KEMENTERIAN PENDIDIKAN MALAYSIA

SINGLE DISCIPLINARY PROJECT

APPLICATION FORM
SKIM GERAN PENYELIDIKAN FUNDAMENTAL (FRGS)

(Pindaan 1/2012)

JABATAN PENDIDIKAN TINGGI
KEMENTERIAN PENDIDIKAN MALAYSIA

A. Application Details

Application ID	291983-329281
Reference Code	FRGS/1/2019/SKK06/USM/03/6
A(i). Selected Grant	FRGS 2019-1
A(ii). Title Of Proposed Research Project	Understanding and elucidating the cellular mechanism of oral and topical administration of stingless bee honey (SBH) in promoting wound healing in streptozotocin-induced diabetic rat wound models
A(iii). Keyword	Stingless bee honey, diabetic wound rat models, inflammation, oxidative stress

B. Details of Project Leader

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B(xii). Type of Service (Permanent/Contract)	Permanent

C. Research Information

C(i). Research Domain

Research Domain	Sub Research Domain
Clinical and Health Sciences	Health Science

C(ii). Research Cluster

Cluster: Health

C(iii). National Priority Area

Priority Area: Healthcare and Medicine

C(iv). Location of Research

Location
Animal Research Sevice Centre, USM, Health Campus, Kubang Kerian, Kelantan
Department of Chemical Pathology, Department of Pathology, School of Medical Sciences, USM, Kubang Kerian, Kelantan

C(v). Duration of this research

From	September/2019
To	August/2022
Duration	3 years

C(vi). Other Researchers

Researcher Id	Name	IC / Passport Number	Faculty/ School/ Centre/ Unit	Position	Area of Expertise
37170	Wan Amir Nizam bin Wan Ahmad	700506035591	Universiti Sains Malaysia	Senior Lecturer (Dr)	Pharmacodynamics study and natural products pharmacology
48444	Kuttulebbai Nainamohamed Salam Sirajudeen	660602755069	Universiti Sains Malaysia	Professor (Lecturer)	Oxidative stress &Antioxidants; Hypertension; Excitotoxicity & Honey research
53042	Wan Faiziah Binti Wan Abdul Rahman	770328036228	Universiti Sains Malaysia	Senior Lecturer (DR)	breast and endocrine pathology
61701	Tengku Ahmad Damitri Al Astani	800714035981	Universiti Sains Malaysia	Lecturer (Dr)	Molecular Pathology, in vivo & in vitro study on Breast cancer & type II diabetic, Science education

C(vii). Research projects that have been completed or ongoing by project leader for the last three years

Title	Grant Name	Role	Progress (%)	Status	Duration	Start Date	End Date
Determination of serum 25 hydroxycholecalciferol and vitamin D receptor expression among breast cancer patient in HUSM	Incentive Grant	Project Leader	N/A	In Progress	2 years	31/01/2018	30/01/2020
Identification of miRNA as potential biomarker for early detection of breast cancer patients in HUSM	Research University Grant (RUI)	Member	N/A	In Progress	2 years	15/02/2017	14/02/2019
EVALUATION OF VITAMIN D LEVEL AND ITS CORRELATION WITH BONE MINERAL DENSITY BY DUAL ENERGY X-RAY ABSORPTIOMETRY (DEXA) SCAN AMONG MALAY ADULT IN KOTA BHARU, KELANTAN	No grants	Project Leader	N/A	Complete - KPI Achieved	2 years	15/12/2016	14/12/2018
Study on Vitamin D Status and Determination of	Geran Jangka	Project Leader	N/A	Complete - KPI	2 years	01/02/2015	30/01/2017

Optimum Vitamin D Level Based on Bone Turnover Markers in Kota Bharu, Kelantan	Pendek			Achieved			

C(viii). Academic publications that has been published by the project leader for the last five years

Title	Name of Journal	Year
Pre-operative serum total bilirubin level as an inidicator marker of perforated appendicitis	Bangladesh Journal Of Medical Sciences	2019
Hemolyzed Specimens: Major Challenge for Identifying and Rejecting Specimens in Clinical Laboratories	Oman Medical Journal	2019
DETERMINATION OF REQUIRED VITAMIN D LEVEL FOR BONE HEALTH BASED ON BONE TURN OVER MARKERS	The Korean Journal of Clinical Laboratory Science	2017
MULTIPLE ENDOCRINOLOGIC COMPLICATIONS IN THALASSEMIA MAJOR	The Korean Journal of Clinical Laboratory Science	2017
Serum magnesium levels patients with Type 2 diabetes mellitus: comparisons between good and poor glycaemic control	Brunei International Medical Jurnal	2015

C(ix). Executive Summary of Research Proposal

(Please include the problem statement, objectives, research methodology, expected output/outcomes/implication, and significance of output from the research project)

Different types of honey have been investigated for their wound healing properties. Unfortunately, research on stingless bee honey (SBH) addressing the mechanism of wound healing potential is limited. Despite many randomized trials which provide evidence with the effectiveness of honey in diabetic wound healing, the mechanism on how honey works are not well addressed. The healing of the wound is also associated with good glycemic control and the role of honey in controlling glucose level in animal and human study are still a matter of debate. Does the oral administration and topical treatment of honey provide a better systemic environment for diabetic wound healing?

In this study, using two types of diabetic wound (incision and punch biopsy models) in STZ induced diabetic rats, we are aiming to determine cellular mechanism of wound healing with topical (2%) and oral (1g/kg body weight) administration of SBH, influencing the macroscopic (wound contraction percentage), biochemical changes, Total Antioxidant Status(TAS); apoptotic and angiogenesis activity; Inflammatory markers- Interleukins (IL)-6,4,10 and Tumor Necrosis Factor-alpha (TNF-α)] in the serum/granulation tissues and histological changes of wound healing (by light microscopy) in the granulation tissue collected after sacrifice of the rats ie. at the end of 12 days of experiment period in which wound contraction percentage will be observed on day 1,6th and 12th.

The SBH perhaps has good wound healing potential and can be used as a treatment strategy in the management of diabetic wound in our local and international setting. The novelty of this study is the synergistic effect of SBH on diabetic wound healing through oral which is systematic effect and topical treatment that will be enhanced the wound healing process. SBH is widely cultivated in Malaysia and can be a product that will help boost the country's economy.

C(x). Detail Planning

(a) Research background

1. Problem Statement

Diabetes is one of the leading causes of impaired wound healing. The healing processes of diabetic wounds are notoriously slow and it is often associated with infection that leads to tissue necrosis and end-up with amputation. Limb amputation is associated not only with significant morbidity and mortality but also with major psychosocial and financial consequences to the family and country. With the advance of wound healing management, the reported healing rate is increasing. However, over a quarter of diabetic wound failing to heal. Honey from different parts of the world is potential natural products to improve healing process of the diabetic wound through its antioxidants effect, anti-inflammatory and antimicrobial activity. However, there were contradictory finding between clinical trials regarding the efficacy of honey on healing potential.

The stingless bee honey (SBH) has been widely used across time and space. The distinctive feature of this honey is that it is stored naturally in the pot (cerumen) which contains an anti-inflammatory agent such as IL-10 that inhibits synthesis of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, thus contributing to its beneficial properties especially in the wound healing process. Healing of the wound involved 4 overlapping systematic phases including inflammatory phase which is very important to activate the immune response.

However, if the inflammatory response is elongated and exacerbated, the wound healing process will be impaired and affect the healing rate and scar formation. Therefore, anti-inflammatory agents are required to reduce or prevent elongation and exaggeration of the inflammatory phase.

SBH is easier to be managed than other types of honey and its production can be a national economic contributor if it is proven to have significant wound healing properties. However, the wound healing potential by SBH is not well establish since limited research conducted using SBH in vivo as well as in vitro. In addition, the special feature of SBH should prove further at the cellular level. To add further debate, the topical use of honey has been used in a clinical trial, comparing with usual dressing used, however, the mechanism of the action needs to be further investigated. Yet, no study has been done to focus on the combined effect of SBH and metformin that (synergistically) enhanced the wound healing potential.

2. Hypothesis

SBH administration will improve wound healing rate and influences biochemical and histological changes in STZ induced diabetic wound rat groups compared to non-diabetic untreated control

3. Research Questions

1. How SBH administration in STZ induced diabetic wound rats group will improve wound healing rate and outcome compared to non-diabetic untreated control?

2. How SBH administration in STZ induced diabetic wound rats group will influence anti-inflammatory and antioxidant effect, and histological changes on wound healing compared to non-diabetic untreated control?

3. What is the mechanism of action of SBH in promoting diabetic wound healing in STZ induced wound rats model?

4. Literature Reviews

4.1 Alarming incidence of diabetic wound and knee amputation

The prevalence of diabetes in Malaysia is more than double since 1996 till 2015 based on national studies (3). The major concern in diabetes is related to increase its macro- and microvascular complications. Diabetic wound and its consequences are an important cause of morbidity and mortality in diabetes (4). It is often associated with infection that leads to tissue necrosis and end-up with amputation. The number of patients expected to have complications of diabetic wound estimated by consensus group of Ministry of Health (MOH) clinicians Malaysia is 5-15% (5).

Diabetic patients complicated with diabetic wound, 58% of them presented with infected wound at initial presentation (6) and have a 50-fold increased risk of hospitalization and 150-fold increased risk of lower-extremity amputation compared with patients with diabetes and no foot infection (7). In 2001, University of Malaya Medical Centre, Kuala Lumpur reported a total of 13% diabetic patients admitted within 5 days study period and amputation were the common surgical procedure performed in these patients (8). In Kelantan, 66% patients of total amputated patients within 2 years review were diabetic and associated with lower limb amputation (9).Epidemiological reports indicate that over one million amputations are performed on people with diabetes each year. This amounts to a leg being lost to diabetes somewhere in the world every 30 seconds. But the latest prevalence data of 2011 means that nowadays globally every 20 seconds a lower leg is lost due to diabetes (IWGDF, 2011) (10) .Lower-limb amputation is associated not only with significant morbidity and mortality but also with major psychosocial and financial consequences (11). A person that had undergone a lower limb amputation will be greatly affected as they have lost the ability to mobilise and be independent.

4.2 Current problem in managing wound healing using standard care

Diabetic wound is one of great challenge to wound care professional and consume a great deal of healthcare resources around the globe. Diabetic foot problems are among the most serious and costly complications of diabetes. It is well recognized that diabetic wound are notoriously hard to heal. The healing time and risk of amputation are known to vary markedly based on patient and ulcer factors such as infection, ischemia, ulcer size, and ulcer duration, as well as more difficult to quantify extrinsic factors such as the standard of foot care provided and patient adherence to prescribed foot ulcer care (12).
The normal process of inflammation following injury in diabetic patients and neuropathic foot ulcers are disorganized and is believed to contribute to their vulnerability and poor healing wound. An impaired immune response, bacterial burden, and/or ischemia, can interfere with this physiological process and result in a non-healing wound (13). With the advance of current management and evidence-based practice guideline of diabetic wound, the reported healing rates are approximately 66-77%. However, over a quarter of diabetic wound failing to heal (1). Why this occurs is not certain.

Wound care plays a pivotal role in the management of diabetic wound. Although topical treatment is an important aspect of wound care, it is always considered secondary to surgical and systemic care. There are numerous topical regimens and devices available for the management of diabetic wounds including hydrogels, hydrocolloids, alginates, foam, silver impregnated dressings, growth factors, silicon impregnated atraumatic

dressings, vacuum aided devices, hyperbaric oxygen therapy, etc. (14) However, before choosing a regime one should consider factors such as the general health of the patient, the process of tissue repair, assessment of the wound by means of grading, description and classification of the wound, local environment of the wound, knowledge on specific properties of the dressing materials and devices as well as their availability, affordability, and accessibility.

4.3 Wound healing process in diabetic condition

Although often difficult to treat, an understanding of the underlying pathophysiology and specific attention toward managing these perturbations can often lead to successful healing. The process of wound healing is achieved through four temporarily and spatially overlapping phases: hemostasis, inflammation, proliferation, and remodeling phases(15). During hemostasis and inflammation phases, various cytokines are released, recruiting fibroblast, endothelial cells, immune cells, neutrophils and macrophages in initiating wound healing, prevent bacterial contamination and prepare suitable environment for proliferation and remodeling phase (16). The challenges in managing diabetic wounds occur when those cells releasing excessive levels of pro-inflammatory cytokines, proteases, ROS, strong oxidants and senescent cells, as well as the existence of persistent infection which amplifying the inflammation cycle, damaging the extracellular matrix proteins and surrounding tissues, thus delaying the wound healing rate (17).

4.4 Role of honey in wound healing process (topical/local administration versus oral/systemic administration)

Over many years, honeys have been shown to be one of the highest potential natural products, served as potent antioxidants (18). The antioxidants found in honey will reduced the ROS and inflammations (19) and fight against infections at the wound site (20), thus aid in the healing process. It has been reported that topical application of honey associate with scarless healing in cavity wound, and showed less edema, fewer polymorphonuclear and mononuclear cell infiltrations, less necrosis, better wound contraction, improved epithelialization, and lower glycosaminoglycan and proteoglycan concentrations (21). Honey stimulates tissue growth, synthesis of collagen, and development of new blood vessels in the bed of wounds (22).

There have been reported many randomized and clinical control trials which provide considerable evidences indicating the effectiveness of honey in diabetic wound healing. Those studies focusing on topical usage of the honey on wound and comparing with regular dressing such as silver impregnated dressing, povidone iodine, ethoxy-diaminoacridine plus nitrofurazone dressing, iodine/hydrogen peroxide dressing and normal saline dressing(23). The mechanism on how the honey works in promoting wound healing were not addressed well and limited study conducted in vivo to understand the exact mechanism, thus requiring fundamental research to determine the underlying mechanism.

Oral administration of honey was reported to produce lower glycemic response in both diabetic and non-diabetic rabbits (24, 25). However, oral administration of honey for management of diabetic wound to induce better systemic environment which promoting healing process is not well studied base on our reading. Furthermore, there is scarcity evidence from clinical studies on the effect of honey on glycemic control among diabetic patients. The hypoglycemic or health beneficial effects of honey might be dose-dependent and further research is required

4.5 Stingless bee honey

Stingless bee honey is a precious bee product of the stingless bee. The distinctive feature of this honey is that it is stored naturally in the pot (cerumen) which influenced the honey quality by infiltration of phytochemicals from the cerumen. Anti-inflammatory mechanism in the cerumen inhibits the 5-LOX enzyme that is responsible for the synthesis of proinflammatory cytokines (26). MARDI have revealed protocatechuic acid (PCA) and 4-hydroxyphenylacetic acid as major free phenolic acid in stingless bee honey (27). PCA is a strong antioxidant and 4-hydroxyphenylacetic acid is a scavenger of ROS and nitrogen species (28). These special features of stingless bee honey contributing to its beneficial properties, especially in the wound healing process.

Many studies revealed benefit of honey in managing diabetic wound. However, very limited studies conducted in vivo to investigate the mechanism on how the honey influences the healing process. Therefore the aim of this study is to understand the mechanism of wound healing, influenced by stingless bee honey consumptions.

4.6 Biological Parameters: Involvement of inflammatory, anti-inflammatory, oxidant, antioxidant, reactive oxygen species (ROS) and growth factor during diabetic wound healing process

Healing is a systematic process, explained in terms of 4 overlapping phases: hemostasis, inflammation, proliferation, and maturation. Platelets play a crucial role in clot formation during hemostasis that started within hours after injury. Platelets facilitate the formation of a hemostatic plug and secrete platelet derived growth factor (PDGF), which attracts and activates macrophages and fibroblasts. At the cellular level, monocytes infiltrate the wound site and become activated macrophages that release growth factors, such as PDGF and vascular endothelial growth factor (VEGF), which initiate the formation of granulation tissue. Epithelialization, fibroplasia, and angiogenesis occur during the proliferative phase which started within 1 to 2 days after injury. Meanwhile, granulation tissue forms and the wound begin to contract, usually after day 4 of injury. Finally, during the maturation phase, collagen forms tight cross-links to other collagen and with protein molecules, increasing the

tensile strength of the scar.

The inflammatory response following tissue injury plays important roles both in normal and pathological healing (29). Honey significantly increased the inflammatory markers such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 release from monocytes which activate the immune response to infection (30). If the inflammatory response is elongated or exacerbated, it leads to a delay in the subsequent phases of proper wound healing and scar formation (31). Production of anti-inflammatory agents to suppress pro-inflammatory cytokines such as IL-10 is thus required to reduce this inflammatory phase.

Low levels of antioxidants accompanied by raised levels of markers of free radical damage play a significant role in the delay of wound healing. In diabetic rats, reduced glutathione levels had a role in delaying the healing process (24, 32). Hydrogen peroxide is one of the mediators of healing responses. It has been assumed that the antibacterial activity of honey is due to H2O2 (24). H2O2 is an oxidizing agent released by the action of the enzyme oxidase that is added by bees to nectar.

5. Relevance to Goverment Policy (if any)

Health Public Policies - Wellness Policy (Non Communicable diseases).

Potential biological activities of SBH may create a new therapeutic choice from the current honey and represent an interesting advance in the search for promising applications in the pharmaceutical industry for the wound healing area.

(b) References

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(c) Objective (s) of the Research

General objective:

To investigate the cellular mechanism of oral and topical administration of SBH in promoting wound healing in STZ induced diabetic wound (skin incision and punch biopsy) rat model.

Specific objective

1. To assess the wound healing rate and outcome of SBH (topical / oral) administration in STZ induced diabetic wound rat groups when compared to untreated non-diabetic control.

2. To determine the wound healing mechanism of SBH (topical / oral) administration by assessing the biochemical parameters (fasting blood glucose, pro and anti-inflammatory markers, oxidative stress) in STZ induced diabetic wound rat groups compared to untreated non-diabetic control.

3. To assess the mechanism of action of SBH (topical / oral) administration using microscopic and immunohistochemical (apoptosis, inflammatory and angiogenesis markers) studies in STZ induced diabetic wound rat groups compared to untreated non-diabetic control.

(d) Methodology:

1. Description of Methodology

Experimental animals and experimental design:

Eight-weeks-old male Sprague-Dawley rats (200 - 250 g) will be used as experimental animal in this study and a total of 98 male rats will be obtained from the Animal Research and Service Center (ARASC), Health Campus USM, Kota Bharu, Kelantan, Malaysia. Animals will be individually housed in a well-ventilated room maintained at 21 ± 2 oC under a 12-h light/dark cycle and will have free access to drinking water and food pellets ad libitum.

The animals will be acclimatized for a week period before starting the experiments. The ethical approval for this proposed study will be obtained from the Institutional Animal Ethic Committee, Universiti Sains Malaysia (USM) . All procedures performed in this study will be in accordance with the Institutional Guidelines for the Care and Use of Animals for Scientific Purposes.

The rats will be randomly divided into seven groups with n=12/group as follows:

1. Group 1: Control rats (untreated non diabetic)
2. Group 2: Diabetic rats (without any treatment)
3. Group 3: Diabetic rats + oral SBH
4. Group 4: Diabetic rats + topical SBH
5. Group 5: Diabetic rats + oral metformin
6. Group 6: Diabetic rats + oral metformin + oral SBH
7. Group 7: Diabetic rats +oral metformin + topical SBH

From each group 6 rats will be used for incision wound study and another 6 rats for punch biopsy wound study.

Induction of diabetes:

Group 2 to Group 7 rats will be weighed before induction of diabetes. After an overnight fast, diabetes will be induced by intraperitoneal administration of Streptozotocin (50 mg/kg BW dissolved in normal saline) using single injection. Three days after Streptozotocin injection, development of diabetes will be confirmed by measuring glucose level in fasting blood samples taken from tail vein. Glucose measurement will be performed with an Accu-Chek glucometer (Roche, Germany). Rats with blood glucose concentration of 8.0 mmol/L or higher will be considered as diabetic and included in this study (M Sheykhzade, GT. Dalsgaard, T Johansen & NCB Nyborg (2000) The effect of long-term streptozotocin-induced diabetes on contractile and relaxation responses of coronary arteries: selective attenuation of CGRP-induced relaxations. British Journal of Pharmacology, 129: 1212 - 1218).

Wound healing study:

The following procedure will be carried out on the above group of both and control and diabetic rats.
The rats will be anaesthetized with intraperitoneal sodium phenobarbital 50 mg/kg bodyweight before surgical positioning for full thickness wound (6 mm diameter) creation by punch biopsy and 3 cm incisional wound on the dorsum. The rats will be kept back into the cage, so that the rats will not become agitated. Anesthesia will take effect after 5–10 min. The rat's tail will be pinched to make sure that whether the rats are fully anesthesized. If there is no reaction, then the rats will be confirmed to be anesthetized and then the following wound healing study will be carried out.

Excision wound model:

The dorsal site of the rats will be fully shaved by an electric razor. This is important to allow precise removal of skin areas from the backs of the rats and, moreover, easy handling on the collection of skin wound biopsy

specimens. The dorsal area will be swabbed with 70% alcohol and after that scrubbed with iodine to disinfect the skin and remove the dust out. All the process will be carried out under sterile condition. Excisional wounds will be created using standardized sterile biopsy punch about 6mm circle diameter.

Resutured Incisional Wound Model:

In the anaesthetized rats, two paravertebral long incisions of 2.5 cm length will be made through the skin and cutaneous muscles at a distance of about 1.5 cm from midline on either side of the vertebral column using a sharp scalpel. After complete haemostasis, the wounds will be closed by interrupted sutures with the surgical nylon thread and curved needle No.11, 0.5 cm apart. [J.S. Reddy et al, J. of Ethanopharmacology 79 (2002) 249-251.]

Preparation of honey and Metformin:

Honey will be obtained from Lembaga Pemasaran Pertanian Persekutuan (FAMA) Kelantan and freshly prepared with distilled water (1.0 g/kg body weight) and metformin (250 mg/kg body weight) will be dissolved in distilled water just before oral administration on each day. 2% of topical honey will be prepared by diluting 2g of SBH (pure) in 100ml distilled water

Animal treatment:

The wound in group 1 and group 2 will be dressed daily with a normal saline solution until the end of experimental period (12 days). Then, group 3 to group 7 will be treated daily with the respective drug for the period of 12 days as follow: Group 3 will be administered with stingless bee honey (SBH) (1g/kg bodyweight) by oral gavage. Then group 4 wound will be topically applied with SBH (2%). Group 5 will be treated with oral metformin (250mg/kg BW). Group 6 will be treated with oral metformin (250mgh/kg BW) and topically applied with SBH (2%). Group 7 will be treated with metformin (250mg/kg BW) and SBH (1g/kg BW) orally.

The dosage of honey at 1.0 g/body weight was selected based on the previous studies that demonstrated the protective effect of honey in an animal model od diabetic (1), menopause (2) and excitotoxicity (3).

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Macroscopic analysis (on 1, 6 & 12th day) by measuring wound contraction diameter:

On day 1, 6 and 12, the rats will be measured for wound contraction percentage according to the formula as below:
% wound contraction = (Ao – At) x 100
Ao
Ao = original wound area
At = area of wound at the time of measurement

All the rats will be sacrificed at the end of the experimental perid on day 12 after macroscopic observation using guillotine. Blood samples will be collected in sodium oxalate tube for measurement of fasting plasma glucose as well as in plain gel tube. Then serum/plasma samples will be separated from the blood and stored at −80 °C until measurement of biochemical parameters. The granulation tissue will be collected from the wound edges of the rats for the biochemical and histological studies.
The granulation tissues will be homogenized with Phosphate buffer and the serum/tissue homogenates will be used for the biochemical (MDA, TAS and inflammatory markers) assays.

Biochemical study:

1.Fasting plasma glucose
The glucose will be measured in Architect AU800 base on hexokinase (HK) principle. Glucose is phosphorylated by HK in the presence of adenosine triphosphate (ATP) and magnesium ions to produce glucose-6-phosphate (G-6-P) and adenosine diphosphate (ADP). Glucose-6-phosphate dehydrogenase (G-6-PDH) specifically oxidizes G-6-P to 6-phosphogluconate with the concurrent reduction of nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide reduced (NADH). One micromole of NADH is produced for each micromole of glucose consumed. The NADH produced absorbs light at 340 nm and can be detected spectrophotometrically as an increased absorbance.

2.Malondialdehyde (MDA)
The oxidative stress marker MDA will be estimated using Abcam's kits according to the manufacturer instructions. The MDA in the sample reacts with thiobarbituric acid (TBA) to generate an MDA-TBA adduct. The MDA-TBA adduct can be easily quantified spectrophotometrically at a wavelength of 532nm and expressed as nmol/mg protein.

3.Total Anti-oxidant Status (TAS)
TAS will be will be estimated using Abcam's kits according to the manufacturer instructions. Cu2+ ion is converted to Cu+ by both small molecule and protein. The Protein Mask prevents Cu2+ reduction by protein, enabling the analysis of only the small molecule antioxidants. The reduced Cu+ ion is chelated with a colorimetric probe giving a broad absorbance peak around 570 nm, proportional to the total antioxidant capacity and expressed as nmol/mg protein

4.Inflammatory markers:
The pro-inflammatory markers IL-6 and TNF-α will be measured with kits following immunoassay principle using Cobas e601.

The anti-inflammatory markers such as IL-10 and IL-4 will be measured using Rat ELISA (Enzyme-Linked Immunosorbent Assay) kits according to manufacturer instructions. In general, these assays involves an antibody specific for Rat inflammatory cytokines coated on a 96- well plate. Standards and samples will be pipetted into the wells and the cytokines present in a sample is bound to the wells by the immobilized antibody. The wells will be washed and biotinylated anti-Rat antibody of respective cytokines will be added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin will be pipetted to the wells. The wells will be again washed, a tetramethylbenzidine (TMB) substrate solution will be added to the wells and color develops in proportion to the amount of respective cytokines bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm in a microplate reader and expressed as pg/mL or ng/mL or ng/mg protein.

Histological study:

The tissues will be fixed in 10% buffered formalin for the microscopic studies. The fixed tissue will be processed, embedded in paraffin wax and sectioned (3-5 µm). These sections will be stained with hematoxylin and eosin as well as Masson's trichome staining. Histological analysis of the tissue will be carried out using light microscopy to visualize and semi-quantitate the histomorphological features of wound healing (epithelization, inflammatory cells, fibroblasts proliferation, new vessels formation and collagen organisation) (Gal et al 2008).
Gal, P., Kilik, R., Mokry, M., Vidinsky, B., Vasilenko, T., Mozes, S., Bobrov, N., Tomori, Z., Bober, J. and Lenhardt, L. (2008) Simple method of open skin wound healing model in corticosteroid-treated and diabetic rats: standardization of semi-quantitative and quantitative histological assessments. Veterinarni Medicina., 53 (12): 652-659.
Standard procedure of Immunohistochemistry (IHC) will be adopted for the markers of apoptosis (Bax, BCL-2), angiogenesis (VEGF, BEGF) and inflammation (TNF-α).

Statistical analysis:

Data will be analysed by using two-way ANOVA and Student's t-test to determine significant differences among different groups. $P < 0.05$ were considered statistically significant.

2. Flow Chart of Research Activities — Flow Chart SBH.pdf

3. Research Activities

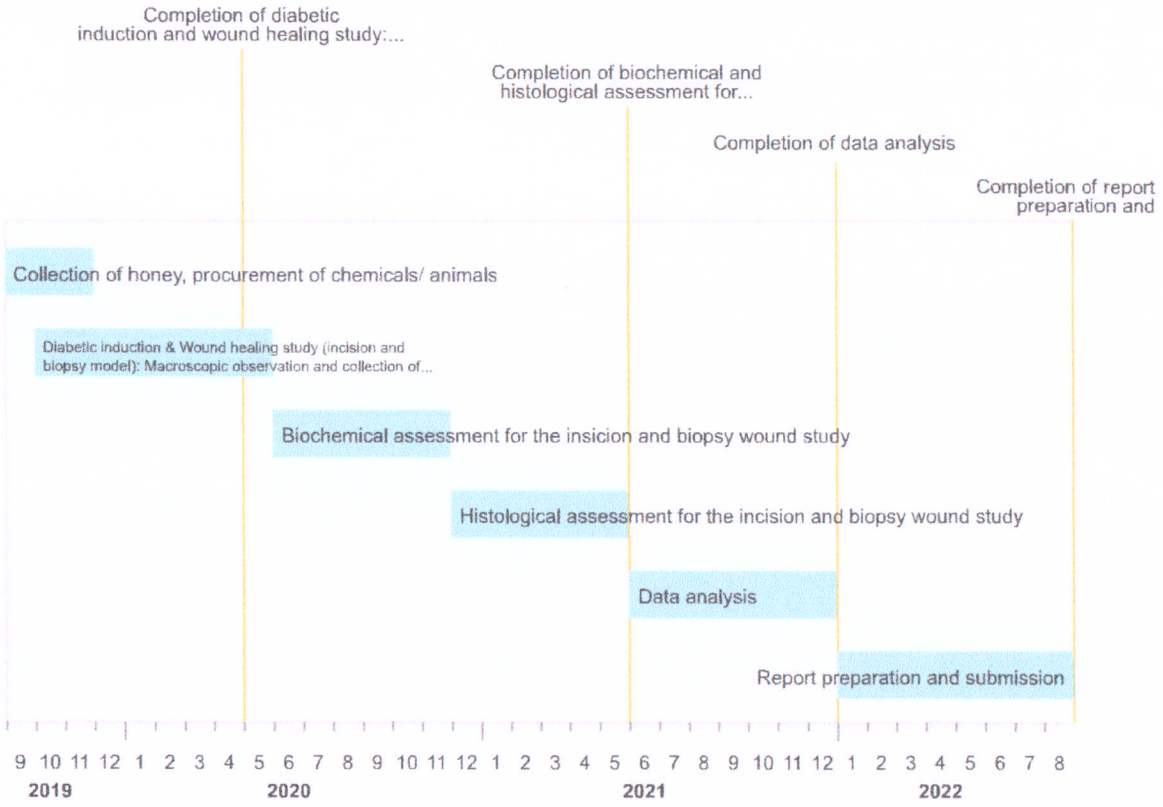
Activity	Start Date	End Date
Collection of honey, procurement of chemicals/ animals	01/09/2019	30/11/2019
Diabetic induction & Wound healing study (incision and biopsy model): Macroscopic observation and collection of tissue/serum samples for analysis	01/10/2019	31/05/2020
Biochemical assessment for the insicion and biopsy wound study	01/06/2020	30/11/2020
Histological assessment for the incision and biopsy wound study	01/12/2020	31/05/2021
Data analysis	01/06/2021	31/12/2021
Report preparation and submission	01/01/2022	31/08/2022

4. Milestones and Dates

Description	Date	Cumulative Project

		Completion Percentage(%)
Completion of diabetic induction and wound healing study: Macroscopic observation and collection of tissue/ serum samples for biochemical and histologic assessment	30/04/2020	45
Completion of biochemical and histological assessment for the wound study	31/05/2021	65
Completion of data analysis	31/12/2021	85
Completion of report preparation and submission	31/08/2022	100

Gantt Chart of Research Activities with Milestones



(e) Expected Results/Benefit

1. Novel theories/New findings/Knowledge

This study will provide better understanding and further insights into the cellular mechanisms of wound healing using oral/ topical SBH in different types of diabetic wounds (incision and punch biopsy wound).

2. Research Publications

1.Evidence based complementary and alternative medicine journal. Hindawi publication. (Q2 journal)
2.BMC complementary and alternative medicine journal. (Q1 journal)

3. Specific or Potential Applications

The scientific evidence from this study will illuminate further understanding and innovations in the management of wounds beneficial to patients with diabetic wound, full thickness burns wound injury or when surgical intervention is contradicted.

Total Number of Applications: 1

4. Number of PhD and Masters (by research) Students

Number of PhD Students:

1

Number of Masters (by research) Students:

Remark (if any):

5. Impact on Society, Economy and Nation

Impact on:

1.Society: This study will reveal scientific discoveries for wound management especially to meet the bottom billion population for a cost-effective, easily available , sustainable natural product.

2.Economy: As a sustainable, evidence based natural product from the cultured stingless bee honey, it will enhance local/international market of that honey and reduce the increased expenditure due to diabetic wound burden.

3.Nation- Generate wellness for people and next generation

6. Intellectual Property(IP)

Nil

Total Number of IP: 0

D. Access to Equipment & Material

Type	Description	Owner	Location	Address
Cobas e 601	For analysis of IL-6 and TNF-alpha	Endocrine Unit, Chemical Pathology Department	Chemical Pathology Department	School of Medical Sciences
ELISA reader	For analysis of anti-inflammatory markers and anti oxidants	Research lab	Chemical Pathology Department	School of Medical Sciences
Microscope	For histological analysis	Pathology	Pathology department	School of Medical Sciences
ARASC, Health Campus, USM, Kubang Kerian	wound healing animal study	Health Campus, USM	Health Campus, USM	Health Campus, USM

E. Budget

Budget Type	Description	Year 1	Year 2	Year 3	Grand Total
11000 - Salary and Wages	GRA for PhD: RM1800 x 12 months x 2 years = RM43200 Year 3 (RM1800 X 7=14400)	21600	21600	12600	**55800**
Vot-Total		**21600**	**21600**	**12600**	**55800**
21000 - Travelling and Transportation **Local**	a. Attending conference MSPP conference 2019 -air ticket for supervisor and 2 students (KL-KB-KL) RM 150X3X3= RM1350 -Hotel fees for supervisor and students: RM 180X3X3= Rm1620 -Subsistence: RM60X3X3=RM540 -Taxi= RM 100		2820		**2820**
Sub-Total		**0**	**2820**	**0**	**2820**
Overseas	b. Overseas 50th International Scientific Conference on Sport, Medical and Health Sciences at UAE December 2021 -Return flight KB-KL-Bali= RM3800 -3 days conference accommodation= RM800X3=RM2400 -subsistence= RM100 X 3= RM300			7000	**7000**
Sub-Total		**0**	**0**	**7000**	**7000**
Field work					**0**
Sub-Total		**0**	**0**	**0**	**0**
Vot-Total		**0**	**2820**	**7000**	**9820**
24000 - Rental					**0**
Vot-Total		**0**	**0**	**0**	**0**
27000 - Research Materials and Supplies	Animals: Rats: 98 X RM40= RM 3920 Rat's bed and feed: RM1000 Oral feeding tools: RM1000	5920			**5920**
	Wound creation tools: i. Punch biopsy= RM1000 ii. Blades=RM560 iii. Verniar caliper =RM500	2560			**2560**
	Measuring tools for analysis of: 1.Oxidatve stress markers i. Total Antioxidant status (TAS) Assay kit: 80 test/kits (80 test x 3 + (standard)		12300		**12300**

Total 3 kits = rm 2100 x3= rm 6300 ii. Malondialdehyde (MDA) Assay kit: 80 test/kits (80 test x 3 + (standard) Total 3 kits = rm 2000 x3 = rm 6000				
Measuring tools for analysis of: Pro-Inflammatory markers i. Il-6 (Elecsys) assay kit: 100 tests/kit = RM 5000 IL-6 Elecsys Calset= RM950 ii. Tumour Necrosis Alpha-Factor (TNF-α) (Elecsys) assay kit: 100 tests/kits = RM5000 Calset Elecsys= RM810		11740		**11740**
Measuring tools for analysis of: Anti-inflammatory markers i. IL-10 (ELISA) assay kit: 80 test/kits (80 test x 3 + (standard) Total 3 kits = RM3000 X 3= RM9000 ii. IL-4 (ELISA) assay kit: 80 test/kits (80 test x 3 + (standard) Total 3 kits = RM3000 X 3= RM9000		18000		**18000**
Biochemical test: plasma glucose=98 X RM3= RM294	294			**294**
Glucometer (Accu-Chek glucometer (Roche, Germany). = RM120 glucometer strips: RM220	340			**340**
5. Histological test i. immuhistochemical study 1. Apoptotic markers Bax antibody = RM1500 Bcl-2 antibody = RM 1640 2. Angiogenesis & inflammatory markers VEGF antibody = RM2500 TNF-□ antibody = RM2500 3. Silanized Glass Slide 100pk = RM300 4. Dako REAL™ Peroxidase-Blocking Solution 250 ml 1S202386 = RM 986.00 5. Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+,Rb/Mo kit 1K500711 = RM 4,534.00 6. Tris-Buffered Saline (TBS)	24285			**24285**

	(For 6 x 1 L) 6 x 1 L 1S300130 975.00 Total: RM16,524 ii. Histological study (staining) -RTU Hematoxylin = RM 1800 -RTU eosin = RM1700 -Xylene = RM1500 -Alcohol = RM 350 -RTU Masson trichrome = RM2500 -glass slide = RM500 Total : RM 8350				
	Consumable: Plain gel tube=RM650 Disposable glove=RM150 Pippete tips= RM450 Syringe= RM300 Alcohol swab= RM50 Bullet tube=RM 150 Microtube storage box= RM 400 Sample storage box= RM641	2941			**2941**
Vot-Total		**36340**	**42040**	**0**	**78380**
28000 - Maintenance and Minor Repair Services	For maintenance of ELISA reader		1500		**1500**
Vot-Total		**0**	**1500**	**0**	**1500**
29000 - Professional Services			1500	1000	**2500**
Services/Consultancy	Registration fee for local conferences				
	Registration fee for international conferences			3000	**3000**
	Publications fee in indexed journals			7000	**7000**
	English editing fee for manuscript (proofreading)			1000	**1000**
Sub-Total		**0**	**1500**	**12000**	**13500**
Short term course	For the student to attend animal handling workshop	500			**500**
Sub-Total		**500**	**0**	**0**	**500**
Vot-Total		**500**	**1500**	**12000**	**14000**
35000 - Accessories and Equipment					**0**
Vot-Total		**0**	**0**	**0**	**0**
Grand Total		**58440**	**69460**	**31600**	**(100.00%) 159500**

F. Declaration

- [x] 1. All information stated here are accurate, KPM and IPT has right to reject or to cancel the offer without prior notice if there is any inaccurate information given.
- [x] 2. Application of this research is presented for the Skim Geran Penyelidikan Fundamental (FRGS).
- [ ] 3. Application of this research is also presented for the other research grant/s (grant's name and total amount)
- [x] 4. Application of this research is subject to Ethical Committee approval.

Proof of Ethical Committee approval

- [ ] 5. Project Leader have ongoing Skim Geran Penyelidikan Fundamental (FRGS) project.

Name: Tuan Salwani Tuan Ismail Signature: [redacted]

Date: 11/03/2019

Appendix

Flow Chart	Flow Chart SBH.pdf

Appendix	Name	File Name
A	CV Dr Tuan Salwani	CV_wani.pdf
B	CV Dr Wan Amir	Dr Wan Amir CV-USM 2019.pdf
C	CV Dr Damitri	CV Damitri Jan 2019.pdf
D	CV Dr Wan Faiziah	update MARCH 2019 CV Dr Wan Faiziah (1).pdf
E	CV Prof Sirajudeen	CV-KNS Sirajudeen.pdf