3, 6 and 9 hours.

PHA biosynthesis, film casting, sterilization and patch fabrication

PHA used in this study was Poly (3 hydroxybutyrate-*co*-3 hydroxyhexanoate) P(3HB-co-3HHx). PHA was synthesized using *Cupriavidus necator* bacteria. The biosynthesis of PHA involved two main stages. Briefly, the bacteria were activated from glycerol stocks (Merck, USA) and inoculated in nutrient agar plates (Sigma Aldrich, USA) and propagated in an enriched media environment, followed by a biosynthesis process (20). Next, PHA was cast on 12 mm diameter cover slips (Fisher Scientific, Selangor, Malaysia), aged in a vacuum desiccator (Kartell labware, Italy) for seven days. To sterilize the films, it was placed in 24 well plates, incubated in ethanol (Merck, USA) for half an hour, washed and rinsed with DPBS three times, and finally left under the UV light for 30 minutes. DPBS was discarded and 100 µL DMEM: F12 medium was added to the wells for conditioning.

To fabricate PHA for porous wound dressing patches, the salt leaching technique was adapted from a previous protocol (21) using sodium chloride (NaCl) particles within the range of 100 – 200 µm (Sigma, USA) as a leachable porogen in a proportion of 5:95 polymer to porogen ratio. This is to promote macro and microporous structure of the scaffold as suitable for biomedical applications. The mixture forms a thick paste which was cast on a 5 mm diameter glass petri dish (Corning, USA) then left to air dry for 24 hours inside a biosafety cabinet (Esco lifesciences, Singapore), and to a vacuum dryer for 7 days until the solvent had evaporated completely. The porogen was then leached using a running distilled water.

Water contact angle measurement and absorbability test

Water contact angles of PHA were measured using the optical tensiometer apparatus (Biolin scientific, Finland). Measurement of the contact angles was carried out using 3 µL of distilled water which was dropped on the film surface followed by the calculation using the apparatus software. High absorbability is an important property in wound dressing to control wound exudates and to keep a moist environment at the wound site. The absorption property of the dressing was evaluated by incubating PHA-films into simulated wound fluid (SWF) which was constituted from 50% peptone water [8.5 g/L NaCl, 1 g/L peptone] (Sigma Aldrich, USA), 50% fetal calf serum (Sigma Aldrich, USA), according to the previous literature (22) and measured at 24, 48 and 72 h timepoints. Percentage of absorption capability of the films was calculated according to the formula: Wh – Wd /Wd x 100 [Wh = weight of the hydrated samples, Wd = weight of the dry samples]

Scanning Electron Microscopy (SEM)

Round shaped PHA patches measuring 20 mm diameter x 5 mm height were coated with gold for 20 min and viewed under SEM at 3000 X magnification.

Angiogenesis-related gene expression

PCR was performed to assess angiogeneic gene expression in HDF grown on PHA and propolis. All samples were cultured in DMEM:F12 for three days. Experiment was run in a three biological replicates, each one included 3 technical replicates. The steps involved RNA extraction, reverse transcription and real time quantitative PCR (qPCR) using SsoAdvanced Universal SYBR Green Supermix (BioRad, California, USA) according to manufacturer's instructions. The plates were then placed in Step one plus Real time PCR system (Applied Biosystems, California, USA). qPCR was performed at 95°C for 5 minutes for enzyme activation, followed by 40 cycles at 95°C for 10 seconds, 60°C for 30 seconds. Beta Actin (ACTB) was used as the housekeeping gene. The fold change was calculated using ΔΔCt method (by calculating the cycle threshold (Ct values, Delta Ct (ΔCt) and delta Delta Ct (ΔΔCt) and $2^{-\Delta\Delta Ct}$. Statistical analysis was done using Graph Pad Prism 8.0.2 using One way ANOVA test.

Streptozotocin (STZ)-induced diabetic rat model

Ten male Sprague Dawley (SD) rats weighing between 250-350 grams were involved in this study. The rats were purchased and housed at the Animal Research Facility of Advanced Medical and Dental Institute, University Sains Malaysia. The rats were acclimatized for five days and behavioral changes were observed daily. Diabetes was induced by intra-peritoneal injection of freshly prepared STZ solution in 0.05 M sodium citrate (pH 4.5) at the dose of 65 mg/kg and rats were provided with

normal food and 10% sucrose water. On day 2, 10% sucrose water was switched to regular water. Blood glucose level was tested from a tail vein sample using a blood glucometer (Lifescan, USA). After diabetes was established, the dorsal area was shaved and cleansed with chlorohexidine solution. Intramuscular ketamine 50-75 mg/kg + dexmedetomidine 0.25 mg/kg injection was given. A self-designed cylindrical stainless-steel rod with 1 cm diameter was used to inflict burn wounds on four identified sites representing four experimental groups. The rod was immersed in a boiling water at 100°C, and before each burn infliction, the depth of anesthesia was assessed by performing two consecutive toe-pinch tests on two different limbs. The rod was lifted off the flask and placed perpendicular onto the skin for 5 seconds. The wounds were treated with propolis and covered with PHA patches.

Morphometric analysis

The wound size was assessed by tracing the wound area on a transparent tracing paper on Day 3, 6, 9 and 12. The wound borders were traced and placed on a sheet of 2 mm2 graph paper and calculated as surface area. The percentage of wound closure was calculated using the following formula = The sum of wound area on day 0 - Wound area on day 12 / Wound area of day 0 x 100%

Histopathological sample collection

After 12 days, the animals were euthanized using CO2 chamber. Wound area was excised using a 10 mm punch (Robbins instruments, USA) following a previously published protocol (23), placed in PBS for homogenization or in 10% buffered formalin for 6 hours. Tissues were cleaved into half, fixed again for 24 hours, then embedded in paraffin and processed by a tissue processor (Sakura, Netherlands), following the routine steps of dehydration, wax clearing, and wax infiltration. Tissue blocks were sectioned by microtome (Thermoscientific, USA), cut at 5 µm thickness and sections were transferred to water bath (Memmert, USA) at 39°C. Floating section were fished out using frosted end of glass slides (Thermoscientific, USA) and then dried in an oven (Memmert, USA) at 50 °C for 15 minutes.

Hematoxylin and Eosin (H&E) staining, Inflammation and Scab Formation Scoring

Slides were deparaffinized then hydrated in distilled water. Slides were dipped in hematoxylin for 5 minutes, rinsed twice in D.W to remove the excess of hematoxylin, rinsed in bluing reagent for 15 seconds, followed by rinsing the sections in D.W, then dipped in absolute alcohol and the excess stain was blotted off. Eosin was applied for 2 minutes, slides were dehydrated in absolute alcohol (70%, 90%, and 100%) and finally mounted by xylene mounting medium. All reagents were manufactured by Abcam UK.

Inflammatory cells infiltration (polymorphonuclear and mononuclear) and scab formation on H&E-stained sections were examined under a direct microscope (Olympus, Japan) under 10X objective magnification. The cellular infiltration scoring system follows a previous protocol (23) where Score 0 is given to absence of any inflammation, Score 1 when there was discrete inflammatory cells, Score 2 for moderate inflammatory changes and Score 3 is given to a severe inflammatory reaction to the tissue. Similarly, the scab scoring system was described as Score 0 (no scab formation, Score 1 = discrete, Score 2 = moderate and Score 3 = severe amount of scab formation).

Haemocompatibility test

To determine the ability of PHA to come into blood without causing lysis, the haemocompatability test was conducted. From cardiac puncture, 3 mL of whole blood samples were added to heparin anticoagulant and mixed with sterilized physiological saline at a ratio of 1:1.25. 2 mL of PHA sample was incubated in a 37° C water bath for 1 hour to be added to the blood. Sterilized physiological saline was used as the negative control and distilled water as the positive control. The absorbance of the supernatant was recorded at 545 nm using a microplate reader (BMG Labtech, Germany). The haemolysis ratio percentage was calculated using the formula: HR (%) = $\frac{PHA - NC}{PC - NC} \times 100\%$ [PHA, NC, and PC refer to absorbance of PHA samples, negative controls, and positive controls]

RESULTS

Propolis cytotoxicity, EC50 and PHA cytotoxicity in human dermal fibroblast

Different concentrations of propolis between 10% to 100% dissolved in 0.1% DMSO were tested using Presto Blue assay which showed that 10 µg/mL of propolis gave 100% cell viability as shown in Fig. 1A. PHA cytotoxicity tested at three endpoints gave the highest rate at 72 hours (86%), while at 48 hours cell viability achieved 85% and at 24 hours achieved 62%, which was not statistically significant as compared to control surface (TCP); 100% viability , $P > 0.05$ (Fig 1B). The propolis concentration that allowed 50% growth of HDF (EC50) was 47.0 µg/ml (Fig 1C).

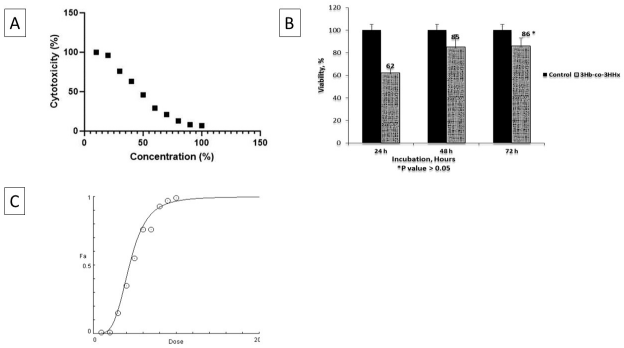

Fig. 1: Cytotoxicity assay of propolis and PHA in human dermal fibroblast and Propolis EC50. Different concentrations of propolis between 10% to 100% dissolved in 0.1% DMSO showed that 10 µg/mL of propolis gave 100% viability, N=3 (A).

Migration assay

Migration and proliferation of HDF was tested using the scratch assay. A significant increase in migration of HDF was observed after exposure to DMEM high glucose + 10 µg/ml propolis as compared to control media (DMEM+DMSO), 100% wound closure rate was observed at 3 hours, 104% rate was achieved at 6 hours and the highest wound closure rate was demonstrated at 106% at the 9 hours end point, (Fig. 2).

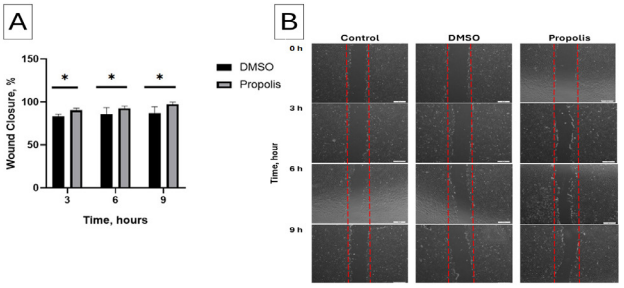

Fig. 2: Scratch migration assay of HDF in propolis media. HDF cell migration was higher in comparison to control media at 3 hours, 6 hours, and 9 hours' endpoints. P < 0.05, N=3 (A). Representative images from in vitro scratch wound healing assays demonstrating that cell migration into the cell-free region (outlined) is significantly accelerated in the presence of 10 µg/ml Propolis compared to controls (B).

Scanning electron microscopy (SEM)

SEM was employed to examine the surface structure of the PHA used in the wound dressing patch. Both types of dressing materials—PHA alone and PHA combined with propolis—displayed interconnected porous structures, with an average pore size of 150 µm (Fig. 3A and Fig. 3B). EDX analysis revealed peaks for carbon and oxygen only, confirming total elimination of the porogen, NaCl, in both PHA and PHA + propolis patches (Fig. 3A1 and Fig. 3B1).

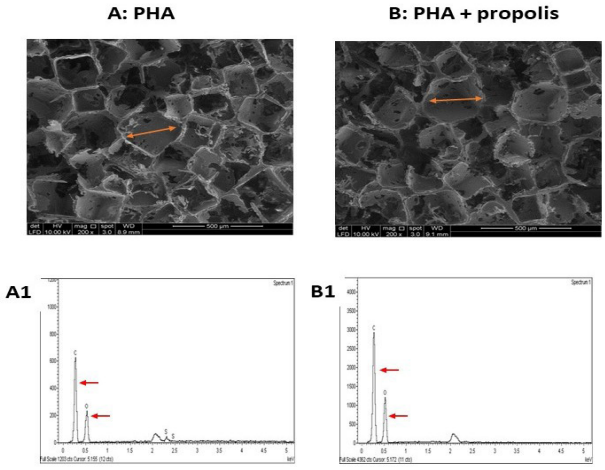

Fig. 3: SEM analysis of the dressing patches. Pore size in both samples (PHA alone and PHA with propolis) showed an average size of 150 µm (yellow arrows) (A and B). EDX analysis showed complete absence of the porogen (NaCl) in both samples A1 and B1 with only carbon and oxygen peaks shown (red arrows).

PHA patch absorbability test and surface wettability

The PHA polymer patch exhibited high absorption properties as shown in Fig. 4A whereby the absorption percentage had increased to 10 times its dry weight in two hours, using the formula described above. The surface wettability demonstrated the surface chemistry of PHA films. In the present study, the water contact angle of PHA was 79.9° at the left and 80.2° at the right (Fig. 4B), reflecting that the surface was hydrophobic, poorly wettable with observable roughness.

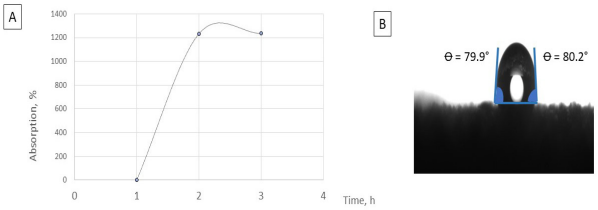

Fig. 4: PHA polymer absorbability test and surface wettability. High absorption capacity of PHA patch (1300 %) was demonstrated after two hours and stabilized at three hours (1200%), N=3 (4A). Representative figure of a water contact analysis on PHA wound dressing patch surface. The surface was poorly wettable with observable roughness: (left – 79.9° and right – 80.2°), N=3 (4B).

Angiogenic gene expression

Angiogenesis is essential in wound healing because it leads to the formation of new blood vessels. Angiogenesis gene expression study was conducted *in vitro* because there were limitations in doing any fluorescence

studies to detect the angiogenic antibodies due to PHA autofluorescence. *VEGF* and b-FGF expression of HDF cultured on different surfaces were studied (N=36). There was no significant difference in the gene expression on TCP, PHA, propolis , PHA + propolis; after three days, as presented in Fig. 5A and 5B.

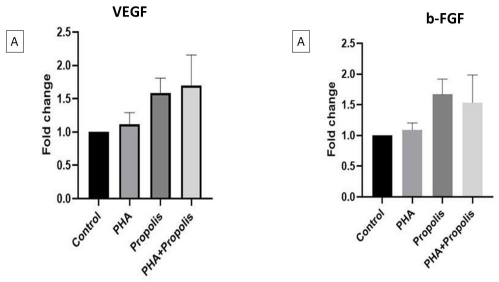

Fig. 5: Angiogeneic gene expression in HDF cultivated on different surface materials. Higher gene expression was demonstrated in the propolis and PHA+propolis groups, compared to control, PHA and propolis alone, although the difference was not statistically significant, P > 0.05. (One-way ANOVA) (n=3), for VEGF (5A) and b-FGF (5B).

Haemocompatibility test

Haemocompatibility test, also known as haemolysis test, showed the stability of red blood cell when contacting with a foreign object, when no lysis is observed. In this study, haemolysis was 3.3% (Table I). According to the national biological safety guideline, the haemolysis rate of less than 5% is considered as non-haemolytic, ensuring the materials are safe for biomedical applications.

Table I: Haemolysis test after exposure to PHA (3PHB-C-3PHHx)

Samples	Optical Density at 545 nm	HR (%)
Normal Saline (NC)	0.483 ± 0.046	0
Distilled Water (PC)	2.926 ± 0.118	100
PHA (3PHB-C-3PHHx)	0.553 ± 0.030	3.3

Abbreviations: HR: haemolysis rate, NC: negative control, PC: positive control.

In vivo wound morphometry assessment, inflammatory cells infiltration scores and scab formation

Progressive changes in wound healing were assessed by tracing the wound area on transparent tracing paper on day 3, 6, 9 and 12 post-wound induction. Figure 6 showed wound healing area percentage. The rate for PHA + propolis group gave the highest result (80%) followed by the propolis group (60%) and PHA only (58%). For the PHA + propolis group, the wound contracted rapidly from day 6 to day 9 and slowed down until day 12. The wound contraction for propolis- only group was slower in response but better than the PHA-only group. All test samples showed better performance as compared to control group (53%) as displayed in Fig 6A. For histopathologic analysis, H&E staining showed similar pattern with the best inflammatory and scab scoring were evident in the propolis group and propolis + PHA group (Figure 6B). For inflammation score, propolis samples gave the highest score (3), followed by PHA only (2), and PHA + propolis groups (2), as compared to control group (1). As for the scab formation, PHA + propolis group gave the lowest score (0) AS compared to the other groups (1). PHA + propolis samples gave the best overall results based on the inflammation and scab formation scores in comparison to other groups. Results were analyzed by one way ANOVA, p-value < 0.05 Fig 6C).

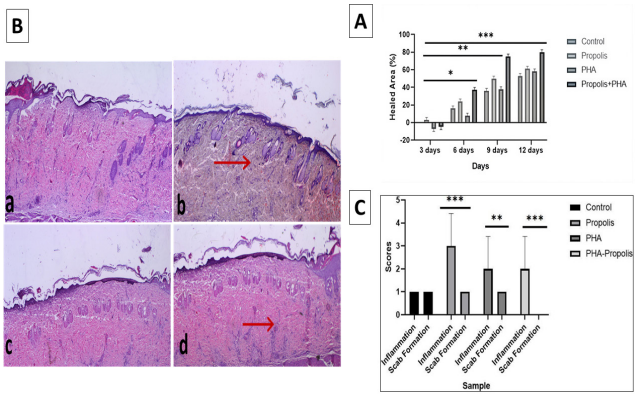

Fig. 6: *In vivo* mound healing area percentage, inflammatory cells and scab formation. Wounds treated with PHA + propolis patches have the highest wound area healing percentage, *P < 0.05, ** P <0.01 and ***P <0.001 (6A). H&E-stained sections showing scab formation in control section (a) propolis group (b) PHA group (c) and propolis+PHA group which showed the best results for inflammatory cells infiltration and scab formation (d). Inflammatory cells are indicated with red arrows, Fig 6B shows scores for inflammatory cells infiltration and scab formation. PHA with propolis patches showed the best result for inflammatory cells infiltration and scab formation, *P< 0.05 (One-way ANOVA) (6C).

DISCUSSION

The aim of the present study is to evaluate the use of dressing patches made of PHA alone or in combination with local propolis from stingless bees *Trigona thoracica* in the treatment of burn wounds in a diabetic rat model. There are many synthetic biocompatible polymers, PHA being one of the largest groups with more than 150 monomers. The attractive characteristics of PHA such as biocompatibility and biodegradability play a major role in making PHA a good candidate for medical applications. In the management of surgical wounds, PHA had been used as suture materials, nerve cuffs, skin substitutes, staples, and swabs (24).

In this study, propolis was used as an adjunct to PHA as a therapeutic wound dressing strategy. It was extracted, purified, and optimized using human dermal fibroblast (HDF) cells. It has many components which might differ according to the type and geographical area from which the propolis is harvested. These components have biological functions like antioxidant, antimicrobial and antifungal effects (25). Wound healing is a process that involves many stages like haemostasis, inflammation, proliferation, and remodeling. If there is any alteration or defect in any of these stages, complications like extreme scars and chronic non-healing wounds would

ensue (26).

The safest concentration of DMSO in the current study was 0.1% (v/v), as conducted during the optimization step of DMSO in HDF cultures. At this concentration, HDF cell viability was 100%, as evident from the Presto Blue assay. These results are in accordance with a previous study where high concentration of DMSO lowered the cell viability in a dose dependent manner and highly toxic to cells when it exceeded 3% (27).

To conform to the requirements of a medical device, the wound dressing materials were further investigated for its potential toxicity to the cells and fluid at the site of its intended action. Here, the interaction of PHA and propolis in HDF cultures was evaluated. HDF viability grown on PHA is comparable to tissue culture plastic (TCP) as the surface control, demonstrating its biocompatibility. EC50 of stingless bee propolis was 47.0 µg/mL, a value that indicates propolis works optimally at a low concentration. EC50 is the half-maximal repressing concentration, and it is used to determine the efficacy of a compound in reducing biological role.

PHA that was used in this study was hydrophobic, as shown in the water contact angle data. Hydrophobic surface has an advantage during changing of dressing materials by not disrupting newly forming granulation tissue. Hydrophobic dressings reduce antimicrobial resistance by preventing the formation of biofilm on the wound (28). On the other hand, extremely hydrophilic polymer in which the wettability is more than 150° is not suitable for the mammalian cells attachment and growth (29). However, the hydrophobicity of PHA in the wound dressing used in this study poses least undesirable effect of the patch because of its sponginess and high porosity, therefore, allowing for a moist environment. A good balance between the two is an essential property of a biomaterial. While the water contact angle of PHA differs between its monomers and according to the modifications on the polymer surface (30).

Previous studies had shown encouraging results on the use of PHA in HDF. A study conducted by Kim and colleagues which used PHBV electrospun scaffolds had demonstrated good cell growth. A mesh of PHA electrospun nanofibers composed of P(3-hydroxybutyrate-co-3-hydroxyvalerate) were used in their study. HDF growth and proliferation were observed showing good cell attachment on the polymer surface. In a previous *in vivo* mouse wound model, PHA meshes showed a good regenerative ability of the new tissues without scarring or scab formation (31). In another study, HDF adhered and proliferated on the electrospun scaffolds of a PHA polymer, P(3HB-co-3HV) (32).

Composition of the polymers, chemicals used in biosynthesis and different sources of bacteria were factors to be considered when studying the cell performance on different PHAs. As far as possible, the dressing material should closely resemble the characteristics of normal skin. It has to be mechanically suitable and must provide protection against microbes; allowing the gas exchange process. The mechanical properties of PHA beside its biocompatibility and biodegradability features, make this polymer a preferred candidate for wound dressing (33).

In the migration assay, the propolis improved HDF migration significantly compared to control (tissue culture media). This result agrees with the previous findings by Jacob and colleagues who tested the Malaysian and Brazilian propolis ability in enhancing the migration of the cells. In their study, the effect of Malaysian and Brazilian propolis on cellular migration was examined using human fibroblasts CRL-7522 cell line). The Malaysian propolis improved the migration rate of the human fibroblasts, while Brazilian propolis showed a slight increase in the migration of fibroblasts. Cellular migration is an important step during the proliferative phase of wound healing.

New blood vessels formation, or angiogenesis, plays a significant role in wound healing and scar formation processes. It provides essential nutrients and oxygen at the wound bed to enhance repair and regeneration of the injured tissue (34). Several key factors involved in angiogenesis such as Vascular endothelial growth factor (*VEGF*), Fibroblast growth factors (FGF), Platelet-Derived Growth Factor (PDGF), Hypoxia-inducible factor (HIF) and nitric oxide (NO) act by increasing endothelial cell proliferation, migration, and blood vessel maturation. Previous study showed that *VEGF*, FGF and combined therapy result in significant acceleration of many histological parameters, including fibroblast migration, collagen deposition, and angiogenesis (35).

Previous angiogenesis gene expression studies on PHA were commonly conducted *in vitro* because of the limitations due to PHA autofluorescence. In the current study, propolis upregulated both *VEGF* and b-FGF gene expression *in vitro* but the increment was not statistically significant as compared to control. This upregulation was also observed in groups containing combined PHA + propolis. Propolis was reported as a factor that upregulates the *VEGF* gene expression when combined with bovine bone graft (36). The combination of propolis extract together with bovine bone graft (BBG) and polyethylene glycol (PEG) upregulated the *VEGF* and FGF-2 expression and increased the number of osteoblasts. The results supported the fact that the flavonoids of propolis upregulated the formation of FGF-2 and *VEGF* which had hastened the wound healing process (37).

On the other hand, Brazilian propolis was tested for the antiangiogenic activity with sponge implants of mice

in 14 days and found to demonstrate antiangiogenic effect which was believed to be due to cytokines modulation (38). Suppression of angiogenesis has been described earlier, and the reports showed some possible mechanisms, which are likely to be the induction of the apoptotic mechanisms in vascular cells. Another view is that it occurs as a result of downregulation of the *VEGF* gene expression (39)

Propolis promotes wound healing through its antimicrobial activity by reducing the growth of any microbes in the wound area and blocking the biofilm formation. This is due to the flavonoids and phenolic acids in the propolis (40). Antioxidant properties of the propolis also have an effect on the wound healing mechanism by reducing the oxidative stress that occurs mainly after burns. Oxygen free radicals produced by macrophages and neutrophils give an adverse effect on the process of cellular regeneration. The antimicrobial and anti-inflammatory properties of propolis play a role in reversing this mechanism (41).

The *in vivo* study was aimed to enhance wound healing in STZ-induced diabetic rats. In this study, DM was induced successfully in SD rats using streptozotocin protocol which had been used by previous investigators. Cheng and colleagues induced diabetes in SD rats to study the enhancement of wound healing in diabetic rats. They used 60 mg/kg of STZ, which is considered a high dose (42). Several protocols of diabetes induction with STZ have been used before, depending on the aim and purpose of the research, the biological condition and monitoring of the SD rats.

Type I diabetes was induced in mice by using low doses of STZ repeatedly. A dose of 40 mg/Kg was administered for 5 consecutive days and this model was used for studying the effectiveness of antidiabetic agents. Another method is by giving a moderate STZ dose to rats after a high fat diet consumption and the protocol successfully caused hyperglycaemia and hyperinsulinaemia (43).

Burn wound induction is a well-established technique by previous investigators, especially in wound healing studies. The type of burn wound in this study is a contact burn wound at the back of male Sprague-Dawley rats. The burn wound completely healed in 35 days. The time taken for a complete healing differed between studies because it largely depended on the size and depth of the wound (44).

In this study, the PHA + propolis combination group showed mean wound healing area of more than 80%. This was the highest percentage among the experimental groups. This result agrees with a previous study which showed rodent wound contraction by up to 80% (45). In this study, measurement of contraction and re-epithelization was done in mice strains and the study reported that simple excisional wounds in murine are a good model for healing by contraction and re-epithelization.

Propolis also has proven to be a good wound healing agent owing to its composition and its non-toxic properties. In this study, the presence of both propolis and PHA have the combined effects on the wound contraction percentage; superior to PHA alone or control patches. The wound closure method used in this study followed the technique described by Jahandideh and colleagues who evaluated the healing effect of a herbal paste in a rat wound model (46).

Inflammatory cells, scab formation, tissue granulation and epithelisation were parameters used to evaluate wound healing in this study. Histopathological analysis showed inflammatory cells were highly abundant in the propolis group and the presence of PHA was anti-inflammatory. This is not unexpected as diabetic wounds present with more inflammation (47) and the anti-inflammatory effect of propolis might be delayed in this situation. An extension of the study time will reduce the inflammation in the propolis group.

Scaffolds containing PHA had been tested for promoting wound healing process previously, capitulating on PHA characteristics such as its biocompatibility, biodegradability and mechanical strength (48). In the current study, PHA with or without propolis showed reduction in inflammatory cells infiltration at the wound site. The scab formation in these groups were also comparable, leading to promotion of the wound healing process. Scab is a part of wound healing process, formed after the wound stops bleeding by blood clotting. Dry clots form a scab which protects the wounds from microbial invasion. The onset of the clotting system within minutes of injury is a crucial factor in scab formation.

Both PHA and propolis were shown to show a favorable response in scab formation. However, in this study, the combination of PHA + propolis was found to contract wound with no scab formation. This finding was similar to a previous *in vivo* study using P(3HB-co-4HB) mixed with collagen peptide which demonstrated excellent wound contraction without scab formation (49). The results of the current study were also in agreement with Kim and colleagues who found that PHA had regenerated the skin tissue without forming any scab (31).

To provide more evidence on the biocompatibility of PHA, the hemocompatibility or hemolysis test was conducted. The hemolysis index is measured based on the release and amount of free hemoglobin. P(3HB-co-3HHx) hemolytic index was 3.3% which indicated

the RBCs were stable when introduced to PHA; based on the standard of the American Society of Testing and Materials (ASTM F) 756-00 guidelines. Hemolytic rates vary depending on the type of polymers, methods of synthesis, composition and other properties like biodegradability. For example, the hemolytic activity of chitosan nanoparticles in human red cells had previously shown a rate of 186 - 223%. This study implicated that synthesis of the chitosan nanoparticles, and the pH needs to be tailored to be appropriate for a human medical device (50). Our study results further proved that PHA was unlikely to cause acute hemolysis when used in the mammalian body.

CONCLUSION

Diabetic wounds present significant challenges due to their complex nature, often leading to prolonged healing times, reduced quality of life, and increased socio-economic impact. The field of wound healing continues to evolve, with advances in tissue engineering focusing on biocompatible materials, cellular therapies, and growth factors to promote tissue regeneration and restore function. In this study, we leverage on PHA, a biocompatible and biodegradable polymer, with adaptability for biomedical applications as a therapeutic solution. Combining PHA with locally-sourced stingless bee propolis, this study showed enhanced wound healing in experimental diabetic rats. However, further optimization, including exploring alternative PHA synthesis and advanced fabrication methods like electrospinning, could enhance the patch's performance. Standardizing propolis extraction and composition will also be critical for clinical trials, which represent the next step in translating this technology into a viable solution for diabetic wound care in clinical settings.

ACKNOWLEDGEMENT

Universiti Sains Malaysia Short Term grant, awarded to Hasmah Hussin (304/CIPPT/6315158) and Research University (Individual) research grant awarded to Bakiah Shaharuddin (1001/CIPPT/8012263).

REFERENCES

1. Armstrong DG, Boulton AJM, Bus SA. Diabetic Foot Ulcers and Their Recurrence. N Engl J Med. 2017;376(24):2367–75. doi: 10.1056/NEJMra1615439.
2. Stern D, Cui H. Crafting Polymeric and Peptidic Hydrogels for Improved Wound Healing. Adv Healthc Mater. 2019;8(9):1–17. doi: 10.1002/adhm.201900104.
3. Mogoşanu GD, Grumezescu AM. Natural and synthetic polymers for wounds and burns dressing. Int J Pharm. 2014;463(2):127–36. doi: 10.1016/j.ijpharm.2013.12.015.
4. Schilrreff P, Alexiev U. Chronic Inflammation in Non-Healing Skin Wounds and Promising Natural Bioactive Compounds Treatment. Int J Mol Sci. 2022;23(9). doi: 10.3390/ijms23094928.
5. Dasari N, Jiang A, Skochdopole A, Chung J, Reece EM, Vorstenbosch J, et al. Updates in Diabetic Wound Healing, Inflammation, and Scarring. Semin Plast Surg. 2021;35(3):153–8. doi: 10.1055/s-0041-1731460.
6. Spampinato SF, Caruso GI, Pasquale R De, Sortino MA, Merlo S. The Treatment of Impaired Wound Healing in Diabetes : Looking among Old Drugs. 2020;1–17. doi: 10.3390/ph13040060.
7. Maleki H, Gharehaghaji AA, Dijkstra PJ. A novel honey-based nanofibrous scaffold for wound dressing application. J Appl Polym Sci. 2013;127(5):4086–92. doi: 10.1002/app.37601.
8. Chen GQ, Wu Q. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials. 2005;26(33):6565–78. doi: 10.1016/j.biomaterials.2005.04.036.
9. Mukherjee A, Koller M. Microbial PolyHydroxyAlkanoate (PHA) Biopolymers—Intrinsically Natural. Bioengineering. 2023;10(7):1–16. doi: 10.3390/bioengineering10070855.
10. Volova TG, Shumilova AA, Nikolaeva ED, Kirichenko AK, Shishatskaya EI. Biotechnological wound dressings based on bacterial cellulose and degradable copolymer P(3HB/4HB). Int J Biol Macromol. 2019;131:230–40. doi: 10.1016/j.ijbiomac.2019.03.068.
11. Kalaoglu-altan OI, Baskan H, Meireman T, Basnett P, Azimi B, Fusco A, et al. Silver Nanoparticle-Coated Polyhydroxyalkanoate Based Electrospun Fibers for Wound Dressing Applications. 2021;14(17):4907. doi: 10.3390/ma14174907.
12. Marcucci MC. Propolis: chemical composition, biological properties and therapeutic activity. Apidologie. 1995;26(2):83–99. doi: 10.3390/molecules28010141.
13. Choudhari MK, Punekar SA, Ranade R V., Paknikar KM. Antimicrobial activity of stingless bee (Trigona sp.) propolis used in the folk medicine of Western Maharashtra, India. J Ethnopharmacol. 2012;141(1):363–7. doi: 10.1016/j.jep.2012.02.047.
14. Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R, Popov S. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J Ethnopharmacol. 1999;64(3):235–40. doi: 10.1016/s0378-8741(98)00131-7.
15. Teixeira ÉW, Message D, Negri G, Salatino A, Stringheta PC. Seasonal variation, chemical composition and antioxidant activity of brazilian propolis samples. Evidence-based Complement Altern Med. 2010;7(3):307–15. doi: 10.1093/ecam/nem177.
16. Umthong S, Puthong S, Chanchao C. Trigona laeviceps propolis from Thailand: Antimicrobial, antiproliferative and cytotoxic activities. Am J

Chin Med. 2009;37(5):855–65. doi: 10.1142/S0192415X09007338.
17. Pahlavani N, Malekahmadi M, Firouzi S, Rostami D, Sedaghat A, Moghaddam AB, et al. Molecular and cellular mechanisms of the effects of Propolis in inflammation, oxidative stress and glycemic control in chronic diseases. Nutr Metab. 2020;17(1):1–12. doi: 10.1186/s12986-020-00485-5.
18. Syed Salleh SNA, Mohd Hanapiah NA, Ahmad H, Wan Johari WL, Osman NH, Mamat MR. Determination of Total Phenolics, Flavonoids, and Antioxidant Activity and GC-MS Analysis of Malaysian Stingless Bee Propolis Water Extracts. Scientifica (Cairo). 2021;2021. doi: 10.1155/2021/3789351.
19. Kisiel MA, Klar AS. Isolation and culture of human dermal fibroblasts. Methods Mol Biol. 2019;1993:71–8. doi: 10.1007/978-1-4939-9473-1_6.
20. Sudesh K, Abe H, Doi Y. Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. Prog Polym Sci. 2000;25(10):1503–55. doi: 10.1016/S0079-6700(00)00035-6.
21. Zubairi SI. Porous Three Dimensional (3-D) Scaffolds of Poly (3-Hydroxybutyric Acid) (PHB) and Poly (3-Hydroxybutyric-co-3-Hydroxyvaleric Acid) (PHBV): Determination of Salt Leaching Efficiency of Solvent-Casting Particulate-Leaching (SCPL)." Advances in Environmental Biology 2014; 8(10):925-932.
22. Panico A, Paladini F, Pollini M. Development of regenerative and flexible fibroin-based wound dressings. J Biomed Mater Res - Part B Appl Biomater. 2019;107(1):7–18. doi: 10.1002/jbm.b.34090.
23. Estevro L, Cassini-Vieira P, Leite AG, Bulhxes A, Barcelos L da, Evκncio-Neto J. Morphological Evaluation of Wound Healing Events in the Excisional Wound Healing Model in Rats. Bio-Protocol. 2019;9(13):1–12. doi: 10.21769/BioProtoc.3285.
24. Karmann S, Follonier S, Bassas-Galia M, Panke S, Zinn M. Robust at-line quantification of poly(3-hydroxyalkanoate) biosynthesis by flow cytometry using a BODIPY 493/503-SYTO 62 double-staining. J Microbiol Methods. 2016;131:166–71. doi: 10.1016/j.mimet.2016.10.003.
25. Irigoiti Y, Navarro A, Yamul D, Libonatti C, Tabera A, Basualdo M. The use of propolis as a functional food ingredient: A review. Trends Food Sci Technol. 2021;115:297–306. doi: 10.1016/j.tifs.2021.06.041.
26. Ridiandries A, Tan JTM, Bursill CA. The role of chemokines in wound healing. Int J Mol Sci. 2018;19(10). doi: 10.3390/ijms19103217.
27. Singh S, Young A, McNaught CE. The physiology of wound healing. Surg (United Kingdom). 2017;35(9):473–7. doi: 10.1016/j.mpsur.2011.06.011.
28. Morilla-Herrera JC, Morales-Asencio JM, Gómez-González AJ, Díez-De Los Ríos A, Lupiácez-Pérez I, Acosta-Andrade C, et al. Effectiveness of a hydrophobic dressing for microorganisms' colonization of vascular ulcers: Protocol for a randomized controlled trial (CUCO-UV Study). J Adv Nurs. 2020;(March):2191–7. doi: 10.1111/jan.14412.
29. Fukazawa K, Nakao A, Maeda M, Ishihara K. Photoreactive Initiator for Surface-Initiated ATRP on Versatile Polymeric Substrates. ACS Appl Mater Interfaces. 2016;8(38):24994–8. doi: 10.1021/acsami.6b07145.
30. Chang CK, Wang HMD, Lan JCW. Investigation and characterization of plasma-treated poly(3-hydroxybutyrate) and s(3-hydroxybutyrateco-3-hydroxyvalerate) biopolymers for an *in vitro* cellular study of mouse adipose-derived stem cells. Polymers (Basel). 2018;10(4). doi: 10.3390/polym10040355.
31. Kim HS, Chen J, Wu LP, Wu J, Xiang H, Leong KW, et al. Prevention of excessive scar formation using nanofibrous meshes made of biodegradable elastomer poly(3-hydroxybutyrate-co-3-hydroxyvalerate). J Tissue Eng. 23;11:2041731420949332. doi: 10.1177/2041731420949332.
32. Esmail A, Pereira JR, Zoio P, Silvestre S, Menda UD, Sevrin C, et al. Oxygen plasma treated-electrospun polyhydroxyalkanoate scaffolds for hydrophilicity improvement and cell adhesion. Polymers (Basel). 2021;13(7):1–17. doi: 10.3390/polym13071056.
33. Shishatskaya EI, Nikolaeva ED, Vinogradova ON, Volova TG. Experimental wound dressings of degradable PHA for skin defect repair. J Mater Sci Mater Med [Internet]. 2016;27(11):0–1. doi: 10.1007/s10856-016-5776-4.
34. Shi Z, Yao C, Shui Y, Li S, Yan H. Research progress on the mechanism of angiogenesis in wound repair and regeneration. Front Physiol. 2023;14(November):1–11. doi: 10.3389/fphys.2023.1284981
35. Adas G, Percem A, Adas M, Kemik O, Arikan S, Ustek D, et al. *VEGF*-A and FGF gene therapy accelerate healing of ischemic colonic anastomoses (experimental study). Int J Surg. 2011;9(6):467–71. doi: 10.1016/j.ijsu.2011.05.002
36. Shishatskaya EI, Nikolaeva ED, Vinogradova ON, Volova TG. Experimental wound dressings of degradable PHA for skin defect repair. J Mater Sci Mater Med [Internet]. 2016;27(11):0–1. doi: 10.1007/s10856-016-5776-4
37. Zarei M, Jafarian AH, Harandi A, Javidi M, Gharechahi M. Evaluation of the expression of VIII factor and *VEGF* in the regeneration of non-vital teeth in dogs using propolis. Iran J Basic Med Sci. 2017;20(2):172–7. doi: 10.22038/ijbms.2017.8243..
38. Kresnoadi U, Widjaja J, Laksono H. Ethanol extract

of propolis-bovine bone graft combination as a prospective candidate for socket preservation: Enhancing BMP7 and decreasing NFATc1. Saudi Dent J [Internet]. 2021;33(8):1055–62. doi: 10.1016/j.sdentj.2021.05.003

39. Daleprane JB, Abdalla DS. Emerging roles of propolis: Antioxidant, cardioprotective, and antiangiogenic actions. Evidence-based Complement Altern Med. 2013;2013. |doi: 10.1155/2013/175135.
40. Wojtyczka RD, Dziedzic A, Idzik D, Kepa M, Kubina R, Kabała-Dzik A, et al. Susceptibility of *Staphylococcus aureus* clinical isolates to propolis extract alone or in combination with antimicrobial drugs. Molecules. 2013;18(8):9623–40. doi: 10.3390/molecules18089623.
41. Jacob A, Parolia A, Pau A, Davamani Amalraj F. The effects of Malaysian propolis and Brazilian red propolis on connective tissue fibroblasts in the wound healing process. BMC Complement Altern Med. 2015;15(1):1–10. doi: 10.1186/s12906-015-0814-1.
42. Cheng KY, Lin ZH, Cheng YP, Chiu HY, Yeh NL, Wu TK, et al. Wound Healing in Streptozotocin-Induced Diabetic Rats Using Atmospheric-Pressure Argon Plasma Jet. Sci Rep. 2018;8(1):1–15. doi: 10.1038/s41598-018-30597-1.
43. Furman BL. Streptozotocin-Induced Diabetic Models in Mice and Rats. Curr Protoc Pharmacol. 2015;70(1):5.47.1-5.47.20. doi: 10.1002/0471141755.ph0547s70.
44. Karina K, Biben JA, Ekaputri K, Rosadi I, Rosliana I, Afini I, et al. *In vivo* study of wound healing processes in Sprague-Dawley model using human mesenchymal stem cells and platelet-rich plasma. Biomed Res Ther. 2021;8(4):4315–23. doi: 10.15419/bmrat.v8i4.670
45. Chen L, Mirza R, Kwon Y, DiPietro LA, Koh TJ. The murine excisional wound model: Contraction revisited. Wound Repair Regen. 2015;23(6):874–7. doi: 10.1111/wrr.12338.
46. Jahandideh M, Hajimehdipoor H, Mortazavi SA, Dehpour A, Hassanzadeh G. Evaluation of the wound healing activity of a traditional compound herbal product using rat excision wound model. Iran J Pharm Res. 2017;16(April 2015):153–63.
47. Tsalamandris S, Antonopoulos AS, Oikonomou E, Papamikroulis GA, Vogiatzi G, Papaioannou S, et al. The role of inflammation in diabetes: Current concepts and future perspectives. Eur Cardiol Rev . 2019;14(1):50–9. doi: 10.15420/ecr.2018.33.1.
48. Oryan A, Alemzadeh E, Moshiri A. Potential role of propolis in wound healing: Biological properties and therapeutic activities. Biomed Pharmacother. 2018;98(December 2017):469–83. doi: 10.1016/j.biopha.2017.12.069.
49. Vigneswari S, Murugaiyah V, Kaur G, Abdul Khalil HPS, Amirul AA. Simultaneous dual syringe electrospinning system using benign solvent to fabricate nanofibrous P(3HB-co-4HB)/collagen peptides construct as potential leave-on wound dressing. Mater Sci Eng C. 2016;66:147–55. doi: 10.1016/j.msec.2016.03.102.
50. Lima JM De, Sarmento RR, Souza JR De, Brayner FA, Feitosa APS, Padilha R, et al. Evaluation of hemagglutination activity of chitosan nanoparticles using human erythrocytes. Biomed Res Int. 2015;2015:3–8. doi: 10.1155/2015/247965.