EFFECTS OF STINGLESS BEE PROPOLIS ON OXIDATIVE STRESS AND STRUCTURAL INTEGRITY OF HEART IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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LIST OF ACRONYMS AND ABBREVIATIONS

β-MHC	beta myosin heavy chain
AGE	advanced glycation end products
AgRP	agouti gene-related peptide
ANOVA	analysis of variance
ARASC	animal research and service centre
ANF	atrial natriuretic factor
BCA	bicinchoninic acid
BMI	body mass index
BSA	bovine serum albumin
BW	body weight
CAT	catalase
CEL	N-ε-carboxy-ethyl-lysine
CI	colour index
CML	N-ε-carboxy-methyl-lysine
CVD	cardiovascular diseases
CVF	collagen volume fraction
cRAGE	cleaved receptor for advanced glycation end products
DCCT	diabetes control and complication trial
DiaMond	Diabetes Mondiale
DiCARE	diabetes in children and adolescent registry
DM	diabetes mellitus
DNA	deoxyribonucleic acid
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EEP	ethanolic extract of propolis

ELISA	enzyme-linked immunosorbent assay
esRAGE	endogenous secretory receptor for advanced glycation end products
EURODIAB	European Diabetes
FBG	fasting blood glucose
FFI	2-(2-Furoyl)-4(5)-(2-furanyl)-1H-imidazole
FI	food intake
HbA1c	haemoglobin A1c
IDF	International Diabetes Federation
IHME	Institute for Health Metrics and Evaluation
GLUT 2	glucose transporter 2
GOLD	glyoxal-lysine dimmer
GPRD	General Practice Research Database
GPx	glutathione peroxidase
GPx-1	glutathione peroxidase 1
H&E	hematoxylin and eosin
HDL	high density lipoprotein
HRP	horseradish peroxidase
HSV-1	herpes simplex virus 1
LDL	low density lipoprotein
MAPK	mitogen-activated protein kinase
MET-REMODEL	metformin and its effects on left ventricular hypertrophy in normotensive patients with coronary artery disease
MARDI	Malaysian Agricultural Research and Development Institute
MDA	malondialdehyde

MOLD	methylglyoxal-lysine dimer
MRSA	Methicillin-resistant *Staphylococcus aureus*
MT	Masson's trichrome
NF-κB	Nuclear Factor kappa light chain enhancer of activated B cells
NKEA	National Key Economic Area
NKRA	National Key Result Areas
NPY	neuropeptide Y
O^{2-}	superoxide anion
OD	optical density
PBS	phosphate buffer saline
PEPCK	phosphoenolpyruvate carboxykinase
PKC	protein kinase C
POMC	preproopiomelanocortin
PVCA/LA	perivascular collagen area to luminal area ratio
RCS	reactive chlorine species
RHQ	reinventing honey quality
RNS	reactive nitrogen species
ROS	reactive oxygen species
SEARCH	SEARCH for Diabetes in Youth
SOD	superoxide dismutase
SPSS	statistical package of social science
SRLS	Scottish Registry Linkage Study
sRAGE	soluble form of receptor for advanced glycation end products
STZ	streptozotocin
T&CM	traditional and complementary medicine

TBARS	thiobarbituric acid reactive substances
TGF-β	transforming growth factor beta
TGF-β1	transforming growth factor beta 1
UPS	ubiquitin-proteasome system
WI	water intake
WST-1	(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
XO	xanthine oxidase

LIST OF SYMBOLS

$\chi 2$	chi-square
µg/mL	microgram per milliliter
µg/mgprot	microgram per milligram of protein
µL	microliter
µm	micrometer
µmol	micromole
%	percent
◌°C	degree Celsius
®	registered trademark
™	trademark
dH2O	distilled water
g	gram
G	gauge
mg	milligram
mg/dL	milligram per deciliter
mg/kg	milligram per kilogram
mg/kg/day	milligram per kilogram per day
mgprot/mL	milligram of protein per milliliter
mL	milliliter
mm	millimeter
mmol/L	millimole per liter
mL	milliliter
nm	nanometer
ng/mgprot	nanogram per milligram of protein

ng/mL	nanogram per milliliter
pg/mgprot	picogram per milligram of protein
pg/mL	picogram per milliliter
rpm	revolutions per minute
U	unit
U/mgprot	unit per milligram of protein
UV	ultroviolet

KESAN PROPOLIS KELULUT KEPADA TEKANAN OKSIDATIF DAN INTEGRITI STRUKTUR JANTUNG KE ATAS TIKUS DIABETES TERARUH STREPTOZOTOSIN

ABSTRAK

Penyakit Diabetes mellitus (DM) merupakan penyakit tidak berjangkit yang sangat membimbangkan disebabkan impak sosio-ekonomi yang besar ke atas negara terutamanya Malaysia di mana prevalens mengatasi angka global. T1DM adalah sejenis penyakit metabolik kronik yang bercirikan hiperglisemia yang berterusan dan penghasilan tekanan oksidatif yang berlebihan yang menyebabkan diabetik kardiomiopati. Propolis kelulut dihasilkan daripada sebatian resin daripada getah pokok dan saliva kelutut yang kaya dengan kompaun fenolik. Ia mempunyai potensi sebagai antihiperglisemia, antioksida dan antiiskemia. Namun demikian, tiada penyelidikan terdahulu yang melaporkan kesan pengambilan propolis kelulut ke atas jantung pesakit DM. Jadi, kajian ini bertujuan untuk mengkaji kesan rawatan propolis kelulut terhadap stres oksidatif dan histopatologi jantung tikus DM aruhan streptozotocin. Sebatian polar diekstrak daripada propolis kelulut mentah melalui kaedah pengekstrakan etanol. Tikus jantan Sprague Dawley dibahagikan kepada lima kumpulan (n=8) iaitu normoglisemia (non-DM), diabetes tanpa rawatan (DM), diabetik dirawat dengan 300 mg/kg/day metformin (DM+Metformin), diabetik dirawat dengan 300 mg/kg/day propolis (DM+Propolis) dan diabetik dirawat dengan 300 mg/kg/day metformin dan 300 mg/kg/day propolis (DM+Combined) dan rawatan diberi sekali sehari. Satu dos mengandungi 60mg/kg streptozotocin diberikan melalui suntikan intraperitoneal untuk mengaruh DM jenis satu. Rawatan

diberi melalui gavaj oral selepas aruhan DM berjaya. Berat badan, glukosa darah semasa puasa, pengambilan air dan pengambilan makanan diambil setiap minggu. Selepas empat minggu, tikus akan dimatikan dengan 300 mg/kg sodium pentobarbital. Serum darah dan jantung dikumpul untuk menganalisis asai kalorimetri (penanda stres oksidatif dan enzim antioksidan) dan histopatologi. Tikus DM mengalami polidipsia, polifagia dan penyusutan berat badan disebabkan hiperglisemia. Jantung tikus DM menunjukkan perubahan ciri-ciri diabetik kardiomiopati seperti hipertrofi kardiomiosit, fibrosis dan fibrosis perivaskular. Pengambilan metformin atau propolis melindungi histopatologi dan perubahan biokimia yang berlaku semasa diabetik kardiomiopati. Gabungan metformin dan propolis juga menampakkan perubahan yang baik berbanding dengan metformin sahaja. Natijahnya, kajian mengenai propolis kelulut ini boleh memberikan hasil positif ke atas tikus diabetik kardiomiopati melalui sifat antihiperglisemia dan antioksidatif yang dikandungnya.

EFFECTS OF STINGLESS BEE PROPOLIS ON OXIDATIVE STRESS AND STRUCTURAL INTEGRITY OF HEART IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

ABSTRACT

Diabetes mellitus is a concerning non-communicable disease worldwide that has great socio-economic impact especially in Malaysia where the prevalence beats global figure. Type 1 diabetes mellitus is a chronic metabolic disorder characterised by persistent hyperglycaemia leading to overproduction of oxidative stress that causes diabetic cardiomyopathy. Stingless bee propolis is rich in phenolic compounds that is made of resins from plant exudates and stingless bee's saliva. It has antihyperglycaemia, antioxidative and antiischemic potential. Nevertheless, no previous study reported the effect of stingless bee propolis on diabetic heart. Thus, this study aims to determine the effect of supplementation of stingless bee propolis on oxidative stress and histopathology of heart in streptozotocin-induced diabetic rats. The polar antioxidative compounds was extracted from raw stingless bee propolis using ethanolic extract. Adult male Sprague Dawley rats was divided into five groups (n=8): normoglycaemia (non-DM), untreated diabetes mellitus (DM), diabetic treated with 300 mg/kg/day metformin (DM+Metformin), diabetic treated with 300 mg/kg/day propolis (DM+Propolis), diabetic treated with both 300 mg/kg/day metformin and 300 mg/kg/day propolis (DM+Combined) and treatment was given on daily basis. Single dose of 60mg/kg streptozotocin was administered intraperitoneally to induce type 1 diabetes mellitus. Treatment was given for four weeks duration following successful induction of diabetes mellitus via oral gavage.

Body weight, fasting blood glucose, water intake and food intake were taken every week. The rats were sacrificed after four weeks using 300 mg/kg of sodium pentobarbital. Serum and heart were collected for determination of colourimetric assays (oxidative stress markers and antioxidative enzymes) and histopathology. Diabetic rats experienced manifestation of hyperglycaemia such as polydipsia, polyphagia and weight loss. Their heart contains higher oxidative stress markers and alteration in antioxidative enzymes. Heart of diabetes mellitus rats showed features of diabetic cardiomyopathy including cardiomyocyte hypertrophy, cardiac fibrosis and perivascular fibrosis. Metformin or propolis supplementation reversed the clinical manifestation of diabetic mellitus but propolis alleviated histopathology and biochemical alteration of diabetic cardiomyopathy better than metformin. However, combination of metformin and propolis supplementation observed better improvement than metformin alone. In a nutshell, this study of stingless bee propolis managed to produce positive data on diabetic cardiomyopathy in rats through its antihyperglycaemic and antioxidative properties.

CHAPTER 1

INTRODUCTION

1.1 A Concerning Global Health Threat: Non-communicable Diseases

Amid era of globalisation, the life expectancy of population increased worldwide. From 1950 to 2017, life expectancy of men increased from 48.1 years to 70.5 years, whereas life expectancy of women gained from 52.9 years to 75.6 years. The improvement of life expectancy means greater emphasis on health care sector to provide sustainable health care goals. Researchers from the Institute for Health Metrics and Evaluation (IHME) came out with a systematic analysis for the global burden of disease study 2017. The statistic was very concerning. Non-communicable diseases occupied the base of the pyramid in worldwide mortality, accounting for 73.4% death in 2017. For just 10 years (2007-2017), they saw an increased of 22.7% deaths from non-communicable diseases, which translated to an additional 7.61 million deaths. The predicted percentage of mortality from non-communicable diseases was bound to climb in the future as the other causes of mortality such as communicable, maternal, neonatal and nutritional causes and accidental death decrease over time (Roth et al., 2018)

1.2 A Silent Killer: Diabetes Mellitus

According to World Health Statistic 2018, the top four contributors to non-communicable disease mortality include cardiovascular disease with 17.9 million deaths (44%), cancer with 9.0 million deaths (22%), chronic respiratory disease with 3.8 million deaths (9%) and diabetes mellitus with 1.6 million deaths (4%) (World Health Organization, 2018). Although diabetes mellitus accounts for only 4% of non-

communicable diseases mortality, it should not be overlooked. In Framingham study, diabetes mellitus was implicated as precursor of mortality and morbidity in cardiovascular diseases, subsequently listed as major risk factor for cardiovascular diseases (Kannel and McGee, 1979). Furthermore, a series of meta-analyses show that diabetes mellitus increased risk of cancers such as colorectal cancer, breast cancer, liver cancer, pancreatic cancer, bladder cancer and non-Hodgkin lymphoma (Vigneri et al., 2009).

1.3 Landscape of Diabetes Mellitus in the World and Malaysia

Diabetes mellitus is a complex metabolic disorder characterised by presence of persistent hyperglycemia due to defects in insulin secretion, defective insulin activity, insulin resistance or both (American Diabetes Association, 2017). The diagnosis of diabetes mellitus is by the presence of clinical symptoms such as polyuria, polydipsia or thirst, together with laboratory tests such as fasting plasma glucose ≥7.0 mmol/L or 2-hour plasma glucose ≥11.1 mmol/L or glycated haemoglobin A1c (HbA1c) >6.5 %. In the absence of clinical symptoms, repeated abnormal laboratory result is required to warrant diagnosis of diabetes mellitus (Clinical Practice Guideline Malaysia, 2015; Goldenberg and Punthakee, 2013).

Diabetes mellitus is a heterogenous disease and pathogenesis is not fully understood. However, studies identified several risk factors to diabetes mellitus. The risk factors for diabetes mellitus include modifiable and non-modifiable risk factors. The non-modifiable factors are family history of diabetes mellitus and ethnicity such as Asian, Hispanics and African American (Meigs et al., 2000; Shai et al., 2006). Modifiable

factors are obesity, lack of exercise, smoking and unhealthy diet (Menke et al., 2014; Reis et al., 2011).

Diabetes mellitus poses a major health threat worldwide, despite numerous measures to tackle this problem. The data from International Diabetes Federation (IDF) on global prevalence of diabetes mellitus is alarming, with estimated prevalence to rise in the coming years. The prevalence of diabetes mellitus is 151 million in 2000, 285 million in 2009 and 451 million in 2017 (1 in 11 adults)(Cho et al., 2018). This will lead to a huge global economic burden, with expenditure of USD 1.3 trillion in 2015 and expected a substantial increase to USD 2.1 trillion in 2030 (Bommer et al., 2018). The situation of diabetes mellitus in Malaysia is worse compared to global figure, one in five Malaysian adults has diabetes mellitus. Therefore, the health expenditure to manage diabetes and it's complication will be costlier to manage (Kadir Abu Bakar et al., 2015).

1.4 Cardiovascular Disease as a Major Complication of Diabetes Mellitus

Complications that arise from diabetes mellitus are common and responsible for significant morbidity and mortality. It is broadly categorised as microvascular and macrovascular complications with microvascular complication predominating the picture. Microvascular diseases are nephropathy, neuropathy and retinopathy, whereas macrovascular diseases include cardiovascular diseases (Papatheodorou et al., 2018).

Cardiovascular diseases (CVD) are the number one killer worldwide and Malaysia is not spared. Since early 1980s, Malaysia has been plagued with health issue from

cardiovascular diseases. The trend kept increasing over time and caught the attention of ministry of health of Malaysia. In 2010, National Strategic Plan was initiated to combat non-communicable diseases especially cardiovascular diseases. Integrated approach is the missing puzzle. The collaboration from dietitian to clinicians is required to tackle this seemingly uncontrollable situation (Clinical Practice Guidelines, 2017).

Cardiovascular diseases include two major categories which are vascular and heart diseases. Examples of vascular diseases are coronary artery disease, cerebrovascular disease, peripheral vascular disease and disease of aorta. Congenital heart disease, rheumatic heart disease, cardiomyopathy and cardiac arrhythmia are heart diseases (Mendis et al., 2011).

1.5 Cardiovascular Disease in Type 1 Diabetes Mellitus

Type 1 diabetes mellitus is an autoimmune disease mediated by T-lymphocytes, causing destruction in β-cell in pancreatic islets of Langerhans leading to hyperglycemia (American Diabetes Association., 2014). The global prevalence, incidence and trend of type 1 diabetes mellitus varies in geographical location worldwide, rendering small epidemiological studies inaccurate. Several large youth registry studies of type 1 diabetes mellitus such as World Health Organization Multinational Project for Childhood Diabetes (DIAMOND project) (Karvonen et al., 2000), EURODIAB study (Eurodiab Ace Study Group, 2000) and SEARCH for Diabetes in United States Youth study (SEARCH) (Liese et al., 2006). Both DIAMOND project and SEARCH study found out that highest prevalence of type 1 diabetes mellitus are younger age groups especially 10-14 years old. Regarding

temporal trends, all three studies reflected a rise in type 1 diabetes mellitus. DIAMOND, EURODIAB and SEARCH studies reported 2.8%, 2.3% and 3.4% annual rise in type 1 diabetes mellitus respectively. The cause is still unknown to researchers. The sparse data on adult type 1 diabetes mellitus impedes further research. However, type 1 diabetes mellitus is a lifelong condition, the rising number of youths living with diabetes mellitus will grow into adulthood as the treatment improves.

When compared to type 2 diabetes mellitus, type 1 diabetes mellitus has less data on cardiovascular disease. The increment of cardiovascular disease risk in diabetes mellitus is largely derived from cohorts of type 2 diabetes mellitus or undistinguished diabetes mellitus subtypes. Thus, the relation of type 1 diabetes mellitus and cardiovascular disease needs further clarity (De Ferranti et al., 2014). There are two large observational studies which are Scottish Registry Linkage Study (SRLS) and UK General Practice Research Database (GPRD) that demonstrated higher rates of cardiovascular diseases in type 1 diabetes mellitus as compared to general population (Livingstone et al., 2012; Soedamah-Muthu et al., 2006). Both studies reported large proportion of cardiovascular disease that derived from older age group when compared to younger age group. When the glucose control is strictly monitored and adhered in type 1 diabetes mellitus, the risk of cardiovascular disease falls 42% as shown in a landmark Diabetes Control and Complications Trial (DCCT) (Nathan et al., 2005).

In Malaysia, Diabetes in Children and Adolescent Registry (DiCARE) reported 69.2% of type 1 diabetes occurred in children and adolescents as opposed to 90%

worldwide. Majority of type 1 diabetes mellitus patients presented with diabetic ketoacidosis, with 64.7% recorded in Malaysia, 19.4% in Finland, 26.3% in Germany. The proportion of diabetic ketoacidosis in Malaysia is increasing in trend, implying poor awareness of glucose monitoring among public (Hong et al., 2015). Indeed, the glycaemic control of patients with type 1 diabetes mellitus in Malaysia recorded HbA1c of 9.46%, far from the optimal target of 7.5% (Lim et al., 2016). Poor glycaemic control, HbA1c > 9% is associated with increased total cholesterol, LDL cholesterol, triglyceride, reduced HDL cholesterol. Therefore, good glycaemic control is important to minimise risk of cardiovascular disease (Dobrovolskiene et al., 2013).

Only a few studies have reported risk of cardiac failure in type 1 diabetes mellitus. Swidish national diabetes registry found that incidence of cardiac failure is inversely related to glycaemic control. Both type 1 and 2 diabetes mellitus both adversely affect heart structure and function but the mechanism and underlying pathophysiology differs (Hölscher et al., 2016). Diastolic dysfunction is more common than systolic dysfunction in type 1 diabetes mellitus (Patil et al., 2011). However, several small-scale studies found no increased risk of heart failure in type 1 diabetes mellitus. It is noteworthy that patients in those studies are treated with insulin and will normalise blood glucose that attenuate the detrimental effects of metabolic derangement on heart (Hölscher et al., 2016).

1.6 Epidemiological Evidence of Diabetic Cardiomyopathy

Diabetes Mellitus is an atherogenic state leading to myocardial infarction and stroke. It was strongly related to microvascular disease and macrovascular disease. Before

1972, the increased cardiovascular mortality and morbidity in diabetic patients was thought to be only due to vasculature disease. In 1972, Rubler did a postmortem on four diabetic patients with congestive cardiac failure in the absence of coronary, hypertensive or valvular heart disease. The pathology findings of heart such as myocardial hypertrophy, fibrosis and perivascular fibrosis were noted. Rubler proposed a type of cardiomyopathy specific to diabetes mellitus. However, his opinion was refuted on few confounding factors surrounding study subjects. For instance, mitral regurgitation, anemia and renal insufficiency (Fein and Sonnenblick, 1985).

In 1974, Framingham study over 18 years involving 5209 people to determine the incidence of cardiac failure in relation to diabetes mellitus was published. Interestingly, after adjustment for all the confounding variables such as age, blood pressure, weight and serum cholesterol, diabetes mellitus patients have fourfold to fivefold increased risk of congestive cardiac failure (Kannel et al., 1974). Since then, multiple epidemiological studies support the concept of diabetic cardiomyopathy.

Diabetic cardiomyopathy can cause cardiac failure alone or accelerate cardiac failure in the presence of additional cardiac complications such as hypertension and coronary heart disease. A study done using ultrasound to screen diabetic patients for diabetic cardiomyopathy found that 40-60% of patients suffered some degree of diastolic dysfunction (Sharma and McNeill, 2006). Despite the high mortality and morbidity caused by diabetes-associated heart failure, the pathophysiology remains understudied (Russo and Frangogiannis, 2016).

1.7 Oxidative Hypothesis of Diabetic Cardiomyopathy

Prolonged hyperglycaemia promotes endogenous non-enzymatic formation of advanced glycation end products (AGE) known as Millard reaction. Receptor for AGE (RAGE) is a multiligand membrane bound receptor from immunoglobulin superfamily exhibited by numerous cell types. AGE can cross link membrane bound RAGE and causes activation of wide array of pro-oxidative and pro-inflammatory cascade, leading to generation of thiobarbituric acid reactive substances (TBARS) and activation of oxidative stress-sensitive NF-κB. (Goldin et al., 2006). There are two soluble forms of RAGE (sRAGE) which are endogenous secretory RAGE (esRAGE) and cleaved RAGE (cRAGE). esRAGE is formed by alternative splicing of RAGE gene within a cell and secreted into plasma. Whereas cRAGE is produced when metalloproteinases cleave the membrane bound RAGE. Both soluble variant of RAGE can act as decoy receptor to RAGE ligand such as AGE, reducing RAGE signaling (Daffu et al., 2013; Heier et al., 2015a; Tan et al., 2007).

Malondialdehyde (MDA) is formed from peroxidation of lipids (arachidonic, eicosapentaenoic and docosahexaenoic acid) due to oxidants or oxidative stress and accumulation of MDA in tissue and is associated with complications in diabetes mellitus (Negre-Salvayre et al., 2008). MDA classically reacts reversibly and irreversibly to protein and phospholipid causing profound damaging effect in cardiovascular system. In diabetes mellitus, the collagen is first modified by glycation process leading to series of lipid peroxidation to MDA. MDA and AGE both can form intermolecular cross link within protein and lipid. The damaging effect can be prevented through antioxidant therapy (Slatter et al., 2000).

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are first line antioxidative defense system in every cell that convert free radicals to stable and harmless compounds. Superoxide ions and singlet oxygen radical produced in cells will be converted to hydroxyl radical by SOD and then to water and oxygen by both CAT and GPx. Decrease in antioxidative defense system will lead to buildup of oxidative stress reflected by increased lipid peroxidation (MDA) (Ighodaro and Akinloye, 2018). In conclusion, disruption in metabolic and antioxidative function in diabetes mellitus will increase oxidative stress, causing diabetic cardiomyopathy (Jia et al., 2018).

1.8 Justification of Study

Natural products are rich in antioxidants such as polyphenols and flavonoids which can protect against cardiovascular diseases (Jia et al., 2018). Phytochemical analysis of Malaysian stingless bee propolis reveals flavonoids, polyphenols, terpenoids, resins, tannins, saponins and xanthoproteins (Nurhamizah Ibrahim et al., 2016 ; Usman et al., 2016). In addition, Malaysian stingless bee propolis confer antihyperglycaemic, antiinflammatory, antioxidative and cardioprotective benefits. A study of stingless bee propolis supplementation on myocardial infarcted rats was observed to improve antioxidant enzymes in cardiac tissue (Ahmed et al., 2017). However, the cardioprotective effect of Malaysian stingless bee propolis on diabetic heart has not been reported. Thus, this study is aims to determine the bioactivity of Malaysian stingless bee propolis specifically on diabetic cardiomyopathy.

Meliponiculture is seen as a potential area for Malaysia to achieve high income nation. Stingless beekeeping can generate huge agricultural income and relatively safe (Ismail. M & Ismail. W, 2018). World Health Organisation reported 50-80%

population of developing and developed countries utilised traditional and complementary medicine (T&CM). In line with WHO traditional medicine strategy 2014-2023 (Qi, 2013) and Malaysia's T&CM blueprint 2018-2027, the safety and efficacy of any T&CM product should be proven by research. In addition, National Key Result Areas (NKRA) and National Key Economic Area (NKEA) also emphasised on self-sustainable agro-food industry together with food security and food safety policy (Bakar et al., 2012). Therefore, current study conforms to the national policies and represent medical evidence for further development in meliponiculture industry.

1.9 Research Objectives

1.9.1 General Objective

To determine the effects of stingless bee propolis on oxidative stress and structural integrity of heart in streptozotocin-induced diabetic rats.

1.9.2 Specific Objectives

1) To determine the effect of stingless bee propolis on body weight, food and water intake of diabetic rats.
2) To determine the effect of stingless bee propolis on fasting blood glucose of diabetic rats.
3) To investigate the effect of stingless bee propolis on oxidative stress marker (MDA and AGE) and antioxidative defences (SOD, GPx, CAT, esRAGE) in heart homogenate and serum esRAGE.

4) To evaluate the effect of stingless bee propolis on histomorphological changes in heart of diabetic rats.

1.10 Research Hypothesis

Stingless bee propolis protects diabetic cardiomyopathy of streptozotocin-induced diabetic rats by its antioxidative properties.

CHAPTER 2

LITERATURE REVIEW

2.1 Meliponiculture or Apiculture: The Battle of Sustainability in Malaysian Beekeeping Industry

Meliponiculture refers to beekeeping with stingless bee comprising of tribe Meliponini (genus *Melipona* and *Trigona*), whereas apiculture is the beekeeping with stinger honeybee (genus *Apis*) (Razali et al., 2018). Beekeeping industry will continue to bloom globally, with honey as the main production and majority of honey originates from well-established apiculture. The honey from honey bee was traditionally regarded as better quality than honey from stingless bee, the reason being stingless bee honey contains higher water content favorable for fermentation (Chidi and Odo, 2017).

However, Malaysia apiculture was badly hit by colony collapsed disorder due to the *Varroa destructor* parasite mite outbreak in 1996. The imported *Apis Mellifera* bee was infected and spread to other honey bee. Malaysia had observed plunging honey production from 1996 to 2010 and it took nearly 14 years for the beekeeping industry to recover. In contrast, the meliponiculture has expanded over the years, without major disease outbreak within the stingless bee community (Ismail, 2016). This sparked researches on the ecology and behavior of stingless bees.

According to Malaysian Agricultural Research and Development Institute (MARDI), stingless bees with small and dimunitive figure than honeybee, can pollinate numerous crops including small-sized flowers, therefore environment conservation can be achieved. In addition, stingless bees are not chosey in forming a colony hive,

giving huge space for manipulation of artificial hive without jeopardising their ecology. Also, as the name implies, the stingless bees do not sting, so they poses no danger to the surroundings and make extraction of honey, pollen and propolis relatively easier and safer. Besides, honeybees often lost their way back to their colony when foraging as compared to stingless bees. Furthermore, stingless bees are more resistant to diseases from pests and parasites than honeybees (Chidi and Odo, 2017; Jalil et al., 2017).

Despite numerous benefits of meliponiculture, there are setbacks to it. In Malaysia, the production of stingless bee product is smaller per bee colony compared to honey bee product. This can be attributed to limited knowledge of beekeeper about stingless bees (Jaffé et al., 2015). The lack of knowledge also resulted in meliponiculture being less popular choice in beekeeping industry, stingless bee products with shorter shelf life and lower quality (Jalil et al., 2017). In response, Reinventing Honey Quality (RHQ) project was launched in 2012 to improve stingless beekeeping, with a vision of achieving world class stingless bee honey industry by addressing all issues surrounding meliponiculture (Mustafa et al., 2018). In sum, stingless beekeeping industry is a holistic approach to beekeeping in Malaysia which will also benefit socio-economies, survival of stingless bee species and long-term ecological conservation and preservation.

2.2 Biology of Stingless Bee

Stingless bees originate from Africa, then migrated to the north to Europe and North America and subsequently to Asia. They adapt well to tropical countries. There are nearly 500 species of stingless bees known around the globe. In Malaysia,

approximately 38 species of stingless bees identified, but only four species are involved in beekeeping industry including *Heterotrigona itama*, *Geniotrigona thoracica*, *Lepidotrigona terminata* and *Tetragonula leviceps* (Mustafa et al., 2018). Out of the four species, only two species (*Heterotrigona itama*, *Geniotrigona thoracica*) are most notable in Malaysia beekeeping industry (Hassan et al., 2018). Stingless bees had undergone evolutionary changes such as reduction in wing size and sting. Their flight range is shorter than honeybees, around 0.3 to 2 km from beehive depending on their size (Van Veen, 2014).

The caste of stingless bee consists of male and female bees. The male bees are called drones and their job is to mate with queen bees of other beehives. While female bees can differentiate into nurse bees and then worker bees or queen bees. In a beehive, the most nourished female bee will grow into queen bee. Others will differentiate into worker bees. Nurse bee are young worker bees and they are involved in provisioning, construction and cleaning of nests as well as feeding larvae and queen bee. The worker bees are responsible to search for pollen, nectar and plant resin. Pollen, nectar and plant resin can be processed by worker bees to honey, bee bread and propolis respectively. The foraged items by worker bees will be brought into nest to be reorganised into nest cavity. Water is collected more in hot environment to cool the nest cavity and for liquifying honey. Propolis or geopropolis or cerumen is a mixture of beewax and plant-based resins with the addition of processing by mandibular secretion to construct comb and lining of nest cavity (Van Veen, 2014; Wille, 2003).

2.3 History of Propolis

The term “propolis” is derived from ancient Greek writing: pro refers to “in front of” or “at the entrance of” and polis means “city” or “community”, in other words, propolis means substance for hive defense. Indeed, the bees know that there are diseases within their community will spread very fast as their hive is small. Propolis is their antibiotic, preventing bacteria, virus or parasite to lurk in beehive. It is used extensively, from lining of internal layer of nest cavity and reparative works in beehive to embalming dead intruders which are too big to be transported. Also, propolis functions to keep the beehive humid and cool (Bankova et al., 2000; Kuropatnicki et al., 2013; Toreti et al., 2013).

During ancient times, propolis was sold together with honey and became a commodity amongst Greeks, Romans, Persians and Jews due to the strong aroma. People often used propolis externally and consumption. Since then, propolis was found to have multiple medicinal benefit. Propolis can cure bruises, wounds, suppurative sores, ulcers, pain, tumor, eczema, myalgia, rheumatism. During medieval times, propolis disappeared from mainstream medicine. Fortunately, the knowledge of propolis survived in traditional folk medicine and was resurrected in European regions. Propolis was then called “penicillin” and made its way to herbal medicine. In 19th century, the modern research era, alcoholic extract of propolis was established. The development of research was extensive especially in chemistry to identify chemical composition of propolis (Bankova et al., 2000; Kuropatnicki et al., 2013).

A glamourous name “Dr Propolis” emerged in Demark in 1970 named Dr Karl Lund Aagaard who is a Danish biologist. He spent 20 years in propolis collection and research and found even broader benefit of propolis in addition to existing literature. The benefit of propolis extends to cancer, urinary tract infection, gout, sinus congestion, influenza, bronchitis, gastritis, ear diseases, intestinal infection, lung infection, headache, biliary infection, warts and conjunctivitis (Kuropatnicki et al., 2013). Such exhausting list sparks more researches until today. However, most of the researches done were from honey bee propolis. Stingless bee propolis is poorly studied and obviously less data available in the literature as compared to honey bee propolis (Sanches et al., 2017).

2.4 Bioactivity and Chemical Composition of Stingless Bee Propolis

Stingless bee propolis exhibits pharmacological potential such as antioxidant antimicrobial, anticancer, antiinflammatory properties (Sanches et al., 2017). Stingless bees explore different plants to yield propolis and the plants are located within their flight range. It is worth nothing that different species will prefer different plants for harvesting resin and secretion. The plants they foraged are still poorly understood. Therefore, the phytochemical compositions of propolis is largely influenced by bee species, location, vegetation and seasonal factors (Aleixo et al., 2017; Farnesi et al., 2009; Rushdi et al., 2014). When ethanolic extract of propolis from two main stingless bee species in Malaysian meliponiculture are compared, *Heterotrigona itama* propolis contains more chemical constituent than *G. thoracica* propolis. *H. itama* propolis consists of phenolic compounds, terpenoids, saponins, steroids, coumarins and essential oils but *G. thoracica* propolis lacks saponins, steroids and coumarins (Ismail et al., 2016; Nazir et al., 2018).

2.4.1 Stingless Bee Propolis as Natural Antioxidant

The antioxidant nature of stingless bee propolis can help in preventing diseases caused by oxidative stress. The free radical is converted to harmless compound by both enzymatic and non-enzymatic antioxidants. The presence of antioxidant in propolis is attributed to phenolic compounds and flavonoids (Lavinas et al., 2018). Most utilised laboratory methods to determine antioxidant property from propolis extract is free radical capturing DPPH (2,2-diphenyl-1-picrylhydrazyl). *H. itama* propolis showed higher antioxidative activity than *G. thoracica* propolis using DPPH scavenging method (Ismail et al., 2016).

2.4.2 Antimicrobial activity of Stingless Bee Propolis

Studies have reported antibacterial, antiviral and antifungi nature of stingless bee propolis and is a promising natural agent for infectious diseases. Stingless bee propolis possess bacteriostatic and bactericidal action against wide range of Gram positive and Gram negative bacterias. The bacterias implicated are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (MRSA), *Escherichia coli*, *Enterococcus faecalis*, *Proteus mirabilis* and *Klebsiella pneumonia* (Lavinas et al., 2018). The antibacterial effect of stingless bee propolis was stronger in Gram positive than Gram negative bacteria. *H. itama* propolis in Malaysia showed better antibacterial property than *G. thoracica* propolis in all 7 types of bacteria tested such as *Staphylococcus aureus*, *Bacillus subtilis*, *E. faecalis*, *Listeria monocytogen*, *Acinetobacter baumannii*, *Salmonella typhi* and *E. coli* (N. Ibrahim et al., 2016).

Stingless bee propolis can inhibit the growth of *Candida albicans* at a lower concentration and kill them at a higher concentration (Campos et al., 2014). The bioactive substance giving antifungi effect is unknown. Also, stingless bee propolis managed to act against herpes simplex virus 1 (HSV-1) in infected cell cultures. The concentration of virus that was reflected by the number of viral copies significantly fall by about 98% after treatment. C-glycosylflavones, catechin-3-O-gallate, and 3,4-dicaffeoylquinic acid are the main phenolic compounds that offer antiviral activity (Coelho et al., 2015).

2.4.3 Anticancer and Antiinflammatory Property of Stingless Bee Propolis

The anticancer activity of stingless bee propolis has been reported. Some of the cancer cell studied are glioblastoma, breast adenocarcinoma, oral squamous cell carcinoma and osteosarcoma. Stingless bee propolis managed to stop the progression cancer cells (Sanches et al., 2017). Furthermore, flavonoids in stingless bee propolis contains antiinflammatory property both *in vitro* and *in vivo* by immunomodulation of inflammatory mediators. For instance, induction of antiinflammatory cytokines and inhibition of proinflammatory cytokines (Campos et al., 2015).

2.5 Therapeutic Potential of Stingless Bee Propolis in Diabetic Cardiomyopathy

Regarding the bioactivity of stingless bee propolis that exhibited antidiabetic effect, six studies reported glucose lowering effect (Ismail et al., 2016; Mahani et al., 2013; Nna et al., 2018; Usman et al., 2017; Vongsak et al., 2015) and one study advocated its cardioprotective activity through antiischemic property in myocardial infarction (Ahmed et al., 2017). Stingless bee propolis from Indonesia managed to lower blood

glucose in mice with type 1 diabetes mellitus and comparable to insulin after two weeks of supplementation (Mahani et al., 2013). Besides, Malaysian stingless bee propolis showed antihyperglycaemic effect by increasing plasma insulin, reducing plasma glucagon and insulin resistance and restoring degenerated pancreatic beta cells (Nna et al., 2018; Usman et al., 2017). Synergistic glucose lowering effect of metformin and Malaysian stingless bee propolis was observed (Nna et al., 2018). Also, Malaysian and Thailand stingless bee propolis can inhibit α-glucosidase activity (Ismail et al., 2016; Nna et al., 2018; Vongsak et al., 2015). The hypothesis of insulin-like action was reported when reduction of blood glucose was observed after one hour of *H. Itama* propolis supplementation (Nna et al., 2018).

In myocardial infarcted rat model, stingless bee propolis was able to reduce oxidative stress, increase antioxidative enzymes, improve lipid profile and histopathological changes in heart tissue (Ahmed et al., 2017). Whereas diabetic cardiomyopathy is due to the join action of inflammation and oxidative stress. The antioxidative and antiinflammatory activity of stingless bee propolis provide a sturdy concept to study on the cardioprotective effect of stingless bee propolis through the combined antioxidative and antihyperglycaemic activity in diabetic rats.

2.6 Experimental Model of Type 1 Diabetes Mellitus

In 1880s, the first animal model of diabetes was a byproduct of experimental pancreatectomy procedure on a dog to study malabsorption of fat in gastrointestinal system done by Minkowski. The animal developed overt clinical diabetes with polyuria, polydipsia and weight loss (Rees and Alcolado, 2005). In 1920s, Banting and Macleod named the only dog that survived in their experiment “Marjorie” and

subsequently involved in the discovery of insulin. This discovery was a huge leap in physiology and medicine because before that, patients diagnosed with type 1 diabetes mellitus were only awaiting death. Banting and Macleod were awarded nobel prize for physiology or medicine in 1923 (Rees and Alcolado, 2005; Vecchio et al., 2018).

Over the years, several animal models were established with the aim getting to the root of diabetes mellitus. In accordance to the animal ethics principle, animal at the lowest rank on phylogenic scale should be used (Johnson and Besselsen, 2002). For instance, rodents should be used to replace dogs in any experimental procedures unless there is suitable reason to do otherwise. Besides that, there are a few methods to induce diabetes mellitus in animal models such as chemical, genetic alterations and viral induction of diabetes mellitus. Each method has been revised to cater for different experiments. The advantages and disadvantages of each method are simplified in the table below.

2.6.1 Methods of Type 1 Diabetes Mellitus Induction in Animal Models

Table 2.1 Methods to induce type 1 diabetes mellitus in Animal models

Method	Advantage	Disadvantage	Source
Chemical induced diabetes	Well established, simple and cheap model, selective loss of pancreatic beta cell, less mortality and easier to manage.	Possible spontaneous regeneration of beta cell, chemical is cytotoxic to other organs.	(Dufrane et al., 2006; Lee et al., 2010)
Spontaneous diabetic rodents	Destruction of pancreatic beta cells through autoimmune insulitis, mimics type 1 diabetes mellitus in human.	Sophisticated maintenance, expensive, require inbred and high mortality due to ketosis.	(Driver et al., 2011; King, 2012; Lenzen et al., 2001)
Surgery induced	Surgically remove pancreas, not cytotoxic to	Require surgery and postoperative	(Rodrigues, 2016)

diabetes	other organs.	procedure, technically cumbersome, digestive problem due to lack of digestive enzyme, higher mortality.	
Transgenic diabetic rodents	Spontaneous mutation of gene coding for proinsulin, leading to accumulation of misfolded protein in pancreatic beta cell, suitable to study islet transplantation.	Very sophisticated and expensive.	(Mathews et al., 2002)
Viral induced diabetes	Able to induce autoimmune destruction of beta cells, good for studying the pathogenesis of infection causing diabetes.	Not established, varying results, may prevent or cause diabetes.	(von Herrath et al., 2011; von Herrath et al., 1997)

2.6.2 Chemical Induction of Type 1 Diabetes Mellitus

Two most widely used pharmacological agents as chemical for induction of type 1 diabetes mellitus in animal models are alloxan and streptozotocin. Both are glucose analogues known to interfere with physiological function of pancreatic beta cell. Streptozotocin is preferred over alloxan. The reason being alloxan has a half-life of 1.5 minutes as compared to 15 minutes in streptozotocin, rendering it less suitable in in vivo setting (Islam and Code, 2017; Szkudelski, 2001). Also, streptozotocin-induced diabetic model has more sustained hyperglycemia with clinical signs of diabetes without ketosis, thereby reducing mortality (Islam and Code, 2017). Alloxan has more toxic effects to the other organs, mainly liver. Associated biochemical changes, morphological and ultrastructural changes are notable in alloxan-induced diabetes and again increases mortality among study subjects (Lucchesi et al., 2015).

2.6.3 Mechanism of Streptozotocin Action

Streptozotocin, 2-deoxy-2-(3-(methyl-3- nitrosoureido)-D-glucopyranose, is a derivative of *Streptomycetes achromogenes* and used as antimicrobial agent. The deoxy group within the chemical structure attached to glucose molecule represents highly reactive deoxyglucose and methylnitrosurea moieties, acting as powerful cytotoxic agent directed specifically towards pancreatic beta cells (Szkudelski, 2001). Streptozotocin recognises glucose transporter 2 (GLUT 2) receptors and pancreatic beta cells expresses high amount of GLUT 2 in contrast to liver and kidney that express lower amount of GLUT 2. Therefore, mild acute kidney and liver injury is highly reversible. For this reason, streptozotocin-induced diabetes offers good animal model for studying the chronic complications of hyperglycemia in many organs such as liver, kidney, brain, heart and muscle (Wu and Yan, 2015). Inside pancreatic beta cells, streptozotocin's main mechanism of induction of diabetes is in DNA alkylation. The transfer of methyl group from methylnitrosurea to DNA molecule initiate chain reaction of DNA fragmentation and destruction, leading to beta cell necrosis and lack of insulin production (Radenkovic et al., 2016).

2.6.4 Practical Application of Streptozotocin

Single dose with 50-60 mg/kg streptozotocin to induce type 1 diabetes mellitus in adult male Wistar rats resulted in clinical overt diabetic symptoms. Dosage more and less than the range of dosage resulted in death and inadequate hyperglycemia respectively. The mortality rate was 12.5-25% due to the acute hypoglycemia in within 6-24 hours of induction with streptozotocin. So, glucose solution in the first day is recommended to reduce mortality. Streptozotocin-induced type 1 diabetes mellitus in rats achieved stable hyperglycemia even after 17 weeks of induction

(Gajdosik et al., 1999). Therefore, streptozotocin provides a feasible framework for studying chronic complications of type 1 diabetes mellitus including heart.

2.6.5 Diabetic Cardiomyopathy in Streptozotocin-induced Diabetic Rat Model

Rodents are useful model in studying diabetic cardiomyopathy due to the resistive nature to atherosclerosis, effectively rule out coronary heart disease as confounding factor among study subjects. The first scientist who pioneered the method of using rodents in proving the existence of diabetic cardiomyopathy in type 1 diabetes mellitus was Dr. John McNeill. The comprehensive evidence covered decrease in contractile performance of working heart and isolated cardiomyocyte, associated biochemical changes and adrenergic dysregulation (Severson, 2004). Multiple studies managed to replicate the findings using type 1 and even type 2 diabetic rodent models (Boudina and Abel, 2007). Four weeks after induction of type 1 diabetes mellitus with streptozotocin lead to the features of diabetic cardiomyopathy such as cardiac hypertrophy, apoptosis, fibrosis and perivascular fibrosis in rats (Fiordaliso et al., 2000; Miric et al., 2001). Treatment given within two to four weeks after induction of type 1 diabetes mellitus can reverse pathological changes in heart such as cardiac and perivascular fibrosis and manifested as improvement in cardiac function (Miric et al., 2001).

2.7 Structural and Functional Phenotype in Diabetic Cardiomyopathy

There is no universally accepted definition of diabetic cardiomyopathy due to the challenges faced by scientists when dealing with complex pathophysiology. The general definition of diabetic cardiomyopathy is structural and functional abnormalities of myocardium in diabetic patients in the absence of hypertension or

coronary artery disease (Miki et al., 2013). Diabetic cardiomyopathy includes structural abnormalities such as cardiac hypertrophy, interstitial fibrosis and perivascular fibrosis and functional changes such as diastolic dysfunction and in late stage, systolic dysfunction. The most striking feature of diabetic cardiomyopathy is cardiac fibrosis including cardiac fibrosis and perivascular fibrosis. The histological changes such as cardiac hypertrophy and interstitial fibrosis can stiffen the heart ventricles and reduce compliance. In addition, cross-linking of collagen due to uncontrolled hyperglycemia will further impair cardiac elasticity, leading to diastolic dysfunction. (Sharma and McNeill, 2006).

2.7.1 Cardiac Hypertrophy

Cardiac hypertrophy is a non-specific phenomenon in reaction to stress. For instance, cardiac hypertrophy seen in athletes is the initial stage of physiological response to stress. On the other hand, pathological hypertrophy occurs when cell death, fibrosis, altered cardiomyocyte, mitochondrial dysfunction and inadequate angiogenesis in response to stress (Nakamura and Sadoshima, 2018). There is a link between progression of cardiac hypertrophy in diabetes mellitus and heart failure which is pathological. This can be attributed to cardiomyocyte hypertrophy, death and loss of function (Feng et al., 2010; Samak et al., 2016).

2.7.2 Interstitial and Perivascular Fibrosis

The architecture of myocardium is a complex network of tightly and well-arranged cardiomyocytes and extracellular matrix protein. The main component of extracellular matrix is collagen, with 85% of type 1 collagen offering greatest tensile strength and 11% of type III collagen giving elasticity of cardiac tissue. The