EFFECT OF HONEY FROM APIS BEE ON BLOOD GLUCOSE AND INSULIN SIGNALING PROTEINS IN SKELETAL MUSCLE OF STREPTOZOTOCIN-NICOTINAMIDE-INDUCED DIABETIC RATS

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KUALA LUMPUR

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Matric No: **MGN 160005**

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Title of Project Dissertation: **Effect of honey from *Apis* bee on blood glucose and insulin signalling proteins in skeletal muscle of streptozotocin-nicotinamide-induced diabetic rats**

Field of Study: **Physiology**

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ABSTRACT

Honey has been established as a potential anti-diabetic agent by lowering the blood glucose and protects against diabetes-induced damage on the vital organs. However, its effect on the skeletal muscle in diabetes is unknown. Hence, in this study, the effect of honey from *Apis* bee on the skeletal muscle expression of the insulin signalling proteins is investigated. Components of *Apis* bee honey were identified by liquid chromatography–mass spectrometry (LC-MS). Adult male rats were rendered diabetes via intraperitoneal injection of streptozotocin and nicotinamide (STZ-NA). Four days after STZ-NA injection *Apis* bee honey was administered orally by using oral gavage tube and confirmation of diabetes mellitus (T2DM). *Apis* bee honey treatment was given at 5 and 10 mg/kg bw once daily for 28 days. At the end of the *Apis* bee honey treatment, blood was withdrawn for plasma glucose analysis after overnight fasting (16 hours). Then rats were sacrificed via cervical dislocation and skeletal muscle was harvested for histology, immunohistochemistry (IHC)/ immunofluorescence (IF) and protein expression analysis by Western blotting (WB). flavonoids, phenolic acids, monosaccharides and fatty acids were present in the *Apis* bee honey.

The compounds present at highest abundance include (i) flavonoids: (epi)afzelechin-(epi)catechin and isorhamnetin 3-O-rutinoside, (ii) phenolic acids: hydroxybenzoic acid-O-hexoside and syringic acid-hexose and (iii) monosaccharide derivative: gluconic acid. *Apis* bee honey administration was found to lower the blood glucose level after 28 days in diabetic rats. Histological analysis indicates that *Apis* bee honey treatment ameliorated skeletal muscle pathological damage in diabetic rats where only minimal myofiber degeneration and mononuclear cellular infiltration were observed in skeletal muscle of *Apis* bee honey-treated diabetic rats.

In the skeletal muscle, *Apis* bee honey treatment enhanced the expression of insulin signalling molecules insulin receptor β (IRβ), insulin receptor substrate 1 (IRS1), phosphoinositide 3-kinase (PI3K), protein kinase B (Akt) and glucose transporter 4 (GLUT4) in diabetic rats. These findings implied that *Apis* bee honey treatment to diabetic rats might enhance the insulin signal transduction via IRβ/IRS1/PI3K/Akt pathway that can help to augment the GLUT4 expression, thereby improving the glucose uptake in the skeletal muscle of diabetic rats. These effects could either be directly or indirectly via improving the hyperglycemia in diabetic rats, where this needs to be further confirmed. It can also be postulated that phenolic compounds with antioxidant and anti-inflammatory properties in *Apis* bee honey, such as (epi)afzelechin-(epi)catechin and isorhamnetin 3-O-rutinoside, might be responsible for these effects. This study thus suggests the usefulness of *Apis* bee honey as an agent to manage diabetes.

Keywords: Honey; *Apis* bee; Diabetes mellitus; Insulin signalling proteins; Skeletal muscle.

KESAN MADU DARI LEBAH *APIS* PADA GLUKOSA DARAH DAN PROTEIN ISYARAT INSULIN DALAM OTOT RANGKA TIKUS DIABETES YANG DIINDUKSI STREPTOZOTOCIN-NIKOTINAMIDA

ABSTRAK

Madu telah ditubuhkan sebagai agen anti-diabetes berpotensi dengan menurunkan glukosa darah dan melindungi daripada kerosakan yang disebabkan oleh diabetes pada organ-organ penting. Walau bagaimanapun, kesannya terhadap otot rangka pada kencing manis tidak diketahui. Oleh itu, dalam kajian ini, kesan madu dari lebah *Apis* pada ekspresi otot rangka protein isyarat insulin diselidiki. Komponen madu telah dikenalpasti oleh kromatografi cecair-spektrometri massa (LC-MS). Kaedah: tikus jantan dewasa diberikan diabetes melalui suntikan intraperitoneal streptozotocin dan nikotinamide (STZ-NA). Madu diberikan secara oral gavage 4 hari selepas suntikan STZ-NA dan pengesahan diabetes mellitus (T2DM). Rawatan madu diberikan selama 28 hari. Pada akhir rawatan madu, darah telah diambil untuk analisa glukosa plasma 12 jam selepas puasa semalam. Kemudian tikus dikorbankan melalui dislokasi serviks dan otot rangka dituai untuk histologi, imunohistokimia (IHC)/ immunofluorescence (IF) dan analisa ekspresi protein oleh Western blotting (WB). Keputusan: flavonoid, asid fenolik, monosakarida dan asid lemak hadir dalam madu. Komponen yang terdapat pada kelimpahan tertinggi termasuk (i) flavonoid: (epi) afzelechin- (epi) catechin dan isorhamnetin 3-O-rutinoside, (ii) asid fenolik: hidroksibenzoik- derivatif monosakarida: asid glukonik.

Rawatan madu didapati menurunkan tahap glukosa darah selepas 28 hari dalam tikus diabetes. Analisis histologi menunjukkan bahawa rawatan madu melemahkan kerosakan patologi otot rangka pada tikus diabetes di mana hanya degenerasi myofiber dan penyusupan sel mononuklear minsimum diperhatikan dalam otot rangka tikus diabetes diberikan madu.

Dalam otot rangka, rawatan madu meningkatkan ekspresi molekul isyarat insulin insulin reseptor β (IRβ), substrat reseptor insulin 1 (IRS1), phosphoinositide 3-kinase (PI3K), Akt dan glukosa transporter 4 (GLUT4) dalam tikus diabetes. Kesimpulan: Penemuan ini menunjukkan bahawa rawatan madu untuk tikus diabetes boleh meningkatkan transduksi isyarat insulin melalui jalur IRβ/ IRS1/ PI3K/ Akt yang dapat membantu meningkatkan ekspresi GLUT4, sehingga meningkatkan pengambilan glukosa dalam otot rangka tikus diabetes. Kesan ini sama ada secara langsung atau tidak langsung melalui peningkatan hiperglikemia dalam tikus kencing manis, di mana ini perlu disahkan selanjutnya. Ia juga boleh dirumuskan bahawa sebatian fenolik dengan sifat antioksida dan anti-radang dalam madu, seperti (epi) afzelechin- (epi) catechin dan isorhamnetin 3-O-rutinoside, mungkin bertanggungjawab terhadap kesan ini. Kajian ini mencadangkan kegunaan madu sebagai agen untuk merawat diabetes.

Kata kunci: Madu, Lebah *Apis*, Diabetes melitus, Protein isyarat insulin, Otot rangka

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	:	Indication
Akt	:	Protein kinase B
ANOVA	:	Analysis of variance
APS	:	Ammonium persulphate
AS160	:	Akt substrate 160
ATF6	:	Activating transcription factor 6
BAX/Bcl-2/caspase-3	:	BCL2-associated X protein/B-cell lymphoma 2/caspase-3
BCA	:	Bicinchoninic acid
Bis	:	N,N'-methylene-bis-acrylamide
BMI	:	Body mass index
BSA	:	Bovine Serum Albumins
cAMP	:	Cyclic adenosine monophosphate
CAPN10	:	Calpain 10
COX-2	:	Cyclooxygenase-2
CRP	:	C-reactive protein
Cu^{+}	:	Cuprous ion
Cu^{2+}	:	Cupric ion
DAB	:	Diaminobenzidine
DC	:	Non-treated diabetic rat
dH_2O	:	Distilled water
DM	:	Diabetes mellitus
DPP-4	:	Dipeptidyl peptidase-4
ER	:	Endoplasmic reticulum
ESI	:	Electrospray ionization

FBG	:	Fasting blood glucose
FFA	:	Free fatty acid
FTO	:	Fat mass and obesity-associated
g	:	Gram
GLP1	:	Glucagon-like peptide 1
GLUT4	:	Glucose transporter 4
h	:	hour
HPLC	:	High performance liquid chromatography
HRP	:	Horseradish peroxidase
H_2O_2	:	Hydrogen peroxide
H&E	:	Hematoxylin and eosin
H1D	:	Low-dose honey treated diabetic rat
H2D	:	High-dose honey treated diabetic rat
H1N	:	Low-dose honey treated non-diabetic rat
H2N	:	High-dose honey treated non-diabetic rat
IF	:	Immunofluorescence
IHC	:	Immunohistochemistry
IKKβ	:	I kappa B kinase β
IL-6	:	Interleukin 6
iNOS	:	Inducible nitric oxide synthase
IR	:	Insulin receptor
IRS1	:	Insulin receptor substrate 1
JNK	:	c-Jun N-terminal kinase
KCNJ11	:	Potassium voltage-gated channel subfamily J member 11
kDa	:	Kilo Dalton
kg	:	Kilogram

L	:	Litre
LC-MS	:	Liquid chromatography–mass spectrometry
MCP-1	:	Monocyte-chemo-attractant protein-1
mg	:	Milligram
mg/kg/day	:	Milligram per kilogram per day
mg/ml	:	Milligram per millilitre
mins	:	Minsute
ml	:	Mililitre
mM	:	Millimolar
MS	:	Mass spectrometry
MW	:	Molecular weight
m/z	:	*Mass to charge*
NAD^+	:	Nicotinamide adenine dinucleotide
NC	:	Non-diabetic rat
NF-κB	:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGT	:	Normal glucose tolerant
NHMS	:	National Health and Morbidity Surveys
NO	:	Nitric oxide
PBS	:	Phosphate buffer saline
PBST	:	Phosphate buffer saline containing 0.1% Tween-20
PDX-1	:	Pancreatic duodenal homeobox 1
PGE2	:	Prostaglandin E2
pH	:	Logarithmic measure of hydrogen ion concentration
PI3K	:	Phosphoinositide 3-kinase
PKC	:	Protein kinase C

PPARγ	:	Peroxisome proliferator-activated receptor γ
PP2A	:	Protein phosphatase type 2A
PTEN	:	Phosphatase and tensin homolog
PVDF	:	Polyvinylidene difluoride
ROS	:	Reactive oxygen species
RT	:	Retention time
SD	:	Sprague-Dawley
SEM	:	Standard error of mean
SGLT2	:	Sodium/glucose co-transporter 2
SDS-PAGE	:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	:	Standard error of mean
Ser/Thr	:	Serine/threonine
SOCS3	:	Suppressor of cytokine signalling 3
STZ-NA	:	Streptozotocin and nicotinamide
TCF7L2	:	Transcription factor 7 like 2
TEMED	:	N,N,N',N'-tetraacetylethylenediamine
TNFα		Tumour necrosis factor-α
Tyr		Tyrosine
T1DM	:	Type 1 diabetes mellitus
T2DM	:	Type 2 diabetes mellitus
V	:	Voltage
WB	:	Western blot
µL	:	Microlitre
µm	:	Micrometre
nm	:	Nanometre

s	:	Second
ºC	:	Degree Celsius
%	:	Percent
3D	:	Three-dimensional

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Diabetes mellitus (DM) is a complex, chronic metabolic disease that affects 3.5 million Malaysian (Tee & Yap, 2017). International Diabetes Federation has estimated that 629 million people worldwide will suffer from DM in 2045 (Atlas, 2017). Obesity, environmental and genetic factors play a crucial role in the pathophysiology of DM (Ralph A. DeFronzo et al., 2015). In overt type 2 diabetes mellitus (T2DM) patients, the metabolic milieu is drastically altered and the constellation of complications, including hyperglycemia, hyperlipidemia, hyperinsulinemia and excessive cytokine synthesis, establish. These secondary factors cause insulin deficiency and insulin resistance which are widely manifested in the T2DM patients (Cersosimo, Triplitt, Solis-Herrera, Mandarino, & DeFronzo, 2018; Zheng, Ley, & Hu, 2018). Insulin resistance in skeletal muscle emerges as the major physiological defect associated with T2DM, particularly T2DM (Carnagarin, Dharmarajan, & Dass, 2015; DeFronzo & Tripathy, 2009), which is the area of concern in the current study.

Skeletal muscle serves as the predominant site of glucose uptake *in vivo* and is vital for whole body glucose homeostasis. During the postprandial state, 65 – 80% of whole-body insulin-stimulated glucose uptake is mediated in skeletal muscle (Berridge, Bolon, & Herman, 2018; Schiavon et al., 2013). However, T2DM impinges on the skeletal muscle health where the diabetic milieu perturbs the skeletal muscle insulin sensitivity and metabolism. In a T2DM milieu, lipotoxicity (Brons & Grunnet, 2017), glucotoxicity (Zhang et al., 2016), oxidative stress (Di Meo, Iossa, & Venditti, 2017), endoplasmic reticulum (ER) stress (Bohnert, McMillan, & Kumar, 2018) and low-grade systemic inflammation (Rehman & Akash, 2016) are most likely to trigger insulin resistance in skeletal muscle.

Physiologically, in skeletal muscle, binding of insulin to insulin receptor (IR) activates insulin receptor substrate 1 (IRS1), phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt), the key mediators of insulin transduction cascade. Subsequently, activated Akt stimulates the translocation of insulin sensitive glucose transporter 4 (GLUT4) to the sarcolemma, hence promoting the glucose uptake in skeletal muscle (Ferrannini & DeFronzo, 2015). On that account, skeletal muscle is a key insulin target tissue that harbours intrinsic defects that impinges upon whole body glucose homeostasis. In diabetic milieu, the insulin signal propagation via IRS1/ PI3K/ Akt/ GLUT4 is impaired, leading to defective glucose intake and thereby insulin resistance (Deshmukh, 2016; Gutierrez-Rodelo, Roura-Guiberna, & Olivares-Reyes, 2017). In fact, clinical and *in vivo* animal studies have proven that impaired insulin signalling pathway at receptor and/or post-receptor levels are accountable for the insulin resistance in skeletal muscle (Boucher, Kleinridders, & Kahn, 2014).

Among the therapies for T2DM, lifestyle changes and weight loss could help in suppressing hyperglycemia and hyperlipidemia as well as ameliorating insulin resistance (Kahn, Cooper, & Del Prato, 2014; Nomura, Kawae, Kataoka, & Ikeda, 2018). Depending on the severity of T2DM, anti-diabetic drugs are necessitated to govern the glycemic level and other associated metabolic derangements. However, undesirable adverse effects including hypoglycaemia, fluid retention and heart failure followed administration of these drugs, limiting the clinical application of oral anti-diabetic drugs (Tran et al., 2015b). On that account, the medical world is turning more and more on apicultural and natural products with anti-diabetic properties in the management of T2DM.

Honey, a natural substance produced by honey bees, possesses medicinal benefits which is universally acknowledged since time immemorial. With respect to honey bees, genus *Apis* encompasses 11 species where *Apis mellifera* is the most popular subspecies and serves as the source of most of the world's honey (Kek et al., 2014). Honey composition comprises more than 200 components, with fructose, glucose and water as the predominant constituents. In addition to carbohydrates, honey contains protein including enzymes, amino acids, vitamins and minerals, antioxidants such as catalase, peroxidase, alkaloids, polyphenols and flavonoids (El Sohaimy, Masry, & Shehata, 2015; Saranraj, Sivasakthi, & Dire Feliciano, 2016).

Generally, honey consists of variable compositions. These differences depend on floral sources, geographical origin, total phenolic content, water proportion and colour (Moniruzzaman, Khalil, Sulaiman, & Gan, 2013; Nayik et al., 2014). These compositional variations have been reported to influence the antioxidant properties and other therapeutic effects of honey in both *in vitro* and *in vivo* studies (Ahmed et al., 2018).

Honey as an emerging novel anti-diabetic agent has been recently recognised in clinical (Abdulrhman, 2016; Al-Waili et al., 2013; Bahrami et al., 2009) and *in vivo* animal studies (Aziz, Giribabu, Rao, & Salleh, 2017; Obia, Ogwa, Ojeka, Ajah, & Chuemere, 2016), proposing that honey, through its antioxidant (Ahmed et al., 2018; Moniruzzaman et al., 2013), anti-inflammatory (Hussein, Mohd Yusoff, Makpol, & Mohd Yusof, 2012; Kassim, Achoui, Mustafa, Mohd, & Yusoff, 2010), anti-microbial (Shehu et al., 2016; Tirado, Hudson, & Maldonado, 2014) and immunomodulating (Mijanur Rahman, Gan, & Khalil, 2014) effects might have the potential to act as a multi-targeted anti-diabetic agent in T2DM patients.

It is well-established that honey reduces hyperglycemia, ameliorates oxidative stress and exerts organ protective effects in pancreas, liver, adipose tissue and kidney of streptozotocin and nicotinamide (STZ-NA)-induced diabetic rats (Ahmed et al., 2018). Although honey shows promising results in treating T2DM, the effect of honey on skeletal muscle and its underlying mechanisms in the regulation of glycemia have yet to be elucidated. It is highly plausible that the anti-hyperglycemic effect of honey from *Apis* bees might be mediated partly via improving the insulin sensitivity in skeletal muscle by attenuating the defects in insulin signalling pathway. In this study, we hypothesize that:

i. *Apis* bee honey contains bioactive compounds with anti-diabetic effect.
ii. *Apis* bee honey protects against skeletal muscle degeneration in T2DM.
iii. *Apis* bee honey improves skeletal muscle insulin resistance in T2DM by enhancing the expression of protein molecules in the insulin signalling pathway.

1.1 Objectives

i. To identify the bioactive compounds in *Apis* bee honey using liquid chromatography–mass spectrometry (LC-MS).
ii. To investigate the effect of *Apis* bee honey treatment on skeletal muscle architecture in STZ-NA induced diabetic rats by using hematoxylin and eosin (H&E) staining.
iii. To investigate the effect of *Apis* bee honey treatment on expression levels of insulin signalling proteins in skeletal muscle of STZ-NA induced diabetic rats by using immunohistochemical, immunofluorescence and Western blot (WB) analyses.

1.2 Significance of study

Honey has been proven as a natural anti-diabetic agent in human and animals, yet there is no data available on the effect on honey particularly from *Apis* bees on skeletal muscle and its underlying mechanisms. Hence, this study could consolidate the current evidence regarding the claimed benefits of honey in T2DM management.

CHAPTER 2: LITERATURE REVIEW

2.1 Type 2 diabetes mellitus (T2DM)

2.1.1 Epidemiology of T2DM

T2DM is a chronic and progressive endocrine disorder characterized by dysregulation of carbohydrate, protein and lipid metabolisms resulting from impaired insulin secretion, insulin resistance or a combination of both (Ralph A. DeFronzo et al., 2015). The epidemic of T2DM and its complications poses a major global public health concern. Globally, International Diabetes Federation estimated that in 2017, 1 in 11 adults aged 20 – 70 years (425 million adults) worldwide had T2DM, with 79% of those impacted living in low- and middle-income countries. This estimate is projected to escalate to 629 million by 2045. Areas explicitly affected by T2DM are China and India, where the prevalence of T2DM has risen spectacularly despite the relatively low prevalence of obesity (Atlas, 2017).

In Malaysia, National Health and Morbidity Surveys (NHMS) have shown that the prevalence of T2DM rose by more than doubled from 1996 (6.9%) to 2015 (17.5%), there were over 3.5 million adults with DM (Figure 2.1). NHMS 2015 report indicated that there was a significant association between the prevalence of T2DM and ethnicity, where Indians have the highest prevalence (22.1%), followed by Malays (14.6%), Chinese (12%), other Bumiputras (10.7%) and et cetera (7.4%).

On the other hand, there was only minor difference in the prevalence of T2DM between males (16.7%) and females (18.3%), without significant association (Tee & Yap, 2017). This escalating trend is caused by factors including population growth, population aging, urbanization and rising prevalence of physical inactivity and obesity (Animaw & Seyoum, 2017; Wild, Roglic, Green, Sicree, & King, 2004).

The impact of DM on society is substantial. In addition to the costs of productivity losses and the loss of patients' quality of life, a large economic cost to the healthcare system with a base estimate of RM 2.04 billion per year is significant and most likely to be conservative (Mustapha et al., 2017). This high medical cost of managing DM underscores the importance of primary and secondary prevention of DM.

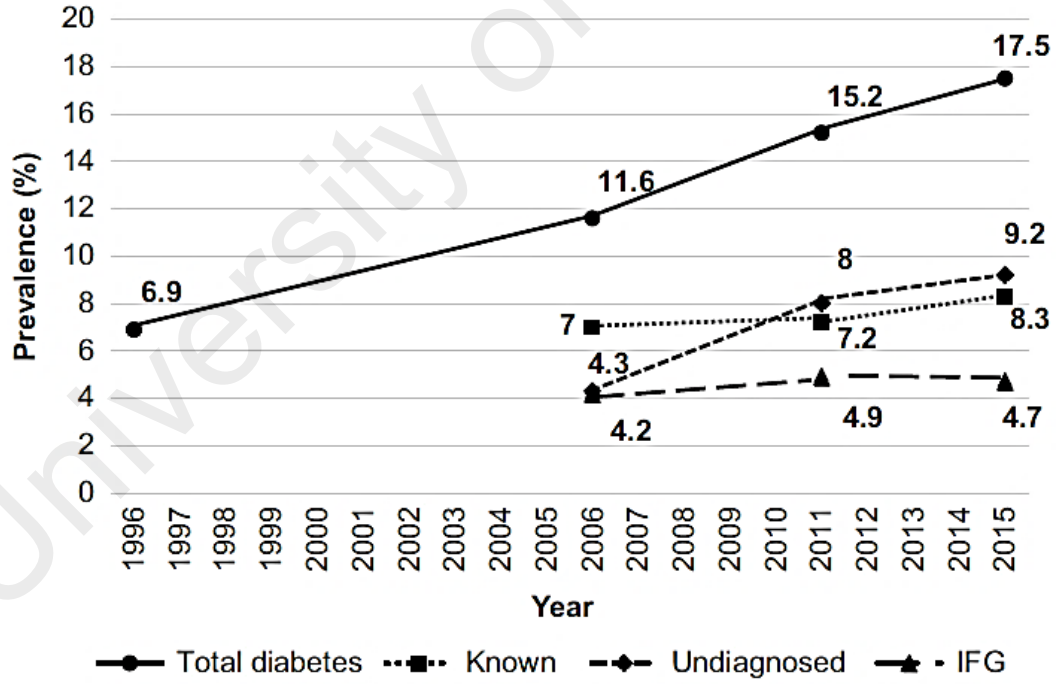

Figure 2.1: Trends in the prevalence of diabetes and impaired glucose tolerance (IFG) in Malaysia from NHMS reports for year 1996 – 2015 (Tee & Yap, 2017).

2.1.2 Pathophysiology of T2DM

In the postprandial state, the maintenance of whole-body glucose homeostasis depends on a normal insulin secretory response and normal tissue sensitivity to hyperinsulinemia and hyperglycemia. Successively, the combined effects of insulin and hyperglycemia in improving glucose disposal depend on the three closely coupled mechanism: (i) suppression of endogenous (primarily hepatic) glucose synthesis; (ii) stimulation of glucose uptake by splanchnic (hepatic plus gastrointestinal) tissues; and (iii) stimulation of glucose uptake by peripheral tissues, predominantly skeletal muscle (Cersosimo et al., 2018).

Over the past few decades, comprehension of the development and progression of T2DM has evolved promptly. The predominant cause of T2DM is gradual impairment of insulin secretion by pancreatic β-cells generally upon a background of antecedent insulin resistance in the skeletal muscle and liver (R. A. DeFronzo, 1988). In addition to the triumvirate of T2DM: pancreatic β-cells (decreased insulin secretion), skeletal muscle (decreased glucose uptake) and liver (increased hepatic glucose production), gastrointestinal tract (decreased incretin effect), adipose tissue (increased lipolysis), kidney (increased glucose reabsorption), brain (neurotransmitter dysfunction) and pancreatic α-cells (hyperglucagonemia), inclusively 8 prominent pathological abnormalities contribute to impaired glucose homeostasis (Defronzo, 2009). Moreover, impaired insulin-regulated vasodilation and activation of inflammation pathways are the recently identified pathological abnormalities that induce skeletal muscle insulin resistance. Collectively, these 10 physiological abnormalities contribute to hyperglycemia in T2DM (Figure 2.2) (Ralph A. DeFronzo et al., 2015).

The symptoms of T2DM include hyperglycemia, unexplained fatigue, blurred vision, polyphagia, polydipsia, polyuria and unexpected weight loss. In contrast to Type 1 diabetes mellitus (T1DM) in which the symptoms begin abruptly in a matter of weeks, T2DM develops gradually many years without causing any noticeable symptoms, hence many people find themselves with T2DM without abnormal symptoms.

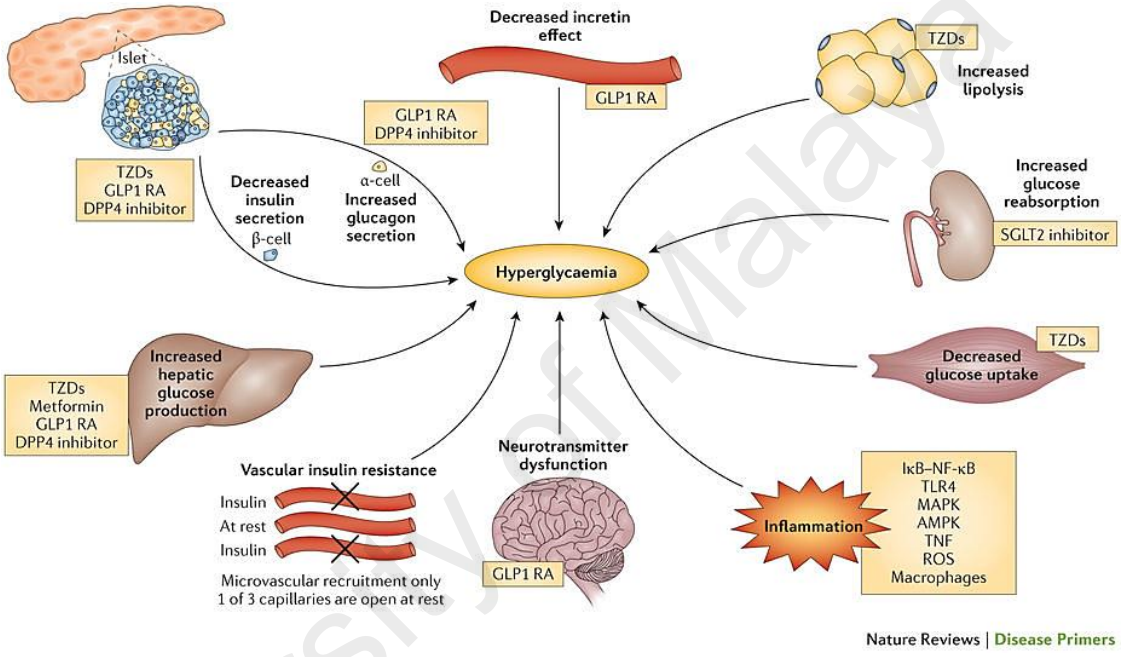

Figure 2.2: Pathophysiology of hyperglycemia in T2DM. The core defects in T2DM are blunt insulin secretion by pancreatic β-cells, insulin resistance in skeletal muscle and liver. Pancreatic β-cell resistance to glucagon-like peptide 1 (GLP1) results in dysfunctional pancreatic β-cell while enhanced glucagon level and hepatic sensitivity to glucagon causes excessive hepatic glucose synthesis. Adipocyte insulin resistance leads to augmented lipolysis and elevated plasma free fatty acid (FFA) levels which subsequently exacerbate insulin resistance in skeletal muscle and liver and promote pancreatic β-cell failure. Enhanced renal glucose reabsorption by the sodium/glucose co-transporter (SGLT2) and heightened threshold for glucose spillage in the urine aggravate hyperglycemia. Resistance to the appetite-suppressive effects of leptin, insulin, GLP1, peptide YY and amylin as well as brain dopamine and elevated brain serotonin levels promote weight gain, thereby aggravating the antecedent insulin resistance (Ralph A. DeFronzo et al., 2015).

2.1.3 Pathogenesis of T2DM

The aetiology of T2DM is multifactorial, involving genetic and environmental factors (Figure 2.3) (Zheng et al., 2018). The predominant feature of T2DM is the impaired insulin sensitivity. Increasing adiposity, as reflected by higher body mass index (BMI), is the single most important risk factor for T2DM. Increasing visceral fat augments fatty acid levels which subsequently results in an elevation of gluconeogenesis and glucose levels (Ma et al., 2018). The elevated glucose levels cause decompensation of pancreatic β-cells, compensation and eventually decompensation of these β-cells leads to impaired glucose tolerance and development of T2DM (C. Chen, C. M. Cohrs, J. Stertmann, R. Bozsak, & S. Speier, 2017).

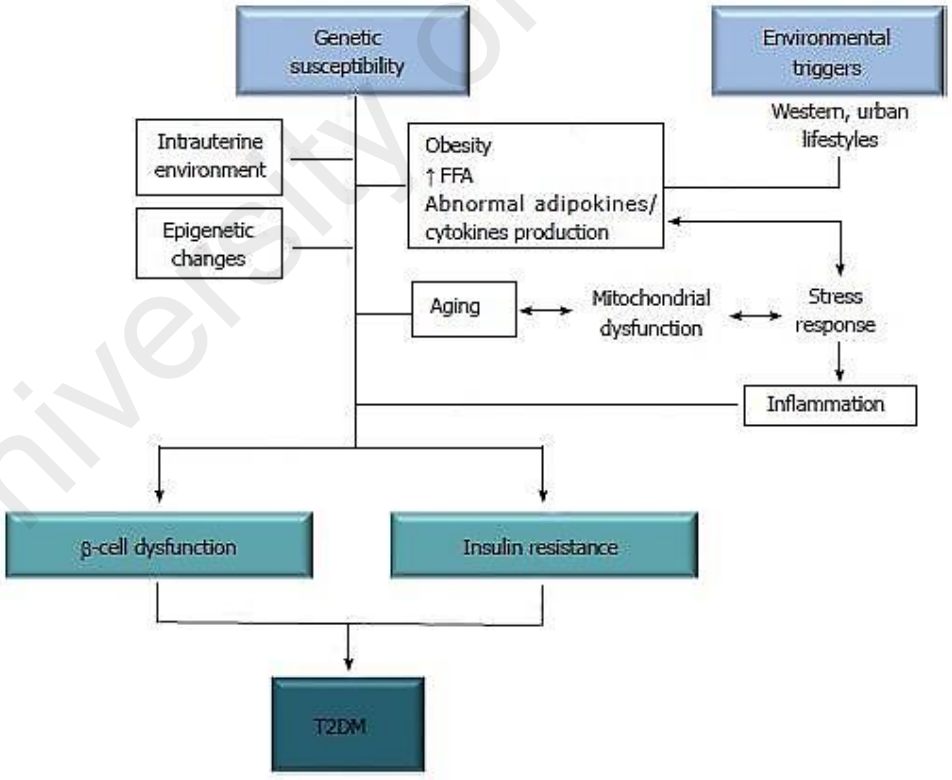

Figure 2.3: Overview of the pathogenic factors contributing to the development of T2DM. A combination of genetic, environmental and lifestyle factors interact together to produce insulin resistance and pancreatic β-cell dysfunction, leading to hyperglycemia (Brunetti, Chiefari, & Foti, 2014).

2.1.3.1 Insulin resistance

Insulin signalling pathway modulates glucose and energy metabolism predominantly via action on skeletal muscle, adipose tissue and liver. Precise regulation of insulin signalling pathway is crucial for adaptation when an individual transits from fed to fasted state (Gutierrez-Rodelo et al., 2017). Physiologically, binding of insulin to IR activates a complex intracellular signalling network via downstream IRS1 proteins and the canonical PI3K cascade which ultimately activates GLUTs for glucose uptake. Impairments at IR and post-receptor level contribute to insulin resistance in insulin-sensitive tissues (Boucher et al., 2014).

Insulin resistance is the predominant cause of T2DM, which defined as a blunted response of target tissues to insulin, as compared with normal glucose tolerant (NGT) individuals without family history of diabetes. The abilities of glucose uptake and metabolism of the body were impaired including reduced insulin sensitivity and decline of the responsiveness (Kahn et al., 2014). In addition to skeletal muscle and liver (key glucose disposal tissues), insulin resistance also present in pancreatic β-cells (C. Chen et al., 2017), gastrointestinal tract (Honka et al., 2013), adipose (Gastaldelli, Gaggini, & DeFronzo, 2017), kidney (Dion et al., 2017), brain (Blazquez, Velazquez, Hurtado-Carneiro, & Ruiz-Albusac, 2014) and vasculature tissues (Mather, Steinberg, & Baron, 2013).

Ample evidences that proved impairments at insulin receptor and post-receptor level contribute to insulin resistance in insulin-sensitive tissues (Table 2.1). Briefly, T2DM patients exhibit 4 altered parameters: (i) decreased concentration of IR, (ii) decreased affinity of IR, (iii) decreased phosphorylation and concentration of mediators (IRS1, PI3K and Akt) and (iv) decreased concentration of functional GLUTs. The combination of several weakly coupling effectors results in decreased insulin sensitivity and signal strength throughout the insulin signalling network, thereby inducing insulin resistance (Cersosimo et al., 2018).

Table 2.1: Molecular mechanism underlying insulin resistance. Inhibited Ser/Thr phosphorylation of insulin signalling molecules, dephosphorylation events, formation of inhibitory complexes and post-translational modifications contribute to insulin resistance.

Causes	Mechanism	Effect	References
Hyperglecemia	Activation of Ser/Thr kinase	Inhibits phosphorylation of insulin signalling molecules	(Gutierrez-Rodelo et al., 2017; Khorami, Movahedi, & Sokhini, 2015; Liang et al., 2015; Nadal-Casellas, Proenza, Llado, & Gianotti, 2012)
Lipotoxicity			
ER stress			
Mitochondrial dysfunction			
Inflammation			
Hyperglycemia	Promotes glycation of insulin signalling molecules	Decreases affinity for IR	(Rhinesmith, Turkette, & Root-Bernstein, 2017)
	Activates PP2A	Decreases insulin secretion	(Arora et al., 2014)
Hyperinsulinemia	Enhances p53 expression	Impairs insulin signalling	(Posa, Selvaraj, Sangeetha, Baskaran, & Lakshmi, 2014)
Lipotoxicity	Enhances PTEN expression	Diminishes PI3K/ Akt signalling	(Wang et al., 2010)
	Activates PP2A	Decreased phosphorylation of IR and insulin signalling molecules	(Bharath et al., 2015)
Inflammation	Cytokines activates SOCS3	Inhibits IRS1 activation	(Jorgensen et al., 2013)
	Cytokines suppresses insulin gene expression	Decreases expression of insulin signalling molecules	(Rehman & Akash, 2016)

Ser/Thr kinase: serine/threonine kinase; PP2A: protein phosphatase type 2A; PTEN: phosphatase and tensin homolog; SOCS3: suppressor of cytokine signalling 3.

2.1.3.2 Impaired function of pancreatic β-cells

Physiologically, pancreatic β-cells constantly synthesized insulin irrespective of blood glucose levels and stored in secretory granules. In response to postprandial blood glucose surge, insulin is secreted from pancreatic β-cells and regulates glucose influx from blood into key insulin-sensitive tissues (skeletal muscle, adipose tissue and liver) via insulin signalling pathway. In addition, insulin serves as the predominant signal for glycogenesis in skeletal muscle and liver. A decline in blood glucose level leads to a reduction in insulin secretion from pancreatic β-cells and an elevation in glucagon secretion from pancreatic α-cells, which stimulate glycogenolysis (Fu, Gilbert, & Liu, 2013).

Generally, unhealthy lifestyle and genetic predisposition cause an elevation in insulin resistance, which is physiologically met by adequate morphological and substantial functional compensation of pancreatic β-cells to preserve normoglycemia, hence intensifying the workload of each pancreatic β-cell (Figure 2.4). In some individuals, functional compensation ceases in spite of prolonged insulin resistance, thereby further increasing the workload of pancreatic β-cell and glucose intolerance. In this pre-diabetic stage, chronic glucose intolerance and hyperglycemia progressively aggravate pancreatic β-cell workload and stress, culminating in cellular exhaustion, apoptosis and clinical manifestation of hyperglycemia. Consequently, uncontrolled hyperglycemia in concert with other cytotoxic factors expedite pancreatic β-cell mass loss and functional deterioration in overt T2DM individuals (Chen, Cohrs, Stertmann, Bozsak, & Speier, 2017). The pathogenic factors and underlying molecular mechanisms contribute to the loss of pancreatic β-cell mass and/or functions are enumerated in Table 2.2.

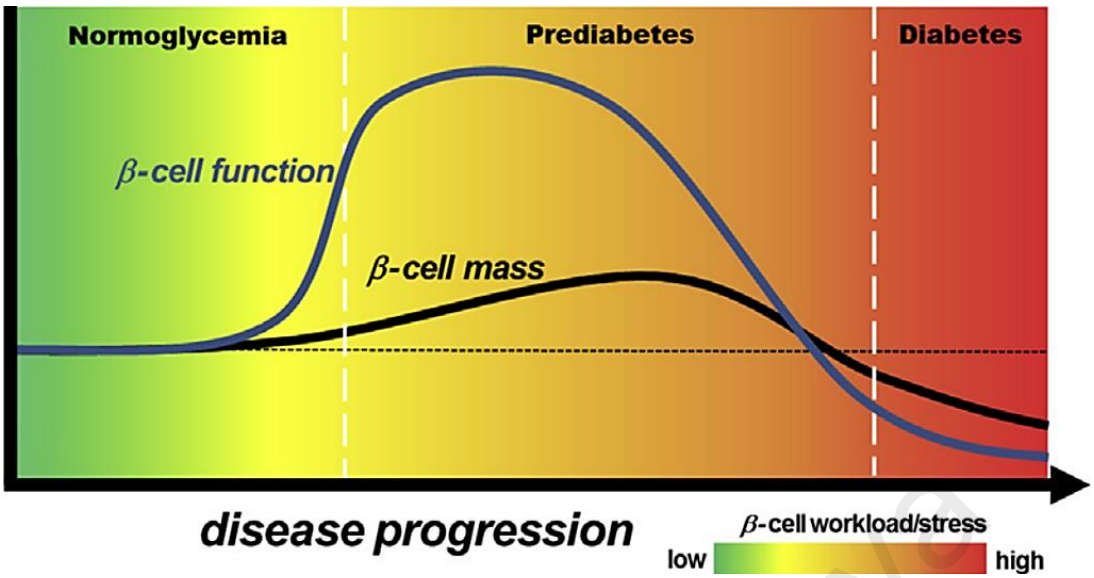

Figure 2.4: Contribution of the pancreatic β-cell mass and function to the pathogenesis of T2DM (Chen et al., 2017).

Table 2.2: Pathogenic factors and underlying molecular mechanisms leading to the loss of pancreatic β-cell mass and/or functions.

Causes	Mechanism	Effect	References
Glucolipotoxicity (hyperglycemia and dislipidemia)	Downregulates PDX-1 and MafA insulin gene promoter activity	Decreases expression of insulin signalling molecules	(D. Gupta et al., 2017)
	Activates PKC	Decreases glucose-stimulated insulin secretion	(Khorami et al., 2015)
	Activates NF-κB pathway	Increases pancreatic β-cell inflammation	(Rehman & Akash, 2016)
	Activates JNK pathway	Diminishes PI3K/Akt signalling	(Solinas & Becattini, 2017)
ER stress	Suppresses insulin gene expression by hyperactivating ATF6	Induces pancreatic β-cell dysfunction and death	(Lenin, Sankaramoorthy, Mohan, & Balasubramanyam, 2015)
Pancreatic islet inflammation	Leptin activates SOCS3	Inhibits insulin synthesis	(Marroqui et al., 2012)
	Activates NF-κB pathway	Induces pancreatic β-cell apoptosis and dysfunction	(Rehman & Akash, 2016)
Pancreatic islet amyloid polypeptide	Induces membrane disorders via physical penetration	Induces pancreatic β-cell death	(Pilkington et al., 2016)
	Activates JNK pathway	Induces pancreatic β-cell apoptosis	(Abedini & Schmidt, 2013)

PDX-1: pancreatic duodenal homeobox 1; PKC: protein kinase C; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; JNK: c-Jun N-terminal kinase; ATF6: activating transcription factor 6; SOCS3: suppressor of cytokine signalling 3.

2.1.3.3 Oxidative stress

Oxidative stress is the outcome of an imbalance between synthesis of reactive oxygen species (ROS) and anti-oxidative defence mechanism against synthesis of ROS. Multiple risk factors (optimal factors: glucolipotoxicity, pro-inflammatory mediators and insulin resistance; partial optimal factors: physical inactivity, hypoxia and impaired glucose tolerance) are involved in provoking oxidative stress. Once the oxidative stress is provoked, it causes further activation of pro-inflammatory mediators and insulin resistance that contribute to the onset of T2DM (Akash, Rehman, & Chen, 2013).

Pancreatic β-cells, adipocytes and other peripheral tissue are more susceptible to the oxidative damage. As depicted in Figure 2.5, overnutrition (one of the risk factors of T2DM) leads to chronic exposure to hyperglycemia and hyperlipidemia which promote synthesis of oxidative stress by activating ROS. Oxidative stress then activates JNK, p53, I kappa B kinase β (IKKβ) and/or NF-κB signalling pathways. JNK, p53, IKKβ activates serine phosphorylation of IRS1 while NF-κB activates inducible nitric oxide synthase (iNOS) expression which subsequently induces S-nitrosylation of IRS1. Both serine phosphorylation of IRS1 and S-nitrosylation of IRS1 suppress the tyrosine phosphorylation of insulin signalling pathway which consequently decreases the insulin-mediated glucose uptake by peripheral tissues. Eventually, impaired insulin signalling pathway induces insulin resistance in skeletal muscles, adipocytes and liver.

In addition, oxidative stress-induced activation of JNK and NF-κB signalling pathways leads to upregulation of pro-inflammatory mediators, including C-reactive protein (CRP), interleukin 6 (IL-6) and tumour necrosis factor-α (TNFα), which induce cellular inflammatory response and apoptosis as well as impair insulin signalling, thereby result in development of insulin resistance. Besides, activation of NF-κB pathway is associated with endothelial dysfunction which can induces insulin resistance.

Taken altogether, it can be suggested that oxidative stress independently exerts its adverse effects on vital organs where endothelial cells and peripheral tissues develop insulin resistance while and pancreatic islets exhibit impaired insulin secretion. Insulin resistance and impaired insulin secretion contribute to the development of postprandial hyperglycemia and overt T2DM, in which both of these outcomes serve as positive feedback mechanism for the development of oxidative stress (Figure 2.6) (Rehman & Akash, 2016).

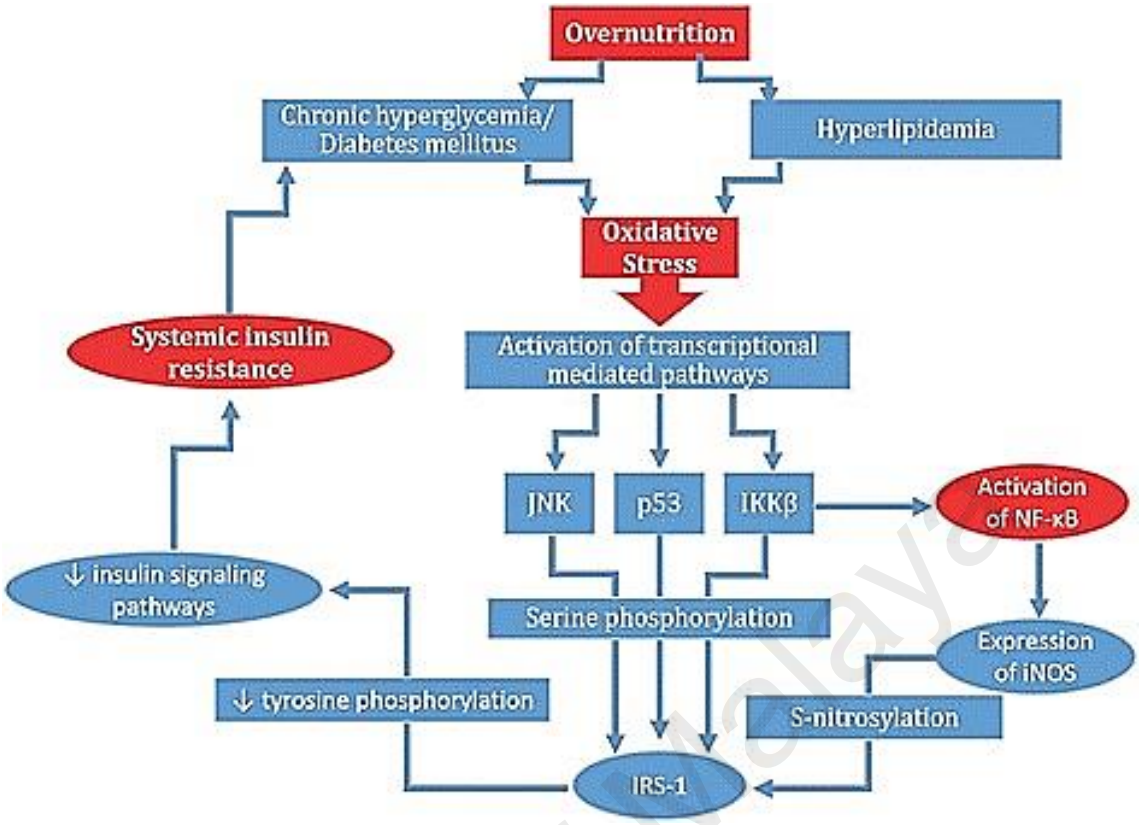

Figure 2.5: Molecular mechanism underlying the oxidative stress-induced insulin resistance (Rehman & Akash, 2016).

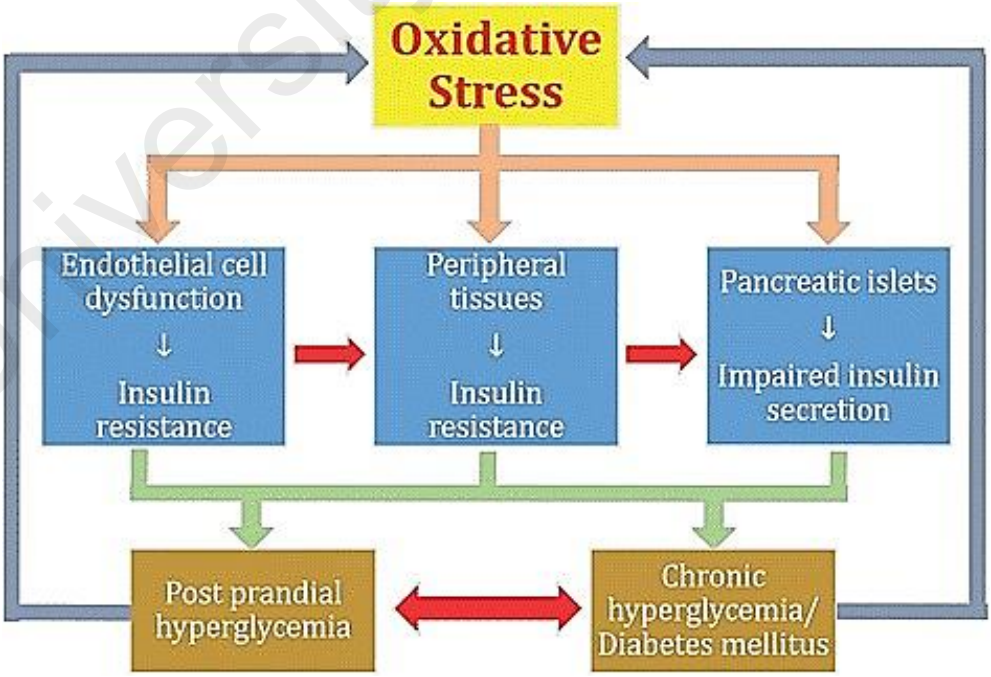

Figure 2.6: Impact of oxidative stress on essential organs of the body (Rehman & Akash, 2016).

2.1.3.4 Inflammation

Human epidemiological and experimental animal studies indicate that inflammation and insulin resistance are directly interlinked with each other during the development of T2DM (Johnson & Olefsky, 2013). As obesity is established and body weight increases with age, a parallel state of chronic inflammation, characterized by an increase of pro-inflammatory cytokines, can trigger changes and switch the metabolic homeostatic set points, leading to T2DM (Kawser Hossain et al., 2016).

In low-grade chronic inflammation state, high level of adipocyte-secreted monocyte-chemo-attractant protein-1 (MCP-1) triggers macrophage infiltration into adipose tissue and the successive secretion of TNF-α impedes insulin signalling pathway. Concomitantly, TNF-α decreases insulin-mediated attenuation of lipolysis and lipid droplet-associated protein perilipin as well as increasing the cyclic adenosine monophosphate (cAMP) pool, all of which enhance FFA release (Guilherme, Virbasius, Puri, & Czech, 2008). Enhanced levels of FFA downregulates the expression of IRS1, hinders the activation of downstream PI3K/Akt signalling in the skeletal muscles and liver, and enhances the expression of JNK signalling in the pancreas.

Eventually, the downregulated expression of PI3K/Akt induce insulin resistance in skeletal muscles and liver, and the upregulated expression of JNK exacerbates apoptosis in the pancreas. Insulin resistance triggers a reduction in skeletal muscle glucose uptake and an elevation in hepatic glucose synthesis, leading to hyperinsulinemia. On the other hand, enhanced apoptosis of pancreatic β-cell causes a reduction in insulin secretion. Eventually, insulin resistance in peripheral tissues and pancreatic β-cell apoptosis lead to overt T2DM (Johnson & Olefsky, 2013; Kawser Hossain et al., 2016).

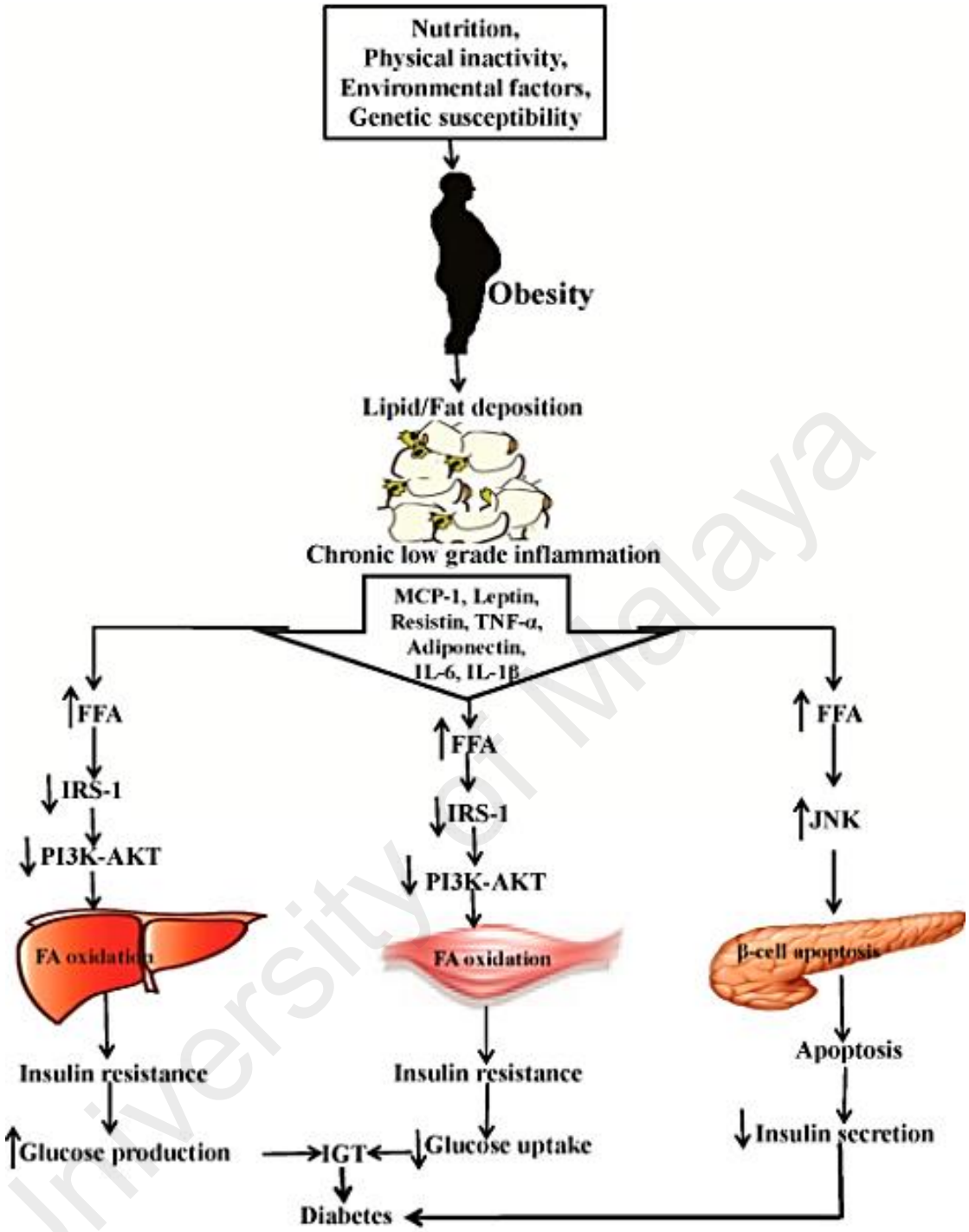

Figure 2.7: The link between inflammation and T2DM (Kawser Hossain et al., 2016).

2.1.3.5 STZ-NA-induced diabetic rat model

Owing to the complexity of T2DM in human, no single animal model is entirely reflective of the major pathogenesis and complications of the human T2DM. Hence, various animal models of different diabetes induction methods have been used, each portraying a different selection of features observed in diabetic patients (King & Bowe, 2016) (Table 2.4). There is no standard protocol of diabetes induction that has been unanimously accepted by the scientific community, hence the most appropriate model will be dependent on the scientific question, type of anti-diabetic agent being sought, institutional financial and facility resources in the T2DM research (Kumar, Singh, Vasudeva, & Sharma, 2012).

In the field of ethnopharmacology, STZ-NA-induced diabetic model serves as the most preferred diabetic models as it highly resembles the structural, biochemical and functional alternations in T2DM patients (Radenkovic, Stojanovic, & Prostran, 2016). The features of STZ-NA-induced diabetic rats, including stable moderate hyperglycemia (8.3 – 10 mmol/L) (Szkudelski, 2012), glucose intolerance (Elamin, Fadlalla, Omer, & Ibrahim, 2018), insulin resistance (Chao et al., 2018), impaired insulin signalling pathway (Chao et al., 2018; Elamin et al., 2018) and responsiveness to anti-diabetic agents (Ghasemi, Khalifi, & Jedi, 2014), make it as a suitable as a model for T2DM for investigating potential anti-diabetic effects of pharmacological and natural compounds on the course of T2DM.

In regard to the mechanism of STZ action, STZ is a *Streptomyces achromogenes*-derived natural diabetogenic agent that induces permanent diabetes in animal models by damaging pancreatic β-cells. The pancreatic β-cell toxicity of STZ is reasoned via alkylation of DNA, generation of free radicals, carbamoylation of proteins and inhibition of O-GlcNAcase (glycoside hydrolase). On that account, STZ leads to DNA fragmentation and necrosis in pancreatic β-cell islets, hence, the rate of insulin synthesis is declined (Figure 2.9) (Goyal et al., 2016).

Concomitantly, NA, a poly-ADP-ribose synthetase inhibitor, protects the pancreatic β-cells via inhibition of PARP-1 and provision of nicotinamide adenine dinucleotide (NAD^+), thus partially retaining the ability of pancreas to synthesis insulin (Szkudelski, 2012). This circumstance averts exacerbation of experimental diabetes induced by STZ and imparts a number of characteristic correspond to T2DM (Figure 2.10) (Ghasemi et al., 2014).

Several factors are affecting the rat sensitivity to STZ and severity of the T2DM. The time of STZ injection plays a critical role in the incidence of T2DM induction where the highest T2DM incidence being observed at 16:00 h and the lowest T2DM incidence at 08:00 h, the reasons on why and how a circadian rhythm may affect T2DM induction rates are yet to be elucidated (Candela, Hernandez, & Gagliardino, 1979)16. Another consideration is the STZ dosage which can vary significantly between gender and strain, < 35 mg/kg are considered a low-dose, 40-55 mg/kg are moderate and > 65 mg/kg are high-dose. A single dose of < 35 mg/kg in rats would not consistently result in T2DM due to spontaneous recovery from diabetic state. A stable and moderate hyperglycemia can be induced by a single dose of 55 – 65 mg/kg (McNeill, 2018).

In addition, the age and gender of rats may affect the sensitivity to STZ. Younger rats being less sensitive to STZ (McNeill, 2018) and male pancreatic islet β-cells are more prone than female to STZ-induced cytotoxicity (Furman, 2015). The common route of STZ administration is intraperitoneal or intravenous injection, other routes of STZ administration includes intramuscular, subcutaneous or even intracardiac. Although intravenous injection of STZ could create a more stable and reproducible T2DM models, it is a difficult approach which requires experience (Ghasemi et al., 2014). In view of those factors, we chose to induce T2DM in 12-week-old male Sprague Dawley (SD) rats consistently in the morning with 55 mg/kg of STZ.

Table 2.3: *In vivo* animal models of T2DM (King & Bowe, 2016).

Aim of the study	Most commonly used animal models	Key characteristics
Understanding the pathogenesis of T2DM: role of insulin resistance and/or obesity in T2DM	High fat feeding rodents KK-A^Y mice $Lep^{ob/ob}$ mice $Lepr^{db/db}$ mice Genetically modified mice Zucker diabetic fatty rats	Insulin resistance Impaired glucose tolerance
Understanding the pathogenesis of T2DM: role pancreatic β-cell compensation in T2DM	Genetically modified mice Neonatal STZ in rats Pancreatectomy in rats Goto Kakizaki rats	Decreased pancreatic β-cell mass and/or declined pancreatic β-cell function
Treating T2DM	Depends on the mechanism of treatment	Hyperglycemia Insulin resistance Impaired pancreatic β-cell compensation Impaired insulin signalling pathway

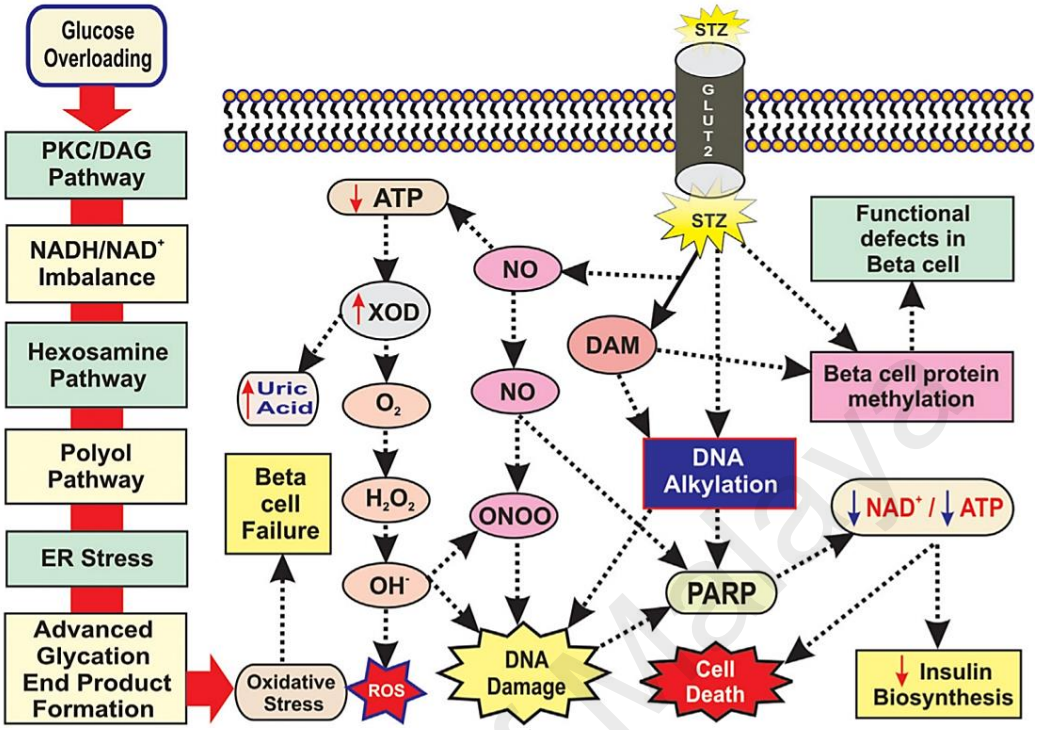

Figure 2.8: Diabetogenic mechanism of action of STZ. Generation of ROS and RNS, oxidative stress, DNA alkylation, DNA damage and glucose overloading causes pancreatic β-cell death, hence resulting in hyperglycemia along with declined biosynthesis and release of insulin (Goyal et al., 2016).

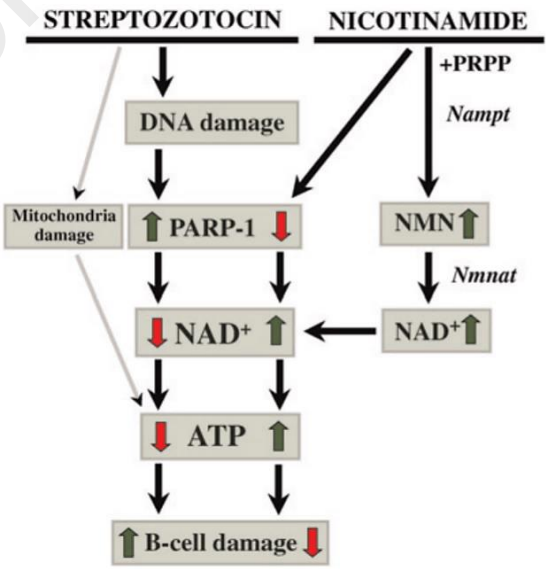

Figure 2.9: **Cytotoxic action of STZ and the protective action of NA on pancreatic β-cells (Szkudelski, 2012).**

2.2 Skeletal muscle

2.2.1 Basic skeletal muscle structure: from whole muscle to individual myofibers

The architecture of skeletal muscle is characterized by myofibers, connective tissues, blood vessels and nerve fibers (Figure 2.11). At the whole muscle level, the size of skeletal muscle is primarily a reflection of individual myofibers. Myofibers are post-mitotic and multinucleated, the multiple nuclei of myofibers allow synthesis of vast amounts of enzymes and proteins necessary for skeletal muscle contraction (Frontera & Ochala, 2015). Epimysium (a sheath of dense and irregular connective tissue) wraps and separate an individual skeletal muscle from other tissues and organs in that area, allowing skeletal muscle to contract and move independently while maintaining its structural integrity (Betts et al., 2013).

Within each skeletal muscle, perimysium (middle layer of connective tissue) organizes myofibers into fascicle. This fascicular organization permits nervous system to trigger a specific movement of a skeletal muscle by activating a subset of myofibers within a fascicle of skeletal muscle. Within each fascicle, myofibers are encased by endomysium (thin connective tissue layer of reticular fibers and collagen) where it consists of nutrients and extracellular fluid to nourish the myofiber (Betts et al., 2013). A single myofiber (approximately 1 cm in length and 100 μm in diameter) is surrounded by sarcolemma where it is associated with a complex of cytoskeletal proteins connected to the internal myofilament, rendering muscle integrity (Nigro & Piluso, 2015). It is estimated that each myofiber is comprises of thousands of myofibrils.

The myofibrils encompasses overlapping thin actin myofilaments and thick myosin myofilaments that confer skeletal myofibers striated appearance (Frontera & Ochala, 2015). The myofibril consists of an alternation of A-I bands, with the H-band in the middle of A-band, in which only thick myofilaments are present; the A-emibands in which both myosin and actin are present to form actomyosin complex; the M line in the middle of H-band where tails of myosin molecules are connected each other; the Z line in the middle of I-band, in which the thin myofilaments of adjacent sarcomeres are linked among them. (Morrison et al., 2015). Each myofibril is composed of sarcomeres of 2 – 3 µm length. Sarcomere, the functional contractile unit of skeletal muscle delimited by two Z lines, is able to contract through the sliding of the thin actin myofilaments on thick myosin myofilaments (Trovato, Imbesi, Conway, & Castrogiovanni, 2016).

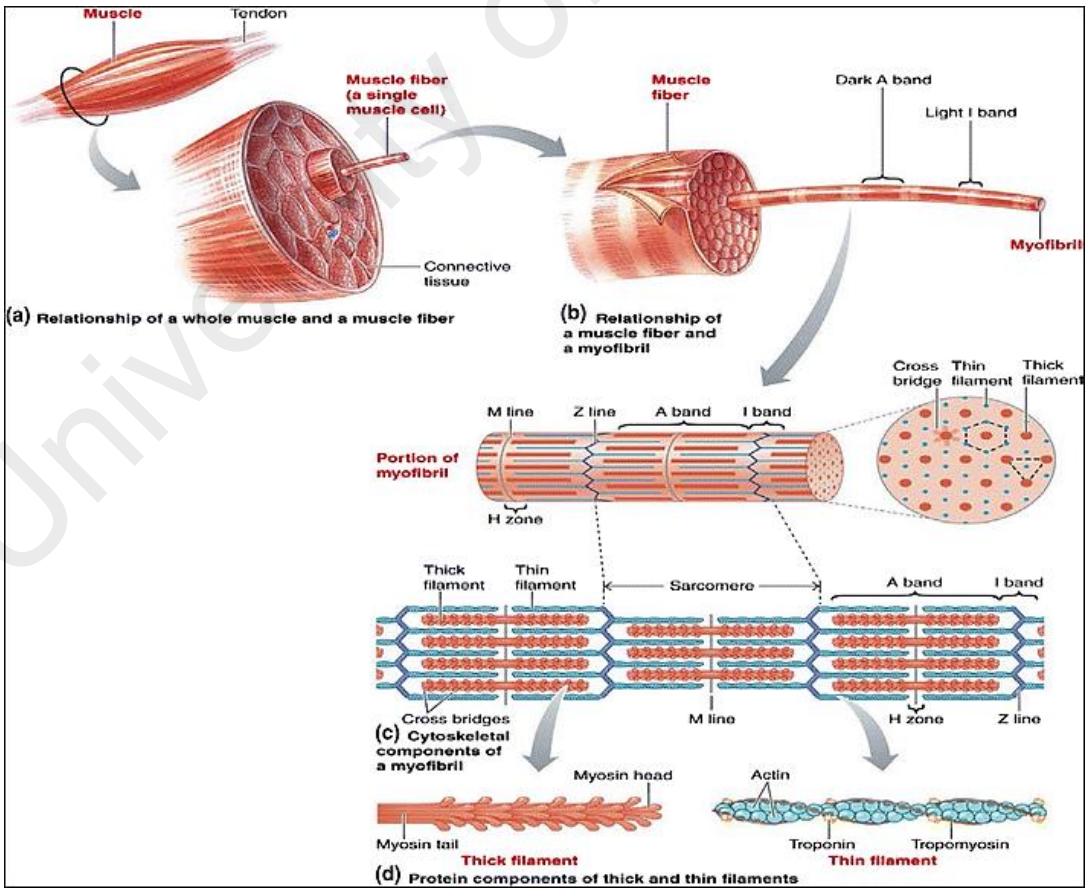

Figure 2.10: Structure of skeletal muscle (Frontera & Ochala, 2015).

2.2.2 Functions of skeletal muscle

Skeletal muscle is a metabolically active tissues which constitutes approximately 40% of total body weight, encompasses 50 – 75% of all body proteins and responsible for 30 – 50 % of whole-body protein turnover. Skeletal muscle is primarily composed of water (75%), protein (20%) and other substances such as carbohydrates, fats, minerals and inorganic salts (5%) (Frontera & Ochala, 2015).

Skeletal muscle has multiple functions. From a mechanical perspective, the primary function skeletal muscle is to convert chemical energy to mechanical energy to produce force and power, generate movement and maintain posture that are essential for enhancing health, participation in occupational and social settings as well as functional independence. From a metabolic standpoint, contribution of skeletal muscle include reservoir of carbohydrate and amino acids, heat generation for maintenance of core temperature, contribution to basal energy metabolism and consumption of fuel and oxygen during physical activity (Berridge et al., 2018).

Of particular interest is the function of skeletal muscle in maintenance euglycemia and in modulating whole-body carbohydrate metabolism (Frontera & Ochala, 2015; Meng et al., 2017). Of relevance to health maintenance, a reduced skeletal muscle mass and/or uptake of glucose impairs the whole body glucose homeostasis and thereby predispose an individual to development of T2DM (Lara Bianchi & Volpato, 2016).

2.2.2.1 Effect of T2DM on structure and function of skeletal muscle

T2DM engenders certain biochemical, contractile and structural alterations in skeletal muscles. The biochemical changes include reduction in glucose and amino acid transport, decrease in protein synthesis and accelerated proteolysis (Ohlendieck, 2012). Besides, the structural alterations comprise disrupted Z-line of some sarcomeres, damage of myofibrils and swollen irregular mitochondria. These structural alterations cause changes in the contractile properties of the skeletal muscles. Also, T2DM subjects exhibit muscle atrophy, reduced capillary density and diminished muscle strength (Imam, Sarwar, Wali, Siddique, & Perveen, 2012). In conjunction, these adverse alterations convert the skeletal muscle to be "metabolically inflexible" as it cannot easily switch between carbohydrate and fat oxidation in response to insulin (Huang, Monu, & Doumanian, 2010).

In the context of functionality of skeletal muscle, T2DM patients exhibited skeletal muscle functional impairments as evident by a significant reduction in muscle strength of upper (Balducci et al., 2014; van der Kooi, Snijder, Peters, & van Valkengoed, 2015) and lower extremities (Ferreira et al., 2017; Nomura et al., 2018). In addition, the loss of skeletal muscle quality and strength would worsen with poor glycemic control and lengthened duration of T2DM (Park et al., 2006). A decline in skeletal muscle strength could leads to T2DM-related physical disability (IJzerman et al., 2012). In fact, epidemiological evidence proves that T2DM is strongly correlates with impaired mobility, particularly in elderly population, and a potential risk factor for future physical disability and loss of independence (L. Bianchi, Zuliani, & Volpato, 2013; de Rekeneire & Volpato, 2015).

Moreover, T2DM elevates the risk of physical disability (Lara Bianchi & Volpato, 2016; Ng, Quinn, Burcu, & Harrington, 2016; Oliveira, Fachin, Tozatti, Ferreira, & Marinheiro, 2012) such that T2DM increases the risk of activities of daily living disability (risk ratio 1.82), instrumental activities of daily living (risk ratio 1.65) and mobility disability (risk ratio 1.51) (Wong et al., 2013).

In the context of pathophysiological mechanism of skeletal muscle dysfunction in T2DM patients, traditional long-term T2DM complications including peripheral diseases and peripheral neuropathy only partially elucidate the T2DM-related dysfunction of skeletal muscles, proposing a direct impact of the metabolic derangements on the intrinsic architecture and functional properties of skeletal muscles (Figure 2.12) (Orlando, Balducci, Bazzucchi, Pugliese, & Sacchetti, 2016).

To date, several pathophysiological mechanisms such as insulin resistance, hyperglycemia, oxidative stress, chronic inflammation, obesity and physical inactivity have been suggested which, at least partially, elucidate the multiple and complex pathways leading to skeletal muscle impairment and thus physical disability. These pathogenic factors cause a reduction in skeletal muscle mass and strength, leading to skeletal muscle dysfunction and hence impaired mobility in T2DM patients (Figure 2.12) (Lara Bianchi & Volpato, 2016).

Of note, the loss of muscle strength and impaired mobility can enhance each other, causing physical inactivity which promote muscle atrophy, thereby results in further diminished muscle strength and vice versa increased muscle weakness. Eventually, this vicious circle leads to a loss of muscle strength and physical disability which erodes health related quality of life (IJzerman et al., 2012).

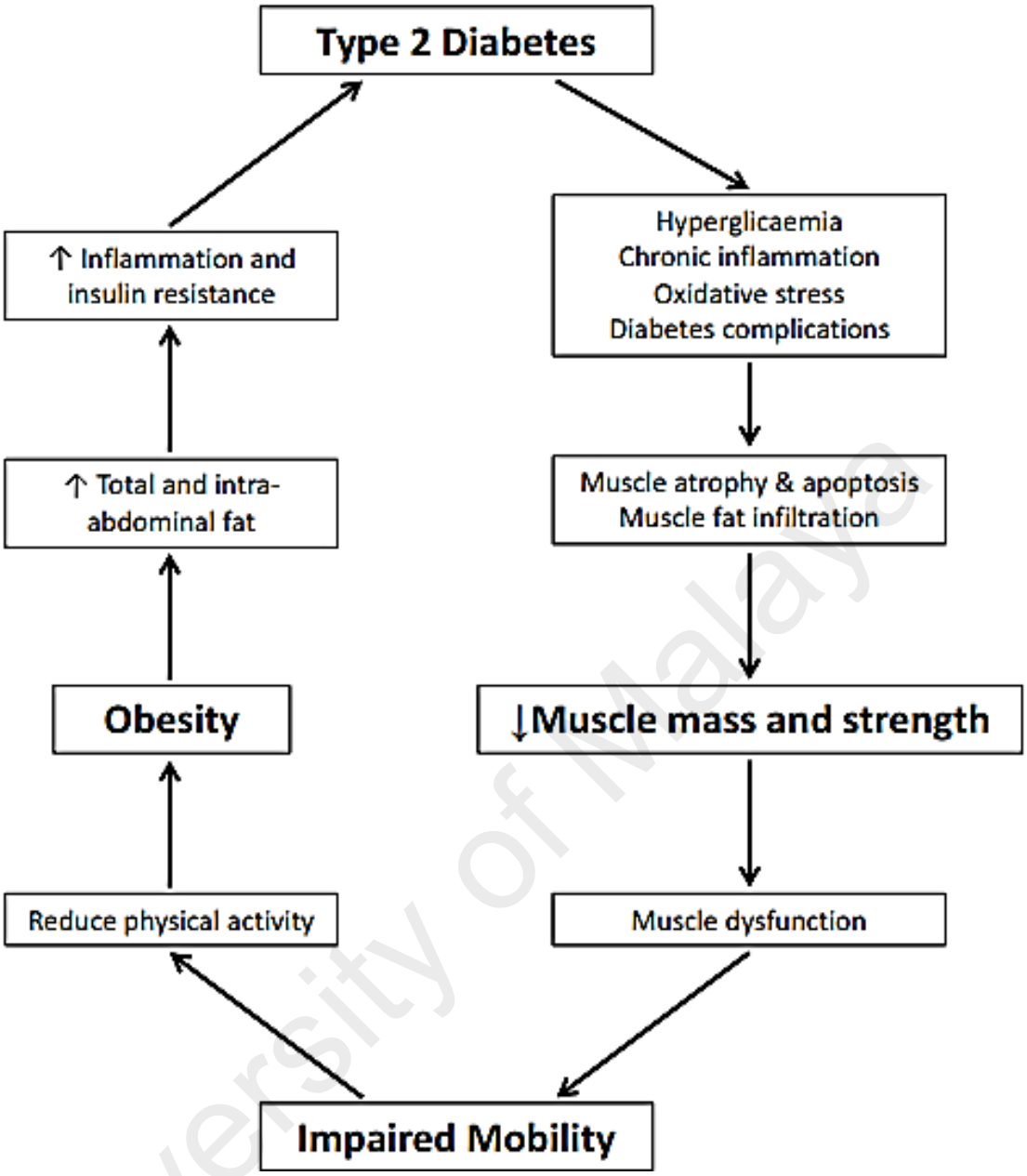

Figure 2.11: Putative model of the pathways from T2DM to dysfunctional skeletal muscle and physical disability (Lara Bianchi & Volpato, 2016).

2.2.3 Role of skeletal muscle in whole body glucose metabolism

2.2.3.1 Glucose metabolism in skeletal muscle

Skeletal muscle responsible for more than 75% of whole body insulin-stimulated glucose uptake, hence plays a crucial role in the regulation of whole body energy homeostasis (Schiavon et al., 2013). The postprandial hyperglycemia stimulates insulin secretion from the pancreas and the elevated plasma insulin concentration stimulates glucose uptake in skeletal muscle via a complex cascade of phosphorylation-dephosphorylation reactions (Carnagarin et al., 2015).

During physiological hyperinsulinemia (80 – 100 µU/ml) in healthy subjects, the muscle glucose intake escalates gradually with time and plateau at approximately 10 mg/kg/min (DeFronzo et al., 1981; Thiebaud et al., 1982). On the contrary, in lean T2DM subjects, the onset of insulin action is impeded and the rate of glucose uptake is decreased by 50% (DeFronzo et al., 1981). Furthermore, in lean healthy subjects, insulin stimulates glucose uptake into skeletal muscle in a dose-dependent manner, with a maximal effect of (EC50) at a plasma insulin concentration ~ 60 µU/ml. However, in insulin resistant states, insulin-stimulated glucose disposal in skeletal muscle is significant decreased with an increased in EC50 to ~ 120 – 140 µU/ml (Groop et al., 1989).

2.2.3.2 Glucose metabolism in skeletal muscle in T2DM

Despite pancreatic β-cell failure is the sine qua non for the development of T2DM, skeletal muscle insulin resistance is considered as the primary defect that is evident decades before β-cell failure and overt hyperglycemia develops. This conclusion is based on the observations of skeletal muscle insulin resistance in NGT subject with family history of T2DM (Figure 2.13) (DeFronzo & Tripathy, 2009). Initially, insulin secretion is augmented to maintain the normal glucose tolerance. Ultimately however, as insulin resistance worsen and accompanied by β-cell deficiency, T2DM will evolve (Chen et al., 2017). Enhanced insulin secretion in response to early development of muscle insulin resistance could exerts negative effects such that elevated and sustained high levels of insulin may induce insulin resistance, as manifested by a diminished glucose uptake and glycogen synthase activity with a concordant reduction in glycogen synthesis in skeletal muscle (Cersosimo et al., 2018).

The aforementioned observations emphasize the role of skeletal muscle as the key tissue in maintaining whole-body glucose homeostasis and insulin resistance in skeletal muscle is a predominant contributor to the pathogenesis of T2DM (Cersosimo et al., 2018). Therefore, insulin signalling pathway underlying the regulation of glucose uptake in skeletal muscle is pivotal because impairments at different levels in the insulin signalling pathway leads to development of skeletal muscle insulin resistance and T2DM (Deshmukh, 2016).

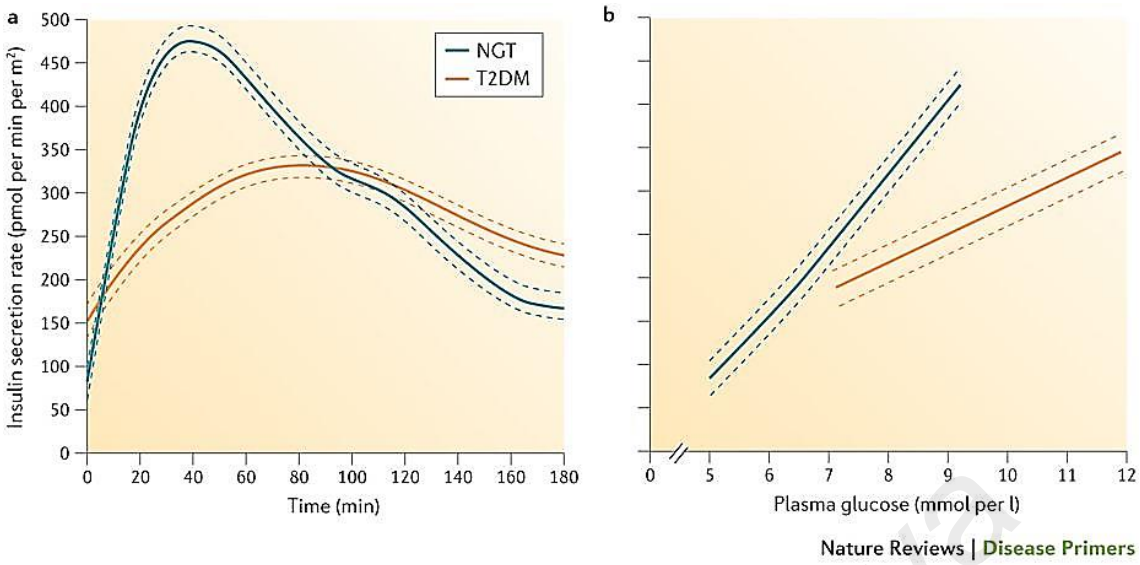

Figure 2.12: Insulin secretion in response to glucose. (a) Characteristics insulin secretory response to oral glucose in T2DM patients and NGT controls (BMI-matched non-diabetic individuals). Note the higher fasting secretion rate, the initial blunted secretory response and the later catch-up phase (due to higher glycemia). (b) Insulin secretion rates against concomitant plasma glucose concentrations to demonstrate the deficit in glucose sensing in T2DM patients versus NGT controls (Ralph A. DeFronzo et al., 2015).

2.2.4 Insulin signalling pathway

2.2.4.1 Insulin signalling pathway in non-diabetic skeletal muscle

As described earlier, insulin is a predominant stimulator of cellular glucose uptake where it enhances glucose uptake and metabolism in skeletal muscles via protein phosphorylation cascade. The effect of insulin initiates upon the binding of insulin to its cognate cellular surface insulin receptor (Figure 2.14) (Carnagarin et al., 2015). IR is a α2β2 heterodimeric transmembrane protein that possesses intrinsic tyrosine kinase activity. Binding of insulin to α subunits causes an autophosphorylation of the β subunits, hence activating the receptor tyrosine kinases which in turn recruits and phosphorylates IRS including IRS1.

Phosphorylated IRS-1 then activates p85 regulatory unit and p110 catalytic unit of phosphatidylinositol-3 kinase (PI3K), leading to an increase in phosphatidylinositol-3,4,5 triphosphate (PIP3). This results in activation of downstream protein kinase B/Akt and phosphorylation of Akt substrate 160 (AS160) which then mediates the translocation of glucose transporter 4 (GLUT4) to the sarcolemma and consequent glucose influx into muscle cells (Alam, Asghar, Azmi, & Malik, 2014).

The free glucose, which has entered the cell, subsequently is metabolized by a series of enzymatic steps that are under the control of insulin. Of these, the most important are glucose phosphorylation (catalysed by hexokinase II), glycogen synthase (controls glycogen synthesis) and phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH) (regulates glycolysis and glucose oxidation respectively) (Ferrannini & DeFronzo, 2015). In essence, the physiological action of insulin is governed by an equilibrium between phosphorylation and desphosphorylation events (Gutierrez-Rodelo et al., 2017).

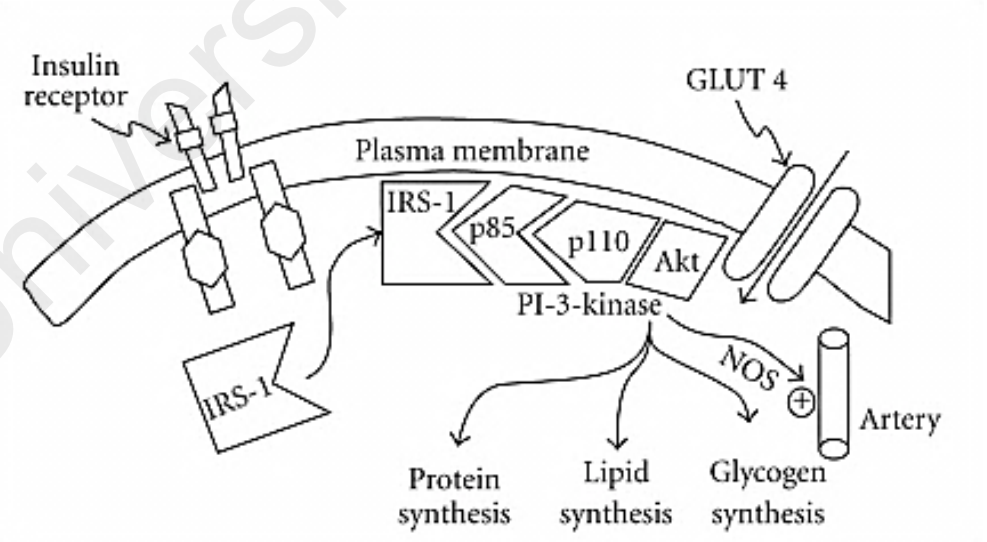

Figure 2.13: Insulin signalling pathway in healthy individuals. Insulin signalling pathway modulates glucose uptake in healthy skeletal muscle. The postprandial hyperglycemia stimulates insulin secretion from the pancreas and the elevated plasma insulin concentration stimulates glucose uptake in skeletal muscle via a complex cascade of phosphorylation-dephosphorylation reactions (Abdul-Ghani & DeFronzo, 2010).

2.2.4.2 Insulin signalling pathway in skeletal muscle in T2DM

Defective insulin-stimulated glucose transport is the rate limiting step for glucose metabolism in T2DM patients, thus defective glucose transport in skeletal muscle is correlated with the impaired whole body glucose uptake in T2DM patients (Olson, 2012). At a molecular level, impaired insulin signal transduction at the level of IR, IRS1, PI3K and Akt were observed in the skeletal muscle from T2DM patients (Figure 2.15) (Deshmukh, 2016). As summarized in Table 2.5, insulin deficient signalling is triggered by various changes including reduced number of IR, elevated Ser/Thr phosphorylation state in IR and IRS1, reduced PI3K and Akt kinases activity as well as impairments in GLUT4 expression and function. These defects within insulin signalling pathway decreases glucose uptake in skeletal muscle and induces insulin resistance, thereby promoting metabolic changes (Gutierrez-Rodelo et al., 2017).

Pioneering research on the insulin signalling pathway in skeletal muscle from T2DM patients has revealed that T2DM is associated with impaired insulin signalling at receptor and post-receptor level. At receptor level, protein expression of IR was found decreased in T2DM patients (Goodyear et al., 1995). Yet, this is not a consistent finding as some studies reported that similar IR protein level (Arner, Pollare, Lithell, & Livingston, 1987; Krook et al., 2000) and concentration-dependent elevation in IR-tyrosine phosphorylation (Klein, Vestergaard, Kotzke, & Pedersen, 1995; Krook et al., 2000) were noted between control and T2DM subjects, implying post-receptor signal transduction defects are probably account for impaired insulin action. Besides, skeletal muscle with unaltered IR protein level but impaired IR-Tyr phosphorylation suggest a functional defects at IR level (Krook et al., 2000).

One of the post-receptor events in insulin signalling is IRS1 phosphorylation. It has been reported that increased IRS1-Ser phosphorylation negatively regulates PI3K regulatory subunit p85 binding to IRS1 (Bandyopadhyay, Yu, Ofrecio, & Olefsky, 2005; Bouzakri et al., 2003; Krook et al., 2000). In addition to the reduced IRS1 protein expression and subsequent IRS1-Tyr phosphorylation (Krook et al., 2000), impaired IRS1-Tyr phosphorylation has been reported in skeletal muscle from morbidly obese insulin resistant subjects and T2DM patients, leading to a nearly blunted interaction with PI3K regulatory subunit p85 (Cusi et al., 2000; Goodyear et al., 1995), this impairment is not associated with altered IRS1 protein levels (Bjornholm, Kawano, Lehtihet, & Zierath, 1997; Cusi et al., 2000; Krook et al., 2000).

The aforementioned Ser/Tyr hyperphosphorylation of IRS1 proteins, in addition to impaired IRS1 interaction with PI3K and subsequent Akt activation, accelerates IRS1 degradation up to approximately 54%, thereby desensitize the insulin signal transduction. Moreover, Ser/Tyr hyperphosphorylation of IRS1 could decrease the Tyr phosphorylation of IRS1 up to 50%. This level of inhibition is adequate to engender glucose intolerance progressing to T2DM, particularly if pancreatic β-cells fail to yield sufficient compensatory hyperinsulinemia. Pathogenic factors including hyperglycemia, FFA, pro-inflammatory cytokines enhance the activity of kinases (JNK stress kinase, mitogen-activated protein kinases and PKC isoforms) that exacerbate Ser/Tyr phosphorylation of IRS1 and further inhibit downstream insulin signalling (Gutierrez-Rodelo et al., 2017).

The PI3K downstream target Akt demonstrated reduced serine phosphorylation in skeletal muscle from T2DM patients (Bandyopadhyay et al., 2005; Cozzone et al., 2008; Krook, Roth, Jiang, Zierath, & Wallberg-Henriksson, 1998). Besides, Cozzone et al. reported defective Akt-serine phosphorylation in skeletal muscle from morbidly obese insulin resistant subjects (BMI > 50kg/m^2). The aforementioned human studies provide evidence that impaired insulin signal transduction in skeletal muscle is associated with insulin resistance and T2DM (Deshmukh, 2016). Consistent with studies in skeletal muscle from T2DM patients, in skeletal muscle from animal models of T2DM, insulin resistance is associated with decreased level of insulin signalling molecules and impairments in insulin action IRS1 phosphorylation, PI3K and Akt activity as well as glucose transport (Cozzone et al., 2008). Taken together, evidence consolidates the importance of functional insulin signalling pathway in maintaining normal glucose homeostasis (Deshmukh, 2016).

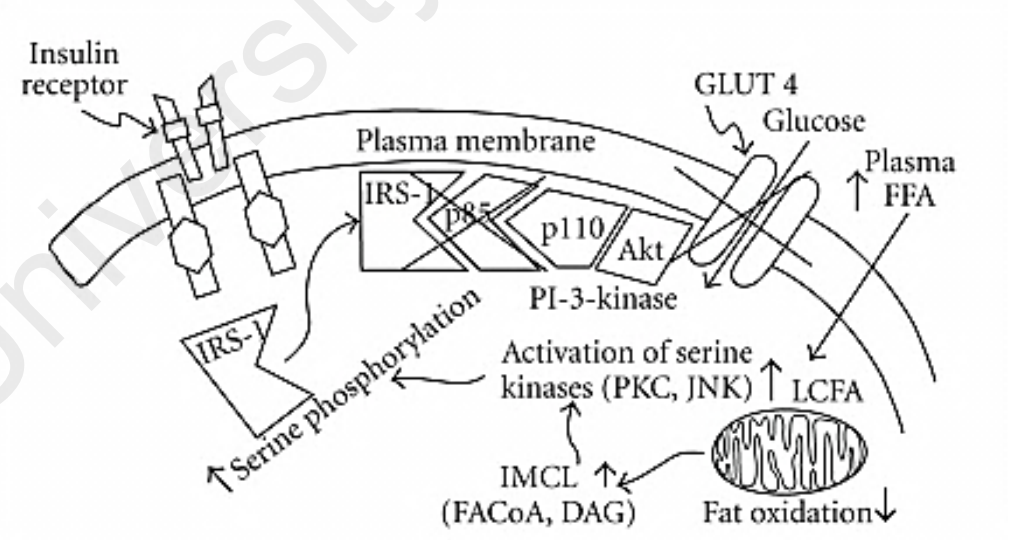

Figure 2.14: Insulin signalling pathway in insulin resistant individuals. Insulin signalling is impaired at the level of IRS1 leading to decreased glucose transport/phosphorylation/metabolism and impaired nitric oxide synthase activation/endothelial function. Increased intramycellar fat and fatty acid metabolite content and mitochondrial dysfunction also exist in skeletal muscle in insulin resistant individuals (Abdul-Ghani & DeFronzo, 2010).

Table 2.4: Modulation of insulin signalling in the skeletal muscles from insulin resistant and T2DM patients.

Insulin signalling molecules	Binding or protein level	Phosphorylation or activity
IR	Decreased IR binding capacity (Maegawa, Shigeta, Egawa, & Kobayashi, 1991) Decreased IR protein level (Goodyear et al., 1995) Unchanged IR protein level (Arner et al., 1987; Krook et al., 2000)	Decreased IR tyrosine phosphorylation (Arner et al., 1987; Cusi et al., 2000; Friedman et al., 1999; Goodyear et al., 1995; Maegawa et al., 1991; Nolan, Freidenberg, Henry, Reichart, & Olefsky, 1994) Unchanged IR tyrosine phosphorylation (Klein et al., 1995; Krook et al., 2000)
IRS1	Unchanged IRS1 protein level (Bjornholm et al., 1997; Cusi et al., 2000; Krook et al., 2000) Decreased IRS1 protein level (Friedman et al., 1999; Goodyear et al., 1995)	Decreased IRS1 tyrosine phosphorylation (Bjornholm et al., 1997; Cusi et al., 2000; Friedman et al., 1999; Goodyear et al., 1995; Krook et al., 2000) Increased IRS1 serine phosphorylation (Bandyopadhyay et al., 2005; Bouzakri et al., 2003; Krook et al., 2000)
PI3K	Unchanged p85 protein level (Krook et al., 2000) Decreased p85 protein level (Cusi et al., 2000; Goodyear et al., 1995)	Decreased PI3K activity (Bandyopadhyay et al., 2005; Bjornholm et al., 1997; Bouzakri et al., 2003; Cusi et al., 2000; Goodyear et al., 1995; Kim, Nikoulina, Ciaraldi, Henry, & Kahn, 1999; Krook et al., 2000)
Akt	Unchanged Akt protein level (Kim et al., 1999) Decreased Akt protein level (Cozzone et al., 2008)	Decreased Akt serine phosphorylation (Bandyopadhyay et al., 2005; Cozzone et al., 2008; Krook et al., 1998)
GLUT4	Decreased GLUT4 protein level (Gaster, Staehr, Beck-Nielsen, Schroder, & Handberg, 2001; Kampmann et al., 2011)	Decreased GLUT4 translocation to plasma membrane (Garvey et al., 1998)

2.3 Pharmacotherapies for T2DM and related challenges

T2DM arises from an impairment in insulin secretion, insulin action and/or both. Therefore, T2DM therapy has advanced from monotherapy using insulin, insulin sensitizers or insulin secretagogues to combination therapy using insulin/insulin secretagogues plus insulin sensitizers and/or incretin-based drugs. The mechanisms of action of these remedies include insulin synthesis, sensitization of the insulin receptor pathway and/or GLP-1 secretion (Tran et al., 2015a, 2015b).

Generally, the most popular approach to treat T2DM is glycemic control in an attempt to decrease the complications and death. Since hyperglycemia is implicated in diabetic complications and death in T2DM patients, better regulation of glycemic maintenance attenuates severity and progression of T2DM (Evans, Balkan, Chuang, & Rushakoff, 2016). Figure 2.16 summarizes the mechanisms of action of the commonly used anti-diabetic drugs in T2DM treatment.

In the past, drugs for T2DM such as insulin, oral anti-diabetic agents and incretin-based drugs have been developed to control blood glucose homeostasis via different mechanisms. Among the oral anti-diabetic agents, insulin secretagogues such as sulfonylurea-type drugs (glibenclamide and glimepiride) directly stimulate pancreatic β-cells to secrete insulin and hence lowering down blood glucose level. Yet, these insulin secretagogues could not rescue pancreatic β-cell atrophy (Hemmingsen, Sonne, Metzendorf, & Richter, 2016; Scheen, 2016). On the other hand, insulin sensitizers such as thiazolidinediones (pioglitazone and rosiglitazone) and biguanide (metformins) directly ameliorate insulin resistance and subsequently blood glucose level (Chen et al., 2017) .

Besides, glucose (re)absorption is an alternative approach to reduce blood glucose level. For instance, SGLT2 inhibitors (empagliflozin and dapagliflozin) diminishing the glucose (re)absorption by inhibiting the activity of SGLT2 in renal tubules (Mosley, Smith, Everton, & Fellner, 2015). Incretin-based drugs such as GLP-1 analogues (liraglutide and exenatide) and enzyme dipeptidyl peptidase-4 (DPP-4) inhibitors (sitagliptin, vildagliptin, saxagliptin, and linagliptin) reduce blood glucose level by leveraging multiple actions of GLP-1, including decreasing glucagon and gastric emptying as well as enhancing insulin secretion (Nauck & Meier, 2016).

Despite the multiple benefits of anti-diabetic drugs, these drugs are still accompanied by inferior efficacy and undesirable side effects (Figure 2.16). For instance, T2DM treatment with insulin secretagogues are associated with weight gain, hypoglycemia and lack of pancreatic β-cell protection from death. Thiazolidinediones and biguanide drugs cause weight gain and kidney toxicity respectively. Incretin-based drugs are linked with gastrointestinal issues such as belching, sour stomach, indigestion, nausea, vomiting and diarrhoea (Chaudhury et al., 2017).

In addition to the inferior efficacy and undesirable side effects of anti-diabetic drugs, the major shortcoming of anti-diabetic drug is that the drugs are designed to ameliorate T2DM but not curing it. Although the anti-diabetic drugs targeting several metabolic pathways showing more favourable results than those that target a single pathway, it should be noted that those drugs that are effective in targeting metabolic pathways are often associated with adverse effects (Tahrani, Barnett, & Bailey, 2016). Therefore, to ensure patients' welfare, there is still an obvious need to develop anti-diabetic medicines.

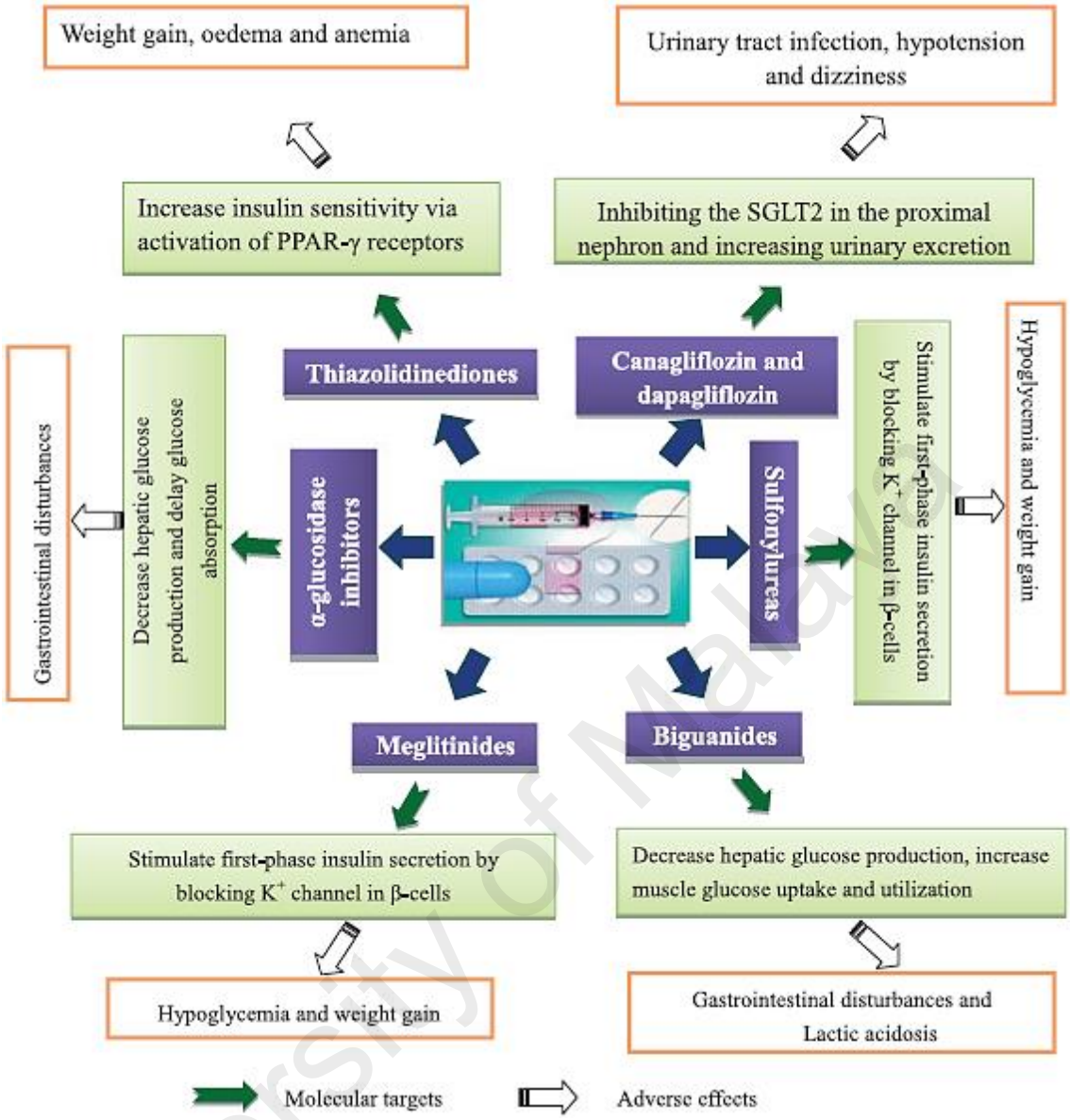

Figure 2.15: The mechanism of action of current anti-diabetic drugs and its adverse effects.

2.4 Honey

2.4.1 Types of honey

Honey, a natural by-product from flower nectar and the aero-digestive tract of the bees from the genus *Apis*, has been known for its nutritive and therapeutic values. It has been widely utilized in innumerable beverages and foods as flavouring and sweetening agent. Among natural products, honey is widely used for various clinical applications, attribute to its biological activities with promising health promoting characteristics (Ahmed et al., 2018).

Generally, honey is named according to the floral source, the trees on which the hives are found or the geographical location where the honey is produced (Ahmed & Othman, 2013). In Malaysia, the well-researched honey includes Tualang honey (*Koompassia excels*), Gelam honey (*Melaleuca cajuputi*), Acacia honey (*Acacia mangium*) and Manuka honey (*Leptospermum scoparium*) (Chua & Adnan, 2014).

2.4.2 Biological bioactive compounds

Honey is constituted of at least 181 compounds and mainly fabricates the fructose (38%) and glucose (31%) as major sugars (Ahmed et al., 2018). Other components include water (15% to 17%), proteins and amino acids (0.1 to 0.4%) and minute amounts of enzymes, vitamins, minerals, and polyphenols, which may contribute to its pharmacological profile (El Sohaimy et al., 2015; Saranraj et al., 2016). The general average constituents and biochemicals of natural honey are presented in Table 2.6 and 2.7 respectively.

In light of the varying concentration of constituents in honeys, International Honey Commission establishes an international honey standard which should be fulfilled by all types of honey. The introduction of a standard for honey is vital for routine honey control including sugar adulteration and honey authenticity (Thrasyvoulou et al., 2018). Regarding the sugar content of honey, fructose and glucose constitute the dominant sugars, with higher fructose/glucose ratio in premium quality honey. Besides, disaccharides (sucrose, maltose, maltulose and nigerose) and trisaccharides (panose, maltotriose, centose and erlose) are commonly identified in honeys (Rahman, Hussain, Ullah, & Zai, 2013). According to the International Honey Commission guidelines, honeys should fulfil a minimum standard for the sum of fructose and glucose of 60 g/100 g as well as sucrose content of less than 5 g/100 g (Bogdanov et al., 1999; Thrasyvoulou et al., 2018).

In honeys, proteins constitute 40 – 60% of total amount of nitrogen, the remaining part of total nitrogen resides in amino acids. The honey proteins are predominantly in the form of enzymes which added by bees during the process of honey ripening. About 8 – 11 proteins are commonly identified in various honey, invertase, diastase and glucose oxidase are the main enzymes in honey (Balasubramanyam, 2013; Chua & Adnan, 2014), glucosidase and catalase present in a relatively lower amount (Assia & Ali, 2015; Borutinskaite, Treigyte, Čeksteryte, Kurtinaitiene, & Navakauskiene, 2018). Besides, several forms of serine proteases are also present in honey (Borutinskaite et al., 2018; Rossano et al., 2012).

Among the 26 amino acids reported, proline serves as the major contributor that constitute of 50% to 85% of the total amino acids (Chua & Adnan, 2014; Moniruzzaman et al., 2013). The minor amount of vitamins cover vitamins B6, pantothenic acid, riboflavin and folic acid. Difference trace elements include calcium, iron, magnesium, potassium and zinc. In addition, organic acids for instance citric, gluconic, malic and butyric acids are other vital group of compounds in honey (Ahmed et al., 2018).

The composition of honey, either from monofloral or multifloral species, is principally depends on the botanical and geographical origin of the nectars, environmental and seasonal conditions, beekeeper ability and honey processing procedure (Moniruzzaman et al., 2013; Nayik et al., 2014). Manuka honey, Tualang honey and stingless bee honey, the few well-studied honeys, are taken as instances to demonstrate the difference in physiochemical properties of honey (Table 2.8).

Moreover, studies reported that fructose content of honey ranges from 21% to 43%, fructose/glucose ratio varies from 0.4 to 1.6 or even higher (Deibert, Konig, Kloock, Groenefeld, & Berg, 2010; Erejuwa et al., 2010; Munstedt, Bohme, Hauenschild, & Hrgovic, 2011; Souza et al., 2006; Stephens et al., 2010) and mineral content ranges from 0.04% (in dark honey) to 0.2% (in light honey) (da Silva, Gauche, Gonzaga, Costa, & Fett, 2016).

Table 2.5: Constituents of honey (data in g/100 g) (Ajibola, 2015).

Proximate analyses	Amount	Proximate analyses	Amount
Water (%)	15.92 (SD 0.07)	Protein (%)	0.42 (SD 0.06)
Carbohydrate (g/100g)	88.97 (SD 1.71)	Fat (%)	0.53 (SD 0.01)
Fructose (g/100g)	43.35 (SD 0.02)	Thiamine (mg/100g)	0.01 (SD 0.00)
Glucose (g/100g)	37.64 (SD 0.01)	Riboflavin (mg/100g)	0.02 (SD 0.00)
Sucrose (g/100g)	2.03 (SD 0.05)	Niacin (mg/100g)	0.15 (SD 0.01)
Maltose (g/100g)	2.75 (SD 0.02)	Vitamin B5 (mg/100g)	0.07 (SD 0.03)
Oligosaccharides (g/100g)	3.11 (SD 0.08)	Pyridoxine (mg/100g)	0.17 (SD 0.02)
Erlose (g/100g)	0.81 (SD 0.06)	Folic acid (mg/100g)	0.006 (SD 0.00)
Melezitose (g/100g)	0.09 (SD 0.03)	Ascorbic acid (mg/100g)	2.35 (SD 0.25)
Glycaemic sugars (%)	76.71 (SD 1.55)	Vitamin K (mg/100g)	0.025 (SD 0.00)
Energy (MJ/Kg)	15.56 (SD 0.21)	Other nutrients (%)	0.53 (SD 0.00)

Table 2.6: Major and trace elements in honey (Ajibola, 2015).

Element	Amount	Element	Amount
Aluminium (Al)	0.01 – 2.4	Magnesium (Mg)	0.7 – 13
Arsenic (As)	0.014 – 0.026	Manganese (Mn)	0.02 – 2
Barium (Ba)	0.01 – 0.08	Molybdenum (Mo)	0 – 0.004
Boron (B)	0.05 – 0.3	Nickel (Ni)	0 – 0.051
Bromine (Br)	0.4 – 1.3	Phosphorus (P)	2 – 15
Cadmium (Cd)	0 – 0.001	Potassium (K)	40 – 3500
Calcium (Ca)	3 – 31	Rubidium (Rb)	0.040 – 3.5
Chlorine (Cl)	0.4 – 56	Selenium (Se)	0.002 – 0.01
Chromium (Cr)	0.01 – 0.3	Sodium (Na)	1.6 – 17
Cobalt (Co)	0.1 – 0.35	Silicon (Si)	0.05 – 24
Copper (Cu)	0.02 – 0.6	Strontium (Sr)	0.04 – 0.35
Fluoride (F)	0.4 – 1.34	Sulphur (S)	0.7 – 26
Iodide (I)	10 – 100	Vanadium (V)	0 – 0.013
Iron (Fe)	0.03 – 4	Zinc (Zn)	0.05 – 2
Lead (Pb)	0.001 – 0.03	Zirconium (Zr)	0.05 – 0.08
Lithium (Li)	0.225 – 1.56		

Table 2.7: Physiochemical properties of Manuka, Tualang and stingless bee honey.

Physiochemical properties	International Honey Commission Guidelines	Manuka honey	Tualang honey	Stingless bee honey
Appearance	Colourless to dark brown	Light brown	Dark brown	Amber brown
Moisture content (%)	< 20.00	18.70	23.30	25.02
pH	3.4 – 6.00	3.20 – 4.20	3.55 – 4.00	3.05 – 4.55
Total reducing sugars (g/ 100 g)	> 60.00	76.00	67.50	55.00 – 86.00
Fructose (g/ 100 g)	20.00 – 42.40	40.00	29.60	31.11 – 40.20
Glucose (g/ 100 g)	23.00 – 32.00	36.20	30.00	8.20 – 30.98
Sucrose (g/ 100 g)	0.00 – 2.80	2.80	0.60	0.31 – 1.26
Maltose (g/ 100 g)	Not available	1.20	7.90	Not available
Calcium (g/ 100 g)	Not available	1.00	0.18	Not available
Magnesium (g/ 100 g)	Not available	1.00	0.11	Not available
Potassium (g/ 100 g)	Not available	1.00	0.51	Not available
Sodium (g/ 100 g)	Not available	0.0008	0.26	Not available
Hydroxylmethylfufural (mg/kg)	< 80.00	40.00	46.17	8.80 – 69.00
Specific gravity	Not available	1.39	1.34	Not available
References	(Bogdanov et al., 1999; Thrasyvoulou et al., 2018)	(Stephens et al., 2010)	(Ahmed & Othman, 2013; Erejuwa et al., 2010)	(Souza et al., 2006)

Polyphenols are the widespread bioactive compounds which can be found in certain plant parts, at specific plant developmental stages or plant families (Cheynier, 2012). Studies confirmed that among the major groups of polyphenols, only flavonoids and phenolic acids are present in honey. Particularly, among the sub-classes of flavonoids, only the flavonols (including quercetin and isorhamnetin), flavones (including apigenin and cinnamic acid) and flavanones (including pinocembrin and pinostrobin) are detected in nearly all honey worldwide.

Among the phenolic acid group, the hydroxybenzoic acids (including benzoic acid, protocatechuic acid and syringic acid) and hydroxycinnamic acids (caffeic acid and vanilic acid) are present in various honey samples (Hossen et al., 2017). Various types of honey along with their source and polyphenol sub-classes are summarized in Table 2.9 and Table 2.10.

Polyphenols are reported to exhibit antioxidant, antimicrobial, anti-inflammatory, anti-diabetic, antiviral, antimutagenic, anticancer and immunomodulatory activities (Ahmed et al., 2018). At organ level, consumption of honey rich in these types of polyphenols drastically attenuates the oxidative and inflammatory damage on brain (Mijanur Rahman et al., 2014), heart (Al-Waili et al., 2013), liver (Oršolić, Jazvinšćak Jembrek, & Terzić, 2017), pancreas (Batumalaie, Qvist, Yusof, Ismail, & Sekaran, 2014; Erejuwa et al., 2010), and kidney-associated diseases (Oršolić et al., 2017).

Each components in honey has unique nutritional and therapeutic properties, and the constituents act synergistically, contribute honey utility in various clinical applications. (Vit, Vargas, & Valle, 2015). In addition to the therapeutic effects of honey, natural constituents of honey such as oligosaccharides (Lazarevic, Jovetic, & Tesic, 2017), amino acids (Chua & Adnan, 2014), mineral and trace elements (Lazarevic et al., 2017), phenolic (Ciulu, Spano, Pilo, & Sanna, 2016) and volatile (Pattamayutanon et al., 2017) compounds have been utilized as a marker for inferring the botanical and geographical origins of honey.

Table 2.8: Sub-classes of flavonoids present in honey.

Flavonoids	Phenolic compounds	Honey types	References
Flavonols	Quercetin	*Acacia*[M], Gelam[M], Nenas[M], Tualang[M] honeys	(Batumalaie et al., 2014; Chua, Rahaman, Adnan, & Eddie Tan, 2013; Hussein, Yusoff, Makpol, & Yusof, 2011; Shehu et al., 2016)
		Almond[A], Lemon[A], Medlar[A], Orange[A] and Prickly pear[A] honeys	(Tenore, Ritieni, Campiglia, & Novellino, 2012)
		Buckwheat[F], Fireweed[F] and Raspberry[N] honeys	(Salonen, Virjamo, Tammela, Fauch, & Julkunen-Tiitto, 2017)
	Isorhamnetin	*Diplotaxis tenuifolia*[A] honey	(Truchado et al., 2010)
		Manuka[NZ] honey	(Adams, Manley-Harris, & Molan, 2009; Chan et al., 2013)
Flavones	Apigenin	Tualang[M], Gelam[M] and Borneo tropical honeys	(Khalil, Alam, Moniruzzaman, Sulaiman, & Gan, 2011)
		Buckwheat[F], Caraway[F], Clover[D], Fireweed[F], Heather[N], Raspberry[N] and Willow[N] honeys	(Salonen et al., 2017)
	Cinnamic acid	Acacia[M], Gelam[M], Tualang[M] honeys	(Chua et al., 2013)
		Buckwheat[F], Dandelion[S], Honeydew[N] and Raspberry[N] honeys	(Salonen et al., 2017)
		Conifer tree honey[G]	(Spilioti et al., 2014)
Flavanones	Pinocembrin	Algarrobo[A] and citrus[A] honeys	(Isla et al., 2011)
		Almond[A], Lemon[A], Medlar[A], Orange[A] and Prickly pear[A] honeys	(Tenore et al., 2012)
		Diplotaxis tenuifolia[A] honey	(Truchado et al., 2010)
		Manuka[NZ] honey	(Chan et al., 2013)
	Pinostrobin	*Diplotaxis tenuifolia*[A] honey	(Truchado et al., 2010)

A: Argentina; C: China; D: Denmark; F: Finland; G: Greece; M: Malaysia; N: Norway; NZ: New Zealand; S: Sweden

Table 2.9 continued: Sub-classes of flavonoids present in honey.

Phenolic acids	Phenolic compounds	Honey types	References
Hydroxybenzoic acids	Benzoic acid	Tualang[M], Gelam[M] and Borneo tropical honeys	(Khalil et al., 2011)
		CloverD, Dandelion[S] and Honeydew[N] honeys	(Salonen et al., 2017)
	Protocatechuic acid	Conifer tree[G], Greek[G] and thyme[G] honeys	(Spilioti et al., 2014)
		Buckwheat[C], Jujube[C], Locust[C], Longan[C] and Vitex[C] honeys	(Cheng, Wang, & Cao, 2017)
		Almond[A], Lemon[A], and Orange[A] honeys	(Tenore et al., 2012)
Hydroxycinnamic acids	Caffeic acid	Acacia[M], Gelam[M], Nenas[M], Tualang[M] and Borneo tropical honeys	(Chua et al., 2013; Hussein et al., 2011; Khalil et al., 2011)
		Almond[A], Lemon[A], Medlar[A] and Orange[A] honeys	(Tenore et al., 2012)
		Buckwheat[C], Jujube[C], Locust[C] and Vitex[C] honeys	(Cheng et al., 2017)
	Vanilic acid	Almond[A], Lemon[A], Medlar[A] and Orange[A] honeys	(Tenore et al., 2012)
		Caraway[F], Dandelion[S], Raspberry[N] honeys	(Salonen et al., 2017)
		Greek honey[G]	(Spilioti et al., 2014)

A: Argentina; C: China; D: Denmark; F: Finland; G: Greece; M: Malaysia; N: Norway; NZ: New Zealand; S: Sweden

2.4.3 Medicinal effects of honey

2.4.3.1 Antioxidant effects of honey

Globally prevalent chronic disease such as T2DM, atherosclerosis, hypertension, Alzheimer's disease is associated with oxidative stress. Oxidative stress causes oxidative damage to cellular compartments and impairs physiological functions. Solid evidence suggests that honey could augment body antioxidant defence system against oxidative stress, counteracting the oxidative damage and thereby exert protective effects on various organ (Erejuwa, Sulaiman, & Wahab, 2012).

As previously described, honey contains various types of flavonoids and phenolic acids which work together to confer a synergistic antioxidant effect (Hossen et al., 2017). The antioxidant activity is strongly correlated with the colour intensity and total phenolic content of honey. Dark honeys (Buckwheat) has a greater total phenolic content and a concomitant greater antioxidant capacity than lightest honey (acacia and lime) (Pontis, Costa, Silva, & Flach, 2014; Sant'Ana, Buarque Ferreira, Lorenzon, Berbara, & Castro, 2014).

Among the Asian honeys, Tualang honey possesses higher phenolic content (83.96 mg gallic acid/100 g of honey) and concomitant stronger antioxidant capacity than Gelam, Indian forest and pineapple honeys. The mechanisms of antioxidant action include (i) scavenging ROS, (ii) suppression of ROS synthesis by chelating trace element or inhibition of enzymes involved in free radical synthesis and (iii) protection or upregulation of antioxidant defences (Ahmed et al., 2018).

In healthy human subjects, following consumption of 1.5 g/kg body weight of Buckwheat honey (containing approximately 1.171 mg of phenolic antioxidants/g), plasma total phenolic content significantly enhanced as did plasma antioxidant and reducing capacities. These findings prove the bioavailability of phenolic antioxidant and the consequent increased antioxidant activity and reducing power of plasma (Schramm et al., 2003). On the other hand, daily consumption of 1.2 g/kg body weight of honey enhances the amount and activity of antioxidant agents such as glutathione reductase, beta-carotene, vitamins C and uric acid (Al-Waili, 2003). Taken together, it is advised to consume honey to enhance the antioxidant defences in healthy adults. Figure 2.17 depicts the mechanisms of antioxidant effects of honey.

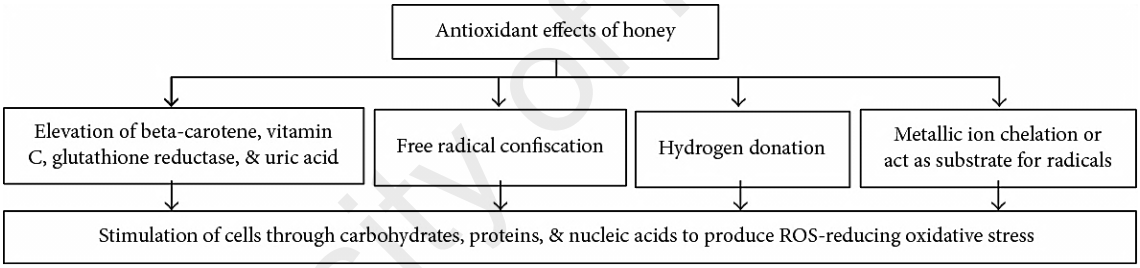

Figure 2.16: Mechanisms of antioxidant effect of honey (Ahmed et al., 2018).

2.4.3.2 Anti-inflammatory effects of honey

Chronic inflammation could inhibit the tissue healing by damaging the tissues. The anti-inflammatory action of honey is well documented, honey attenuates inflammatory response in cell cultures (Candiracci et al., 2012), animal models (Fernandez-Cabezudo et al., 2013) and clinical trials (Leong, Herst, & Harper, 2012). Polyphenols in honey suppresses the lipoxygenases that are responsible for the synthesis of pro-inflammatory mediators. It was found that the flavonoids and phenolic acids such as quercetin, chrysin and galangin suppress activity of cyclooxygenase-2 (COX-2) (Murtaza et al., 2014) and iNOS (Candiracci et al., 2012). Besides, phenolics in honey such as myricetin, chlorogenic, ellagic, ferulic, gallic and caffeic acid can inhibit the overproduction of pro-inflammatory mediators (nitric oxide, NO and prostaglandin, E2 PGE2) and pro-inflammatory cytokines (IL6 and TNFα) (Hussein et al., 2012; Hussein, Mohd Yusoff, Makpol, & Mohd Yusof, 2013; Kassim et al., 2010).

Furthermore, honey ameliorates oedema and pain in inflammatory tissues, attributed to the inhibition of NO and PGE2 synthesis and release (Borsato et al., 2014; Hussein et al., 2013). In inflammatory response, hydrogen peroxide (H_2O_2) production induced by honey stimulates the growth of epithelial cells and fibroblast to repair the inflammatory damage. In addition, honey ceases the release of monocytes, neutrophils and macrophages that promote inflammation (S. S. Gupta et al., 2011; Zbuchea, 2014).

Hence, anti-inflammatory action of honey is supposed to counteract unceasing diseases such as cancer, liver and kidney diseases (Ahmed et al., 2018). Figure 2.18 presents the mechanisms of anti-inflammatory effects of honey.

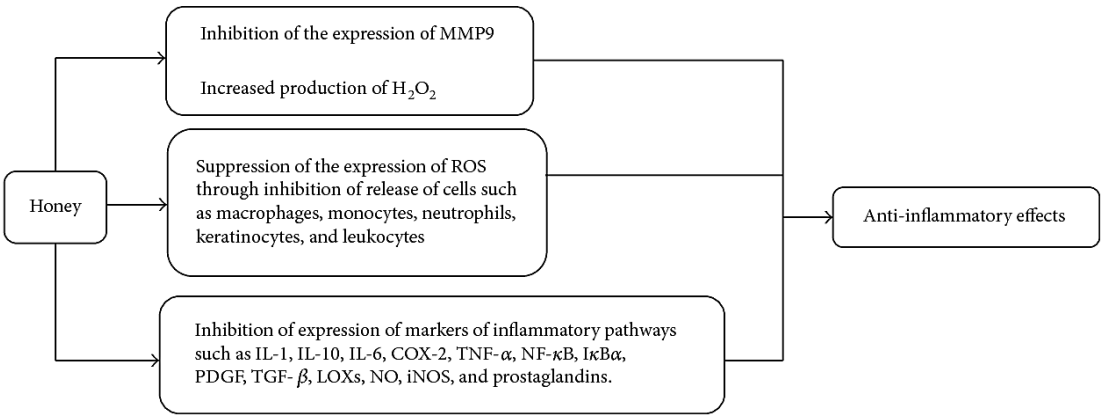

Figure 2.17: Mechanisms of anti-inflammatory effect of honey (Ahmed et al., 2018).

2.4.4 Honey and T2DM

In light of the staggering cost of T2DM and adverse effects caused by anti-diabetic drugs, natural products such as honey have been studied to explore the health benefits of these natural products in T2DM management. Pre-clinical and clinical studies have demonstrated that honey reduces the fasting serum glucose, increases fasting C-peptide and 2 h postprandial C-peptide. In addition, honey has low glycemic index and peak incremental index in T2DM patients. These suggest that honey exert beneficial effects on the glycemic control and metabolic derangements in T2DM (Meo et al., 2017).

Long-term consumption of honey (0.42 to 13.5 years), in addition to weight reduction, results in improved control of blood pressure and cardiovascular status without any anti-diabetic drug in T2DM patients (Abdulrhman, 2016). It was postulated that honey elicits anti-hypertensive effect by inducing synthesis of endothelium derived nitric oxide which results in vasodilation (Aluko, Olubobokun, Atang, & Nna, 2014). Nitric oxide metabolites in honey may be partially responsible for the anti-hypertensive effects of honey (N. S. Al-Waili, 2003). Moreover, honey confer cardioprotective effects against oxidative stress by contributing to endogenous antioxidant enzyme activity via inhibition of lipid peroxidation (Khalil, Tanvir, Afroz, Sulaiman, & Gan, 2015). Hence, it was proposed that honey is comparable to medications in the treatment of T2DM.

Moreover, studies have unscored the beneficial effects of honey in pancreas, liver, adipose, gastrointestinal tract, gastrointestinal microbiota and the mechanisms of action in improving glycemic control and metabolic derangements (Table 2.11) (Erejuwa et al., 2012) . Yet, no study has been carried out to investigate the effect of honey on diabetic skeletal muscle.

Table 2.9: Effects of honey in different tissues. Adapted from Erejuwa et al., 2012.

Tissue/Study design	Oxidative stress status	
	Control	Honey
Liver		
Rats or mice with trichlorfon-, NEM- or CCl_4-induced liver injury or obstructive jaundice	↑ GPx; ↑ CAT; ↓ GSH; ↑ MDA and TAC	↓ GPx; ↓ CAT; ↑ GSH; ↓ MDA and TAC
Pancreas		
Rats with STZ-induced diabetes	↑ SOD; ↑ GPx; ↓ CAT and ↑ MDA	↓ SOD; ↑ CAT; ↓ GPx and ↓ MDA
Kidney		
Rats with STZ-induced diabetes (diabetic SD) or with CCl_4-induced nephrotoxicity	↑ MDA; ↓ TAS; ↓ CAT; ↓ GPx; ↓ GST; ↓ GR; ↑ SOD and ↓ GSH	↓ MDA; ↑TAS; ↑ CAT; ↑ GPx; ↑ GST; ↑ GR; ↓ SOD and ↑ GSH
Rats with hypertension (SHR)	↑ MDA; ↑ GST; ↑ TAS and ↑ CAT	↓ MDA; ↓ GST; ↓ TAS and ↓ CAT
Rats with diabetes (diabetic WKY)	↔ MDA; ↔ CAT; ↑ GPx; ↔ GR; ↓ TAS and ↔ GSH/GSSG	↔ MDA; ↔ CAT; ↔ TAS; ↓ GPx; ↓ GR and ↑ GSH/GSSG
Rats with both diabetes and hypertension (diabetic SHR)	↔ MDA; ↓ CAT; ↓ GPx; ↓ GR; ↓ TAS; ↔ GSH and ↔ GSH/GSSG	↔ MDA; ↔ CAT; ↑ GPx; ↑ GR; ↑ TAS; ↑ GSH and ↑ GSH/GSSG
Plasma/serum		
MNU-induced oxidative stress	↑ MDA and ↑ NO	↓ MDA and ↑ NO
Alloxan- or STZ-induced diabetic rats or non-diabetic rats	↓ GPx; ↓ NO and ↑ formation of glycated products (fructosamine and glycated hemoglobin)	↑ GPx; ↑ NO; ↑ TAS and ↓glycated products (fructosamine and glycated hemoglobin)
Reproductive organs		
Testis of rats exposed to cigarette smoke	↑ MDA; ↓ TAS; ↓ SOD; ↓ CAT and ↑ GPx	↓ MDA; ↓ GPx; ↑ TAS; ↑ SOD; ↑ CAT and ↑ GSH
Seminal oxidative stress in male cyclists undergoing intensive cycling training	↓ TAS; ↓ SOD and ↓ CAT	↓ MDA; ↓ ROS; ↑ SOD, ↑ CAT and ↑ TAS
Other tissues or cells		
Whole blood and erythrocytes of young (2 months) and middle-aged (9 months) rats	Whole blood: ↑ DNA damage; Erythrocytes: ↓ GPx and ↑ CAT	↓ DNA damage ↑ GPx and ↓ CAT
In a cultured endothelial cell line	↑ ROS and ↓ GSH	↓ ROS and ↑ GSH
In inflammation	↑ NO and ↑ prostaglandin E(2)	↓ NO; ↓ prostaglandin E(2) and ↓ inflammation

TNBS, trinitrobenzene sulfonic acid; MPO, myeloperoxidase; NEM, N-ethylmaleimide; TAC or TAS, total antioxidant capacity or status; 8-IP, 8-isoprostane; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; glutathione S-transferase; NO, nitric oxide; ROS, reactive oxygen species; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; ↑ = increased/enhanced; ↓ = reduced/attenuated; ↔ = no significant effect.

CHAPTER 3: METHODOLOGY

3.1 Study design

An overview of the present study design is depicted in Figure 3.1. Diabetes induction and respective *Apis* bee honey treatments to rats as well as investigation of the effect of *Apis* bee honey on hyperglycemia in diabetic rats were completed by previous researcher. This study aimed to identify the components in *Apis* bee honey and examine the effect of *Apis* bee honey on the architecture and expression of insulin signalling molecules in experimental diabetic rats.

In the present study, *Apis* bee honey was sent for liquid chromatography–mass spectrometry (LC-MS) analysis to identify the bioactive compounds in *Apis* bee honey. Same batch of *Apis* bee honey was used to ensure constancy of honey composition.

From the provided skeletal muscle samples, the architecture of skeletal muscle was visualized by hematoxylin and eosin (H&E) staining. Besides, protein expression of insulin signalling molecules was quantitated by Western blot (WB). Immunohistochemistry (IHC) and immunofluorescence (IF) were used to determine the distribution of insulin signalling molecules in skeletal muscle.

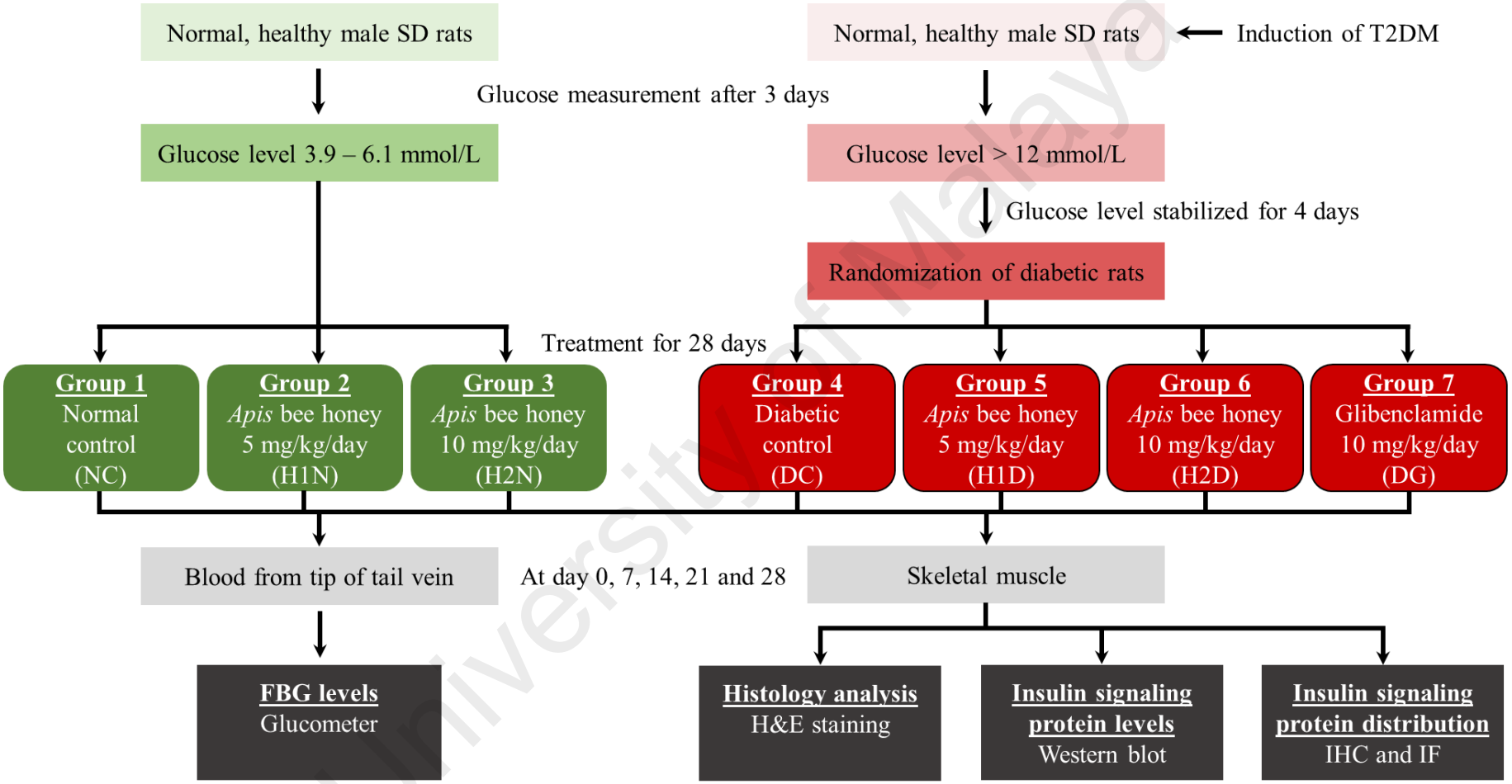

Figure 3.1: Study design of the present research.

3.2 T2DM induction in Sprague Dawley (SD) rats and FBG determination

Twelve-week old male SD rats weighing 200 ± 25 g were housed in a well-ventilated environment with natural light/dark cycle, temperature 25 ± 3°C, 5 – 6 rats per cage. The rats were fed with rodent food pellets and tap water ad libitum.

T2DM was induced in overnight (16 hours)-fasted male SD rats via a single intraperitoneal injection of 110 mg/kg bw of NA (dissolved in normal saline), followed by 55 mg/kg bw of STZ (dissolved in ice-cold 0.1 M citrate buffer, pH 4.5) 15 minutes later. Control rats received citrate buffer only (Aziz et al., 2017). Following these procedures, all rats were given access to standard rat chow diet (Harlan, Rossdoff, Germany) and drink was substituted with 5% glucose for 24 h to prevent hypoglycemia .

Three days after STZ-NA injection, T2DM was confirmed by the FBG level exceeding 12 mmol/L by using the Accu-Chek active glucometer (Accu-Chek, Roche Diagnostic, Meylan, France).

3.3 Experimental design

Four days after T2DM confirmation, heathy and diabetic rats were given *Apis* bee honey once daily for 28 consecutive days by using oral gavage. Experimental SD rats were randomized into 7 groups as follows:

Group 1: Normal, non-diabetic control (NC)

Group 2: Normal, non-diabetic treated with *Apis* bee honey at 5 mg/kg bw/day (H1N)

Group 3: Normal, non-diabetic treated with *Apis* bee honey at 10 mg/kg bw/day (H2N)

Group 4: Diabetic control (DC)

Group 5: Diabetic, treated with *Apis* bee honey at 5 mg/kg bw/day (H1D)

Group 6: Diabetic treated with *Apis* bee honey at 10 mg/kg bw/day (H1D)

Group 7: Diabetic treated with 600 µg/kg bw/day (DG)

On day 0, 7, 14, 21 and 28 of 28-day treatment, blood from tip of tail vein was sampled for FGB measurement. Experimental rats were euthanized, and skeletal muscles were immediately excised and placed in formalin until further use.

3.4 LC-MS analysis of *Apis* bee honey

LC-MS is an analytical technique that integrates high resolution chromatographic separation with specific and sensitive mass spectrum detection. Mass spectrometry in LC-MS is essential in determining the elemental composition and structural elucidation of a sample. Principally, LC-MS is an integration of High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS) using ionization as an interface (Kumar & Vijayan, 2014). LC-MS is widely adopted in chemical, pharmaceutical and food industries for quantitative and qualitative analysis. LC-MS was chosen for the determination of the compounds in *Apis* bee honey in lieu of other methodologies owing to its sensitivity, specificity and ability to handle complex sample (Gautam et al., 2014; Long et al., 2018; Trammell & Brenner, 2013). In addition, LC-MS is more broadly applicable than Gas Chromatography–Mass Spectrometry (GC-MS) ascribed to the wider range of compounds that can be analysed (Pitt, 2009). The *Apis* bee honey was outsourced for an analysis of the compounds in *Apis* bee honey and the details of the analytical condition is depicted in Appendix 1.

LC-MS analysis involves several discrete stages (Figure 3.2). Firstly, the sample components are separated using HPLC column where the analytes are differentially partition between mobile phase (eluent) and stationary phase (coated onto supporting material and packed into the column). The mode of chromatography, surface localisation ion exchange, ion-pair and hydrophobic interaction dictate the mechanism of retention and separation (Qin, Wang, & analysis, 2018). The subsequent separated sample species are sprayed into Atmospheric Pressure Ion Source where the sample species are transmuted into ions in the gas phase and majority of the eluent is pumped to waste (Kang, 2012; Kumar & Vijayan, 2014).

Thereafter, the mass analyser sorts the ions in relation to their mass to charge (*m/z*) ratio. Widely used analyser include Quadruple, Ion Trap, Time of Flight and Magnetic Sector. Also, mass analyser can be adopted to examine all ion *m/z* ratios or to isolate ions of specific *m/z* ratio present in the sample. Following mass analyser, the detector computes the ions emerging from the mass analyser and may also amplify the signal engendered from each ion. Most popular detector types include dynode, electron multiplier, photodiode and multi-channel plate (Kumar & Vijayan, 2014; Ng et al., 2016). Resultantly, mass spectrum portraying ion signal as a function of the *m/z* ratio is built, thenceforth the masses and chemical structures of compounds as well as the elemental isotopic nature of a sample can be determined (Kumar & Vijayan, 2014).

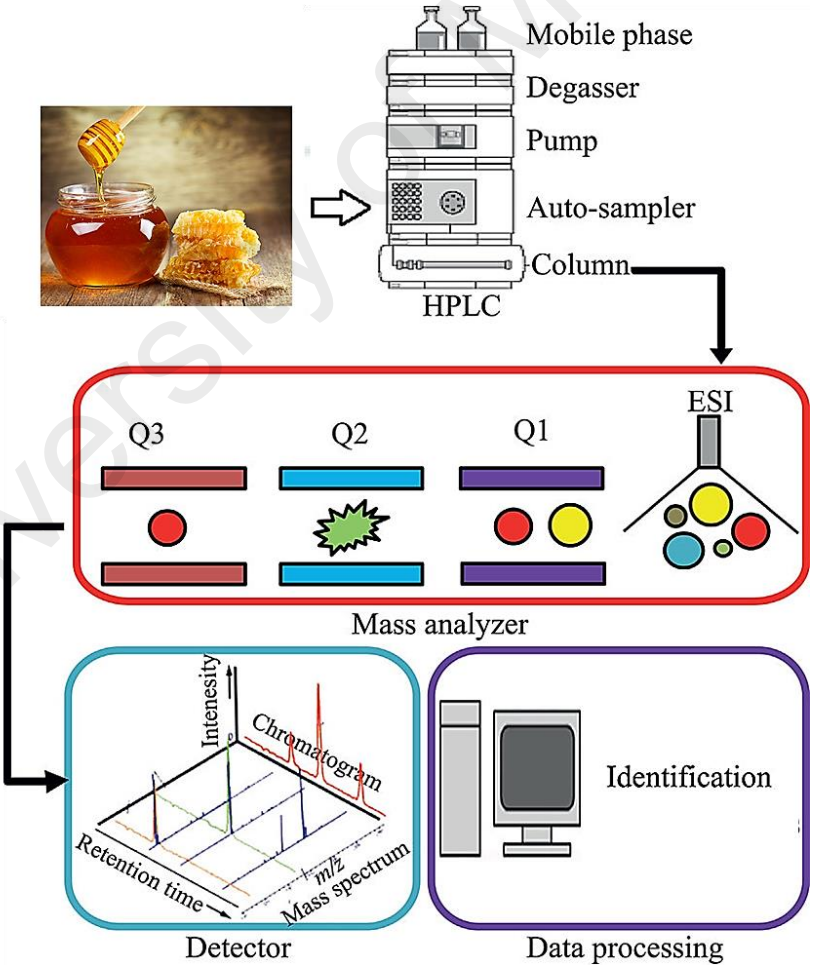

Figure 3.2: LC-MS workflow used in identification of the compounds in *Apis* bee honey.

3.5 Histological study

3.5.1 Skeletal muscle processing and slides preparation

At the beginning of histological studies such as H&E, IHC and IF, skeletal muscles were fixed in 10% formalin overnight to preserve the skeletal muscle, chemical compounds and microanatomic constituents *in vivo* in relation to each other. Subsequently, the tissue processing was performed with gentle agitation overnight using a tissue processor. Tissue processing was carried out according to Aziz et al. and the steps are summarized in Table 3.1.

During tissue processing, the tissues were dehydrated through a series of more concentrated ethanol baths to eliminate water and fixative from the tissues and replacing them with ethanol. For tissue clearing purpose, xylene, a solvent that is miscible with both ethanol and paraffin wax, was used to replace ethanol and making the tissue constituents receptive to the infiltrating paraffin wax. Subsequently, tissues were kept in melted paraffin bath for a total of 2 h so that the tissues were rendered sufficiently firm yet elastic for tissue sectioning. Once the tissues were embedded with paraffin wax, tissues blocks were prepared with moulds, allowed to solidify and stored in -20 °C for 2 days to ease the tissue sectioning(Aziz et al., 2017).

By using rotary microtome, the paraffin blocks of skeletal muscles were cut into thin cross section of 5 μm mounted onto poly-L-lysine coated glass slides in a floatation bath. All slides were incubated at 37˚C for at least 3 days to allow proper adherence of skeletal muscle sections onto the slides. The skeletal muscle sections were kept in room temperature for H&E, IHC and IF staining. All the consumables and chemicals used in the present study is listed in Table 3.2 and Table 3.3.

Table 3.1: Steps for tissue processing.

Procedure	Duration
Dehydration	
70% alcohol	1 h
95% alcohol	1 h
Absolute alcohol I	1 h
Absolute alcohol II	1.5 h
Absolute alcohol III	1.5 h
Absolute alcohol IV	2 h
Clearing	
Xylene I and II	Each xylene 1 h
Tissue impregnation	
Paraffin wax I and II at 58°C	Each paraffin wax 1 h

Table 3.2: List of consumables/ chemicals used in the present study.

No.	Consumables/ Chemicals	Company
1.	Acrylamide	Vivant is Inc., USA
2.	Aluminium Potassium Sulphate Dodecahydrate	Sigma-Aldrich (USA)
3.	Ammonium persulphate (APS)	HiMedia Laboratories Pvt, Ltd, India
4.	Antibodies (primary and secondary)	Santa Cruz Biotechnology, USA
5.	Bis-acrylamide	Vivant is Inc., USA
6.	Blocking One	Nacalai Tesque, Kyoto, Japan
7.	BLUeye Pre-stained Protein Ladder	GeneDrex, Bio-Helix, Co., Taiwan
8.	Bovine Serum Albumin (BSA)	Sigma-Aldrich (USA)
9.	Cover slip	Marienfeld Superior, Germany
10.	Di-Sodium Hydrogen Phosphate Anhydrous (Na_2HPO_4)	Friendermann Schmidt, Australia
11.	DPX mounting media	R&M Chemicals, Malaysia
12.	Eosin	R&M Chemicals, Malaysia
13.	Ethanol	John Kollin Chemicals, UK
14.	Glycerol	Sigma-Aldrich (USA)

Table 3.2 continued: List of consumables/ chemicals used in the present study.

No.	Chemicals/ Consumables	Company
15.	Glycine	HiMedia Laboratories Pvt, Ltd, India
16.	Greiner UV Transparent 96 Well Plate	Thermos Fisher, USA
17.	Haematoxylin	Sigma-Aldrich (USA)
18.	Paraplast (paraffin wax)	Leica Biosystems, Wetzlar, Germany
19.	Peroxidase Stain Diaminobenzidine (DAB) Kit	Nacalai Tesque, Kyoto, Japan
20.	Pierce™ BCA Protein Assay Kit	Thermos Fisher Scientific, USA
21.	Pierce 1- Step Transfer buffer	Thermos Fisher Scientific, USA
22.	Phosphate Buffer Saline (PBS)	HiMedia Laboratories Pvt, Ltd, India
23.	Poly-L-lysine coated glass slides	Thermo Scientific, USA
24.	Polyvinylidene difluoride (PVDF) membrane	Bio-Rad Laboratories Inc., USA
25.	Potassium chloride	Friendermann Schmidt, Australia
26.	Potassium Dihydrogen Phosphate Anhydrous (K_2HPO_4)	Friendermann Schmidt, Australia
27.	PRO-PRE-lysis buffer	iNtRON, Biotechnology, Korea
28.	Sodium chloride	Vivant is Inc., USA
29.	Sodium dodecyl sulphate (SDS)	Vivantis Ine, USA
30.	Tetramethylenediaminse (TEMED)	Sigma-Aldrich (USA)
31.	Tris base	Promega Corporation, USA
32.	Trisodium citrate dihydrate	Friendermann Schmidt, Australia
33.	Tween-20	Croda international Plc, UK
34.	UltraCruz™ Mounting Media (DAPI)	Nacalai Tesque, Kyoto, Japan
35.	Xylene	Friendermann Schmidt, Australia
36.	2-mercaptoethanol	Sigma-Aldrich (USA)
37.	30% H_2O_2	HiMedia Laboratories Pvt, Ltd, India

3.5.2 H&E staining

H&E staining was carried out to visualize the alterations in the architecture of skeletal muscle. The standard procedure of H&E staining is summarized in Table 3.4 (Aziz et al., 2017). The skeletal muscle sections from each experimental group were selected and deparaffinized by 3 changes of xylene. Sections were then undergoing hydration process by a series of less concentrated ethanol before being immersed in distilled water (dH_2O) and stained with filtered hematoxylin.

Following the hematoxylin staining, sections were washed with tap water until no more excess hematoxylin available and further differentiated by acid alcohol for few seconds to eliminate excess background staining. Bluing of sections was completed by using tap water to transform nuclear coloration from reddish purple to crisp blue purple before staining with eosin. Hematoxylin stains the nucleus while eosin stains the cytoplasm and connective tissues. Subsequently, sections were dehydrated by a series of more concentrated ethanol and water residue in sections were cleared by xylene. Slides were left to air dry before being mounted and covered with coverslip.

Table 3.3: Standard procedure of H&E staining.

Procedure	Allocated time
Deparaffinization	
Xylene I, II and III	Each solvent 3 mins
Rehydration	
Absolute alcohol I and II	Each solvent 5 mins
95% alcohol I and II	Each solvent 5 mins
80% alcohol	5 mins
70% alcohol	5 mins
50% alcohol	5 mins
dH_2O	5 mins
Staining	
Hematoxylin	20 mins
Running tap water	Until light to moderate hematoxylin stain is observed on the sections
Differentiation	
Acid alcohol	2 to 5 s
Bluing	
Running tap water	5 mins
Eosin	5 mins
Dehydration	
95% alcohol I and II	Each solvent 5 mins
Absolute alcohol I, II and III	Each solvent 5 mins
Clearing	
Xylene I, II and III	Each solvent 3 mins
Mounting	
DPX media and cover slip	Leave overnight to dry

3.6 Determination of the levels of insulin signalling proteins by WB

WB was used to determine the protein levels of IRβ, IRS1, PI3K, Akt and GLUT4 in skeletal muscles of experimental rats. The work flow of WB is depicted in Figure 3.3.

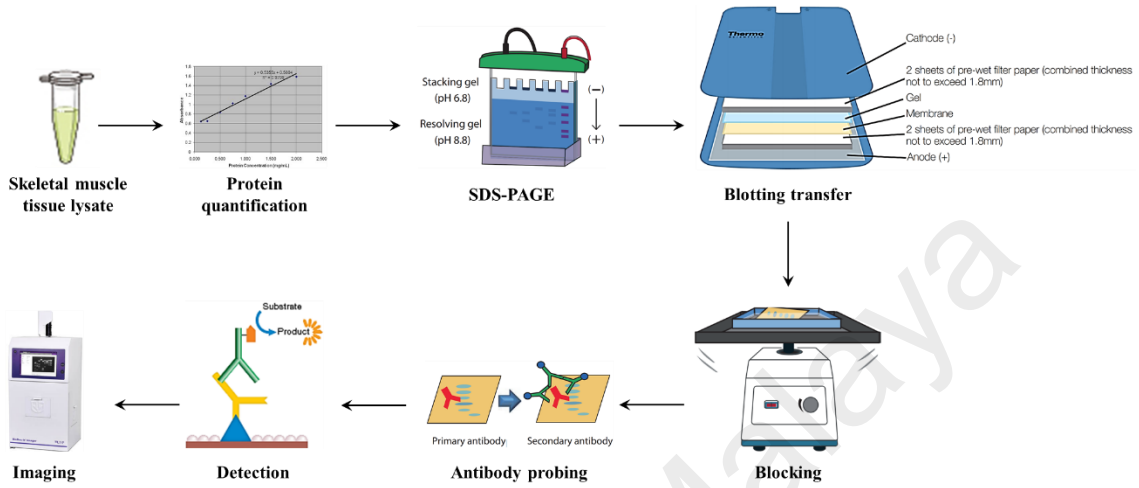

Figure 3.3: Workflow of WB.

3.6.1 Protein extraction

Skeletal muscles were snap frozen in the liquid nitrogen and then stored at -80 °C prior to protein extraction. Approximately 30 mg skeletal muscle was soaked in 400 µl of PRO-PREP extraction solution (Intron, Korea), a protein extraction solution constituted of a cocktail of buffers, detergents and protease inhibitors. The skeletal muscles were then homogenized at room temperature for 5 mins and incubated at -20 °C for 30 mins to induce cell lysis and thereby increasing the protein concentration. Thereafter, tissue lysate was centrifuged at 13000 g and 4 °C for 15 mins to separate the protein pellet from supernatant. Ultimately, the protein samples were stored at -20 °C for future protein analysis via WB.

3.6.2 Protein quantification

Micro BCA Protein Assay Kit (Thermo Scientific^{TM}-US), a detergent-compatible bicinchoninic acid (BCA) formulation, was utilized for the colorimetric determination and quantitation of total protein concentrations in skeletal muscle protein samples. BCA Assay was chosen in lieu of the other dye-binding methods attributed to its stability (less influenced by protein compositional differences) and compatibility with the colorimetric detection and quantification of total proteins.

In an alkaline environment, protein reduces cupric ion (Cu^{2+}) to cuprous ion (Cu^{+}). Following chelation of 2 molecules BCA with 1 Cu^{+} ion generates a purple-coloured reaction product. This water-soluble complex demonstrated a strong absorbance at 562 nm wavelength, corresponding linearly with elevating protein concentrations. Initially, standard concentrations of bovine serum albumin (BSA) were prepared at 0, 0.5, 1, 2.5, 5, 10, 20 and 40 μg/ml. Then the samples were also diluted 1 in 50 with PBS buffer. In a Greiner UV transparent 96 well plates (Thermo ScientificTM-US), 50 μl of standard was added to 50 μl of BSA reagent as a serial dilution. In a same manner, 50 μl of each protein sample was added to 50 μl BSA in triplicate.

After aliquoting the standards and samples in the plate, the plate was sealed with sealing tape and incubated for 2 h at 37 °C. Before measuring the absorbance at 570 nm wavelength, the contents in plate was mixed thoroughly on a plate shaker for 30 s. From the absorbance values enumerated, standard curve and protein concentrations of the skeletal muscle samples can be computed.

3.6.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is an electrophoretic technique extensively utilized for separation of protein, thenceforth determination of the relative abundance of major proteins in a sample. SDS-PAGE works upon the principle that the charge molecules migrate towards the oppositely charged electrode. Under reducing condition, disulphide bonds maintaining the polypeptide subunits and three-dimensional (3D) structures are disrupted. In addition, protein treatments with 2-mercaptoethanol completely denatures and dissociates the proteins, leading to polypeptide backbone unfolding, eventually complexation of proteins with SDS (Mahmood & Yang, 2012).

SDS-polypeptide complexes in an electric field migrate towards anode are simultaneously separately by sieving effect based on size, thereby allowing the MW of the polypeptide subunits to be reasonably calculated with the guide of MW standard. In detail, the sieving characteristics of polyacrylamide gels are contributed by the three-dimensional networks of pores and fibres established by the crosslinking between bifunctional reagent N,N'-methylene-bis-acrylamide (Bis) and adjacent polyacrylamide chains, catalysed by a free-radical initiated vinyl polymerization mechanism constituted of APS and TEMED.

In an inversely proportional relationship, effective pore size of the polyacrylamide gel decreases as the acrylamide concentration increases. A decreasing pore size of the gel limits the migration rate of a protein thorough the gel. In addition, the electrophoretic mobilities of the SDS-polypeptide complexes assume the same functional relationship to their molecular weights (MWs) where higher MW SDS-polypeptide complexes migrate slower than the smaller ones (Brunelle & Green, 2014).

SDS-PAGE utilizes stacking gel and resolving gel of appropriate acrylamide concentration according to the MW range of the proteins to be separated. The stacking gel casted above the resolving gel is slightly acidic (pH 6.8), its lower acrylamide concentration creates a porous gel that separate proteins deficiently but allows the formation of thin and sharply defined bands. The lower resolving gel is basic (pH 8.8), gel with higher acrylamide concentration), its higher acrylamide concentration produces polyacrylamide gel of smaller pore sizes, hence separating the proteins more effectively based on the protein sizes (Brunelle & Green, 2014).

Four percent stacking gel was used for all proteins; 10% resolving gel for IRS1 protein while 12% resolving gel for IRβ, PI3K, Akt and GLUT4 protein identifications (Table 3.5). Practically, a MW marker was loaded at the first well to determine the MW of the sample proteins in the gel. Together with loading dye, sample proteins were denatured at 100°C for 5 mins and subsequently loaded in the remaining wells. The gel was then connected to the power supply and allowed to run for 1.5 h. Appropriate voltage was set as not to overheat and distort the bands.

Table 3.4: Compositions of both resolving and stacking gels.

Components	10% Resolving gel (µl)	12% Resolving gel (µl)	4% Stacking gel (µl)
ddH_20	3800	3200	2975
30% Acrylamide	3400	4150	670
1.5 M Tris (pH 8.8)	2600	2600	1250
10% SDS	100	100	50
10% APS	100	100	50
TEMED	10	10	5

3.6.4 Membrane transfer

Following SDS-PAGE, the gel containing separated proteins was placed in the transfer sandwich (filter paper-gel-PVDF membrane-filter paper) as shown in Figure 3.3. Following the assembly of transfer sandwich, removal of trapped air bubbles using a blot roller was crucial to secure a high-quality transfer. The electrophoretic protein transfer was completed using Thermo Scientific™ Pierce™ Power Blotter where an electric field oriented perpendicular to the surface of the protein-containing polyacrylamide gel, causing a migration of the proteins from the gel onto the PVDF membrane. PVDF membrane was preferred in lieu of nitrocellulose membrane attributed to its hydrophobic nature showing higher binding affinity to proteins (Mahmood & Yang, 2012).

3.6.5 Blocking stage

Following electrophoretic protein transfer, blocking step was performed to inhibit the non-specific binding of antibody probes to the membrane surface hence reduces background signals. Membrane was transferred to 5% BSA blocking solution and agitate for 1.5 h at room temperature. In lieu of non-fat milk, BSA blocking solution is preferred with biotin and alkaline phosphatase antibody labels and it can be utilized to detect phosphorylated proteins. Unlike BSA, milk possesses casein, a phosphoprotein capable of binding to anti-phospho antibodies thenceforth leading to non-specific binding and high background signals (Mahmood & Yang, 2012).

3.6.6 Incubation with primary and secondary antibodies

The transferred proteins on membrane were then complexed with primary antibody at 1: 1000 dilution in PBS-T (PBS containing 1% BSA and Tween-20) with gentle agitation overnight in 4°C. Before incubation with secondary antibodies, the membranes were washed thrice in PBS-T for 5 mins each on a shaker to assure even agitation. The membranes were then incubated with species appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at a dilution of 1: 2000 for 1 h at room temperature. The specific primary and secondary antibodies for detecting IRβ, IR, PI3K, Akt and GLUT4 proteins are listed in Table 3.6.

Table 3.5: List of primary and secondary antibodies used in IHC and WB.

No.	Target protein	Primary antibody	Secondary antibody
1.	IRβ	Rabbit mAb	Goat anti-rabbit IgG-HRP
2.	IRS1	Mouse mAb	Donkey anti-mouse IgG-HRP
3.	PI3K	Goat pAb	Donkey anti-goat IgG-HRP
4.	Akt	Rabbit mAb	Goat anti-rabbit IgG-HRP
5.	GLUT4	Rabbit pAb	Goat anti-rabbit IgG-HRP
6.	GAPDH	Rabbit pAb	Goat anti-rabbit IgG-HRP

3.6.7 Detection of proteins

The membranes were treated with DAB, a colorimetric HRP substrate, from the Peroxidase Stain DAB Kit (Nacalai Tesque, Kyoto, Japan) for 5 mins with gentle agitation. Immediately after the treatment, the signal is the strongest and declines over time ascribed to the reaction kinetics of the HRP-conjugated secondary antibodies and substrate. The visualized protein bands on the membrane were captured by a gel documentation system (UVP Biospectrum 410, USA) and the density of each protein band was quantitated by using ImageJ software (NIH ImageJ, version 1.46j; National Institutes of Health, Bethesda, MD, USA). Thenceforth, the ratio of each protein band/reference protein reflecting the expression level of the target protein was calculated.

3.7 Visualisation of the distribution of insulin signalling proteins by IHC and IF

The standard procedure of IHC and IF immunostaining was summarized in Table 3.7 and Table 3.8, and Table 3.9 respectively. Under standard condition of illumination and the use of NIS-Element AR program (Nikon Instruments Inc, Melville, NY, USA), images of the immunostained skeletal muscle sections were captured with Nikon DS Ri1 12-megapixel camera (Nikon, Tokyo, Japan) on the Nikon Eclipse 80i microscope (SEO Enterprises, Inc, Lakeland, FL, USA), subsequently saved in Tagged Image File Format (TIFF).

Table 3.6: Standard procedure of IHC immunostaining.

Procedure	Allocated time
Deparaffinization	
Xylene I, II and III	Each solvent 5 mins
Rehydration	
Absolute alcohol I and II	Each solvent 10 mins
95% alcohol I and II	Each solvent 10 mins
dH_2O	1 min
Antigen retrieval	
10 mM sodium citrate buffer, pH 6	15 mins at 100°C
Cooling	30 mins
dH_2O	2 mins
Blocking endogenous peroxidase activity	
3% H_2O_2	30 mins
PBS	10 mins
Blocking non-specific sites	
Blocking One	1.5 h
Primary antibody	
Target protein specific-primary antibody	Overnight at 4°C
PBS	15 mins
Conjugated secondary antibody	
Species specific-conjugated secondary antibody	1.5 h at 4°C
dH_2O	15 mins
Chromogen staining	
Peroxidase Stain DAB	5 mins
dH_2O	5 mins

Table 3.6 continued: Standard procedure of IHC immunostaining.

Procedure	Allocated time
Counterstaining	
Hematoxylin	20 s
Running tap water	Until light to moderate hematoxylin stain is observed on the sections
Dehydration	
95% alcohol I and II	Each solvent 10 s
Absolute alcohol I and II	Each solvent 10 s
Clearing	
Xylene I, II and III	Each solvent 10 s
Mounting	
DPX media and cover slip	Leave overnight to dry

Table 3.7: Standard procedure of IF immunostaining for GLUT4.

Procedure	Allocated time
Deparaffinization	
Xylene I, II and III	Each solvent 5 mins
Rehydration	
Absolute alcohol I and II	Each solvent 10 mins
95% alcohol I and II	Each solvent 10 mins
dH_2O	1 min
Antigen retrieval	
10 mM sodium citrate buffer, pH 6	15 mins at 100°C
Cooling	30 mins
dH_2O	2 mins
Blocking non-specific sites	
Blocking One	1.5 h
Primary antibody	
Rabbit pAb	Overnight at 4°C
PBS	15 mins
Conjugated secondary antibody	
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	1.5 h at 4°C
PBS	15 mins
Mounting	
DAPI and cover slip	Immediate imaging

3.8 Statistical analysis

Statistical analysis was conducted by GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Each group constituted of 3 rats. All data are presented as mean ± standard error of mean (SEM). Group means were compared with one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test. Values of $p < 0.05$ were considered significant.

CHAPTER 4: RESULTS

4.1 Detection of the major components in *Apis* bee honey with LC-MS

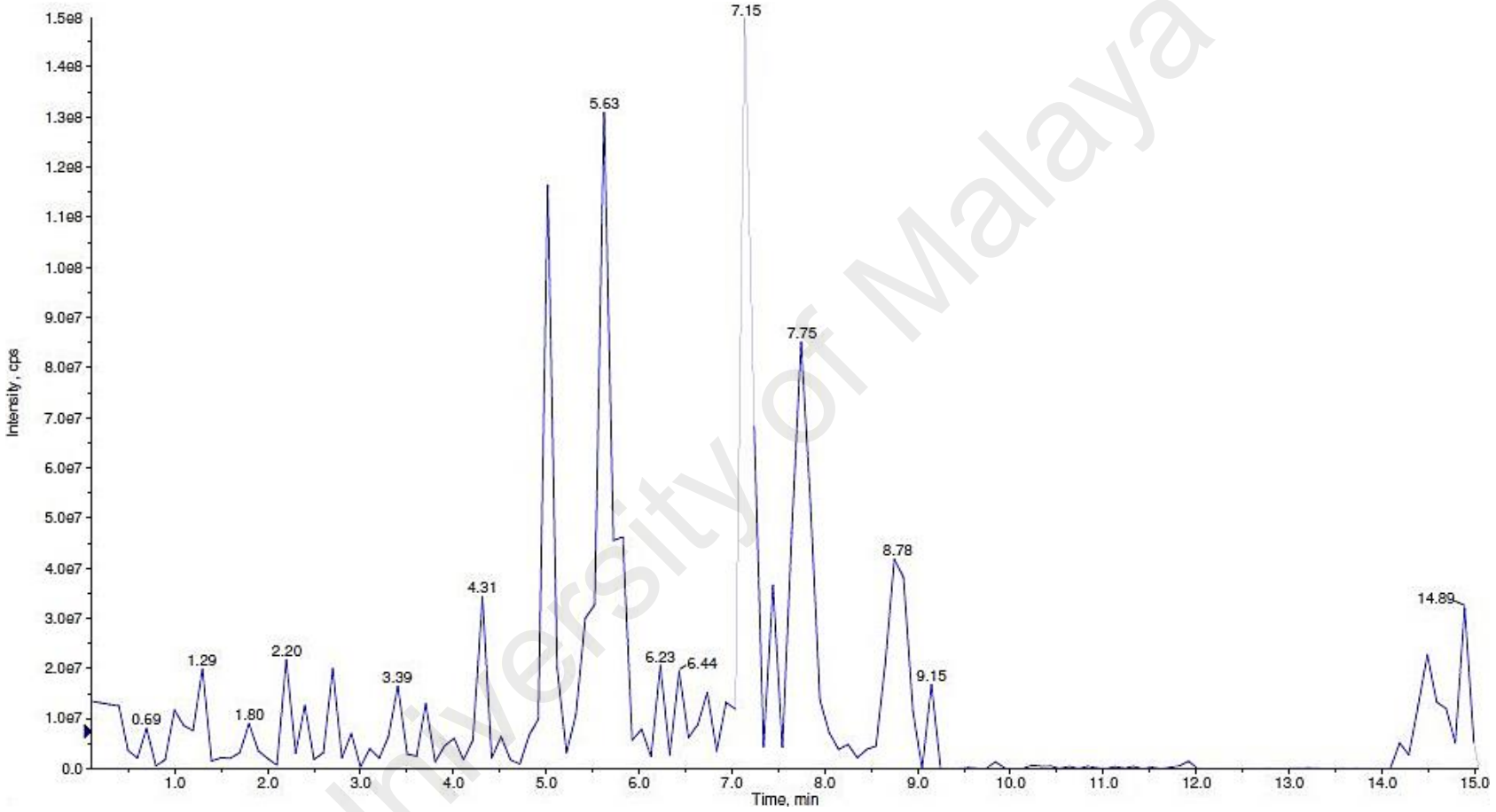

Figure 4.1: LC-MS chromatogram of *Apis* bee honey. The x-axis depicts retention time in mins, the y-axis depicts the intensity.

Table 4.1: Compounds identified in *Apis* bee honey by LC-MS with Electrospray ionization (ESI). RT; retention time; m/z (mass-to-charge ratio).

No.	Compound name	RT (mins)	m/z	ms/mz
1.	(epi)afzelechin-(epi)catechin conjugate	3.401	203.1	161.1 195.1
2.	15,16-dihydroxy- 9Z,12Z-octadecadienoic acid or oxooctadecanoic acid conjugate	6.936	183	119.1 339.2
3.	2',6'-dihydroxy-4'-methoxydihydrochalcone derivative	5.219	271.2	225.1 182
4.	Apigenin derivative	6.330	269.2	197.2 225.2
5.	Benzenoid compound from root conjugate	4.007	201.1	185.1 139.1
6.	Benzoic acid from mushroom derivative	3.100	121.0	181.1 108.1
7.	Caprylic acid conjugate	5.420	142.0	114.0 206.0
8.	Caprylic acid derivative	5.824	142.0	114.0 206.0
9.	Chalcone based compounds derivative	2.700	279.2	-
10.	Cinnamic acid from mushroom derivative	3.703	117.0	119.0 147.0
11.	Gluconic acid derivative	8.150	195.0	129.0 114.0
12.	Gluconic acid derivative	7.342	195.0	131.0 129.0
13.	Gluconic acid	1.297	195.1	129.0 177.0
14.	Hydroxybenzoic acid-O-hexoside conjugate	5.320	136.0	200.0 109.0
15.	Hydroxybenzoic acid-O-hexoside conjugate	2.199	137.0	461.2
16.	Isorhamnetin 3-O-rutinoside or isorhamnetin 3-O-neohesperidoside isomer	4.312	315.1	299.0 624.1
17.	Linolenic acid from extract	6.128	277.2	233.2

Table 4.1 continued: Compounds identified in *Apis* bee honey by LC-MS with ESI.

No.	Compound name	RT (mins)	m/z	ms/mz
18.	Oxooctadecanoic acid derivative	8.250	297.3	183.0 279.2
19.	p hydroxybenzoic acid conjugate	6.734	136.0	109.0 200.0
20.	p hydroxybenzoic acid derivative	4.614	137.1	138.1 121.1
21.	Phenobarbitol conjugate	5.925	186.0	121.0
22.	Phytol derivative	2.900	295.2	251.3 277.2
23.	Phytol derivative	0.696	295.3	276.9 167.1
24.	Protocatechuic acid hexoside conjugate	1.195	297.3	279.3 139.1
25.	Protocatechuic acid hexoside conjugate	14.692	297.3	279.2 253.3
26.	Protocatechuic acid hexoside conjugate	1.095	297.3	279.2 171.1
27.	Syringic acid-hexose derivative conjugate	7.139	831.4	277.3 255.2
28.	Tannic acid std derivative	4.412	183.1	139.0 125.2
29.	Vanilic acid derivative	3.502	167.1	123.1 122.0 152.1
30.	Zerumbone from zinger conjugate	3.906	173.1	111.1 157.1

Table 4.2: Compounds showing highest abundance in *Apis* bee honey by LC-MS with ESI. RT: Retention Time.

No.	Compound name	RT (mins)	m/z	ms/mz
1.	(epi)afzelechin-(epi)catechin conjugate	3.401	203.1	161.1 195.1
2.	Gluconic acid	1.297	195.1	129.0 177.0
3.	Hydroxybenzoic acid-O-hexoside conjugate	2.199	137.0	461.2
4.	Isorhamnetin 3-O-rutinoside or isorhamnetin 3-O-neohesperidoside isomer	4.312	315.1	299.0 624.1
5.	Syringic acid-hexose derivative conjugate	7.139	831.4	277.3 255.2

Table 4.3: Category of compounds identified in *Apis* bee honey.

Polyphenol	Sub-class	Compound
Flavonoid	Proanthocyanidin	**(Epi)afzelechin-(epi)catechin**
	Chalcone	Chalcone based compounds derivative
	Dihydrochalcones	2',6'-dihydroxy-4'-methoxydihydrochalcone derivative
	Flavones	Apigenin derivative
		Cinnamic acid from mushroom derivative
	Flavonol	**Isorhamnetin 3-O-rutinoside or isorhamnetin 3-O-neohesperidoside isomer**
Benzenoid	-	Benzenoid compound from root
Phenolic acid	Benzoic acid	Benzoic acid from mushroom derivative
	Hydroxybenzoic acid	**Hydroxybenzoic acid-O-hexoside**
		p hydroxybenzoic acid
		p hydroxybenzoic acid derivative
		Protocatechuic acid hexoside conjugate
		Syringic acid-hexose derivative
		Vanilic acid derivative
Tannin	-	Tannic acid std derivative
Monosaccharide derivative	Sugar acid	**Gluconic acid** Gluconic acid derivative
Lipid	Polyunsaturated omega-6 fatty acid	Oxooctadecanoic acid
	Polyunsaturated fatty acid	Linolenic acid from extract
	Saturated fatty acid	Caprylic acid
		Caprylic acid derivative
	Long-chain fatty acid	Oxooctadecanoic acid derivative
	Diterpene	Phytol derivative
	Sesquiterpene	Zerumbone from zinger

Compounds found in high abundance in *Apis* bee honey are bolded.

From the analysis of *Apis* bee honey component by LC-MS, a total of 30 components were detected in our *Apis* bee honey sample (Table 4.1 and Table 4.2). Figure 4.1 shows the LC-MS chromatogram of *Apis* bee honey. Among the 30 identified components, 5 components namely (epi)afzelechin-(epi)catechin conjugate, gluconic acid, hydroxybenzoic acid-O-hexoside conjugate, isorhamnetin 3-O-rutinoside or isorhamnetin 3-O-neohesperidoside isomer and syringic acid-hexose derivative conjugate show highest abundance in *Apis* bee honey (Table 4.3).

As shown in Table 4.4, the 30 identified components are further classified into categories of phytochemicals. Among the 5 components showing highest abundance, epi)afzelechin-(epi)catechin conjugate and isorhamnetin 3-O-rutinoside or isorhamnetin 3-O-neohesperidoside isomer are flavonoids, gluconic acid is a monosaccharide derivative, hydroxybenzoic acid-O-hexoside conjugate and syringic acid-hexose derivative conjugate are phenolic acids.

4.2 Effect of *Apis* bee honey on fasting blood glucose (FBG) levels

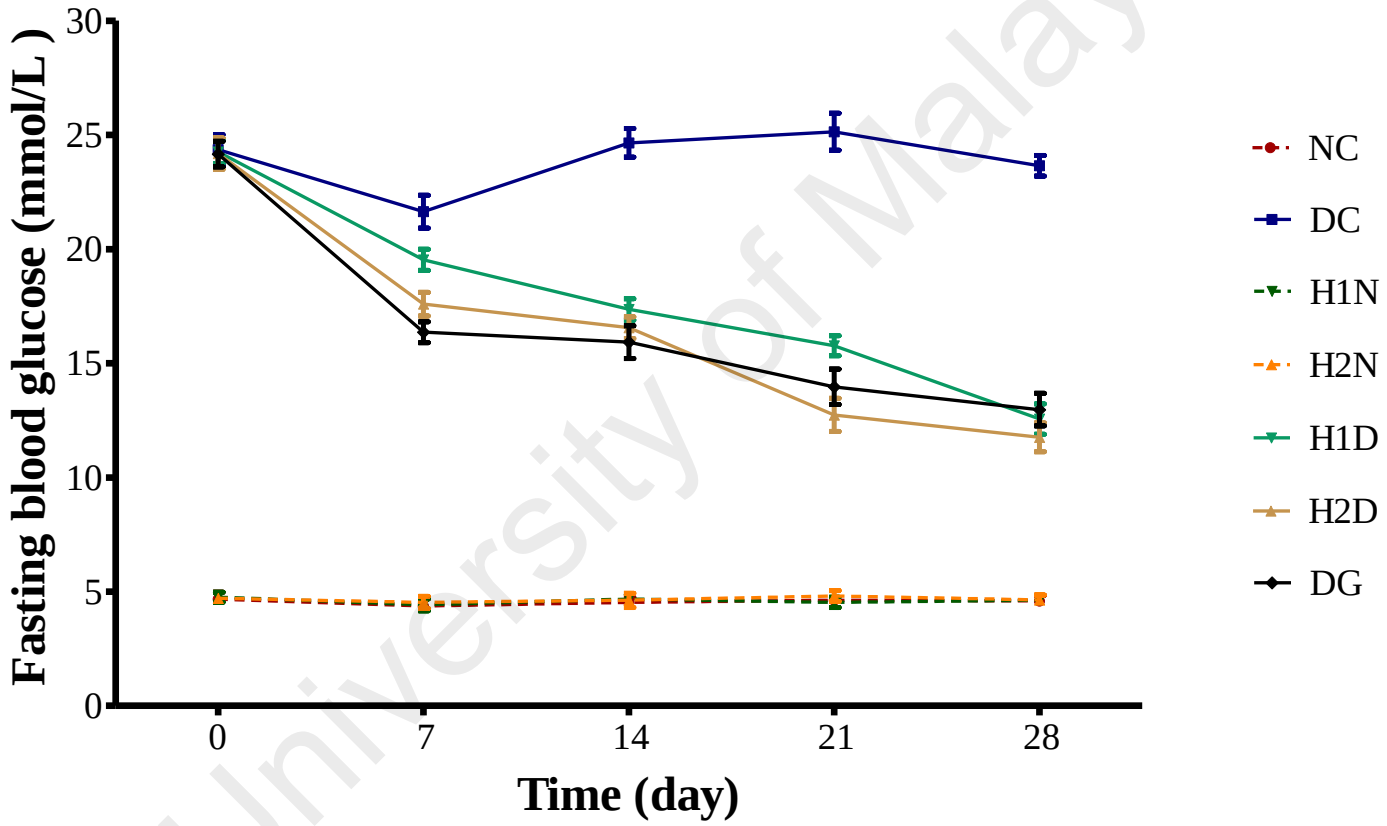

Figure 4.2: Effect of *Apis* bee honey on FBG levels of studied groups (n = 3 in each group). Comparison is made between the non-diabetic rats and all 4 groups of diabetic rats.

On day 0, the FBG levels were significantly higher in the diabetic rats than that in non-diabetic rats ($p < 0.01$), confirming hyperglycemia in diabetic rats. Following a slight decline on day 7, the FBG levels escalated and maintained at a steady state of hyperglycemia in diabetic rats throughout the experiment period. Similar as non-diabetic rats, low- and high-dose honey treated non-diabetic rats did not show pronounced changes in weekly FBG levels.

From day 7 to day 28, glibenclamide, low- and high-dose honey treated-diabetic rats manifested a steady decrease in FBG levels, which were significantly lower than that in diabetic rats at weekly time points. At day 7, glibenclamide-treated diabetic rats showed the lowest FBG levels, followed by high- and low-dose honey-treated diabetic rats. Towards the end of the experiment on day 28, glibenclamide, low- and high-dose honey treated-diabetic rats shared similar levels of FBG.

4.3 Effects of *Apis* bee honey on skeletal muscle histology Effect of *Apis* bee honey on skeletal muscle architecture of non-diabetic rats

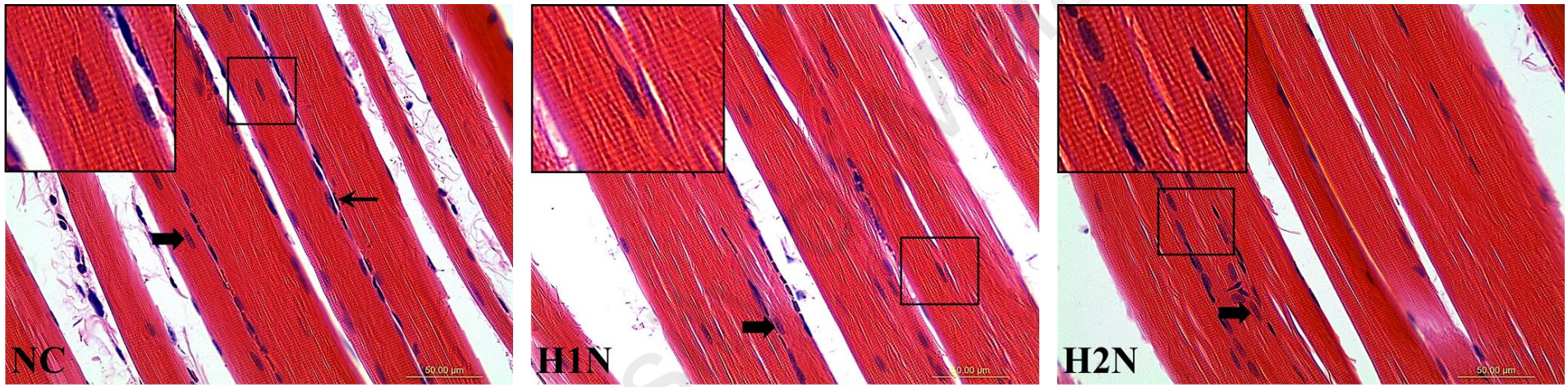

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Figure 4.3: Effects of *Apis* bee honey on skeletal muscle architecture of non-diabetic rats (longitudinal sections). (A) NC, (B) H1N and (C) H2N groups demonstrated normal skeletal muscle architecture. NC: non-diabetic rat; H1N: low-dose honey treated non-diabetic rat; H2N: high-dose honey treated non-diabetic rat. Magnification x400. Scale Bar = 50 µm.

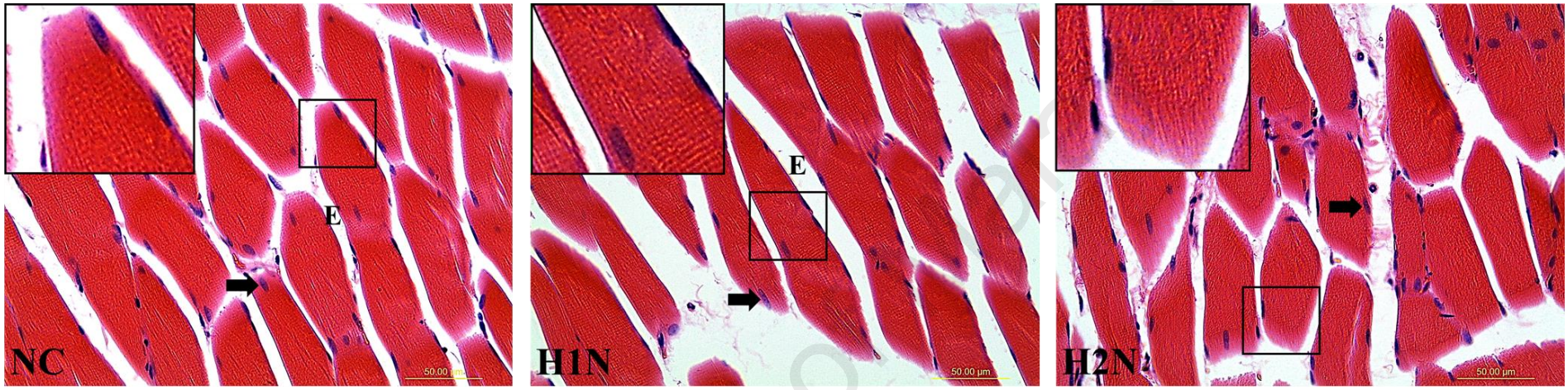

Figure 4.4: Effects of *Apis* bee honey on skeletal muscle architecture of non-diabetic rats (transverse sections). (A) NC, (B) H1N and (C) H2N groups demonstrated normal skeletal muscle architecture. NC: non-diabetic rat; H1N: low-dose honey treated non-diabetic rat; H2N: high-dose honey treated non-diabetic rat. Magnification x400. Scale Bar = 50 µm.

Examination of the H&E-stained longitudinal sections of the skeletal muscle of non-diabetic rats revealed normal architecture of the skeletal muscle as shown by the presence of myofibers separated by perimysium and these myofibers were connected by endomysium (Figure 4.3). The myofibers appeared long, parallel, non-branching, striated and cylindrical with acidophilic sarcoplasm. The nuclei were multiple, elongated, vesicular and peripherally located beneath the sarcolemma.

In transverse sections, the myofibers were polygonal shaped with acidophilic myofibrils and peripherally located nuclei (Figure 4.4). The endomysial connective tissue myofibers composed of some blood vessels. No changes to the skeletal muscle architecture was observed in non-diabetic rats following *Apis* bee honey treatments. The myofibers still possess clear striation and peripherally-located nuclei.

4.3.2 Skeletal muscle architecture in non-treated diabetic rats

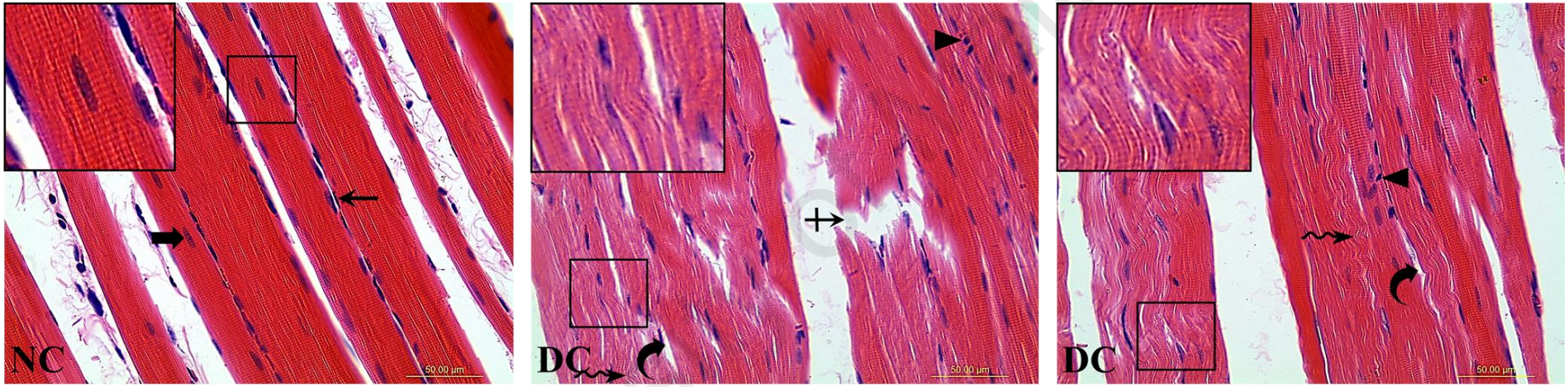

Figure 4.5: Skeletal muscle architecture of non-treated diabetic rats (longitudinal sections). (A) NC group showed bundles of non-branching cylindrical shaped myofibers with acidophilic sarcoplasm and multiple elongated vesicular nuclei (thick arrow) peripherally located beneath the sarcolemma. Notice the flat nuclei of fibroblast (thin arrow) in the endomysium between the myofibers. Enlarged image on the left upper corner shows cross-striated appearance of skeletal muscle. (B) and (C) Diabetic rats exhibited wavy course (wavy arrow) of myofibers with splitting (curved arrow), fragmentation (crossed arrow) of sarcoplasm, pyknotic central nuclei (arrow head) and hyalinisation of the sarcoplasm. NC: non-diabetic rat; DC: diabetic rat. Magnification x400. Scale bar = 50 µm

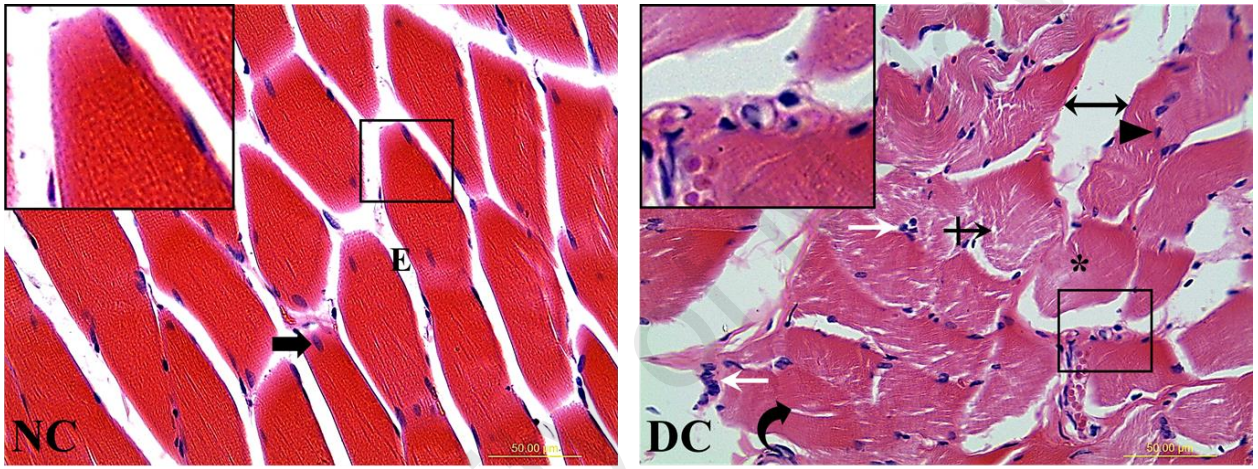

Figure 4.6: Skeletal muscle architecture of non-treated diabetic rats (transverse sections). (A) NC group showed polygonal shaped myofibers with acidophilic sarcoplasm and peripherally located nuclei (thick arrow). Myofiber bundles were separated by perimysium and individual myofibers were separated by endomysium (E). (B) DC group demonstrated vacuolization (enlarged image) and splitting (curved arrow) of myofibers, fragmentation of sarcoplasm (crossed arrow), pyknotic central nuclei (arrow head), mononuclear cellular infiltration (white arrow), hyalinisation of sarcoplasm (asterisk) and increased gap between myofibers (double headed arrow). NC: non-diabetic rat; DC: diabetic rat. Magnification x400. Scale Bar = 50 µm.

Longitudinal sections of the skeletal muscle of diabetic rats depicted degenerative changes including abnormal wavy course of the myofibers with loss of transverse striations, splitting of myofibers and fragmentation of sarcoplasm. In addition, pyknotic central nuclei and hyalinisation of the sarcoplasm were observed (Figure 4.5B). Compared to non-diabetic rats, transverse sections of diabetic rats demonstrated variations in the size and shape of myofibers, associated with cellular infiltration (Figure 4.6A).

4.3.3 Effect of *Apis* bee honey on skeletal muscle architecture of diabetic rats

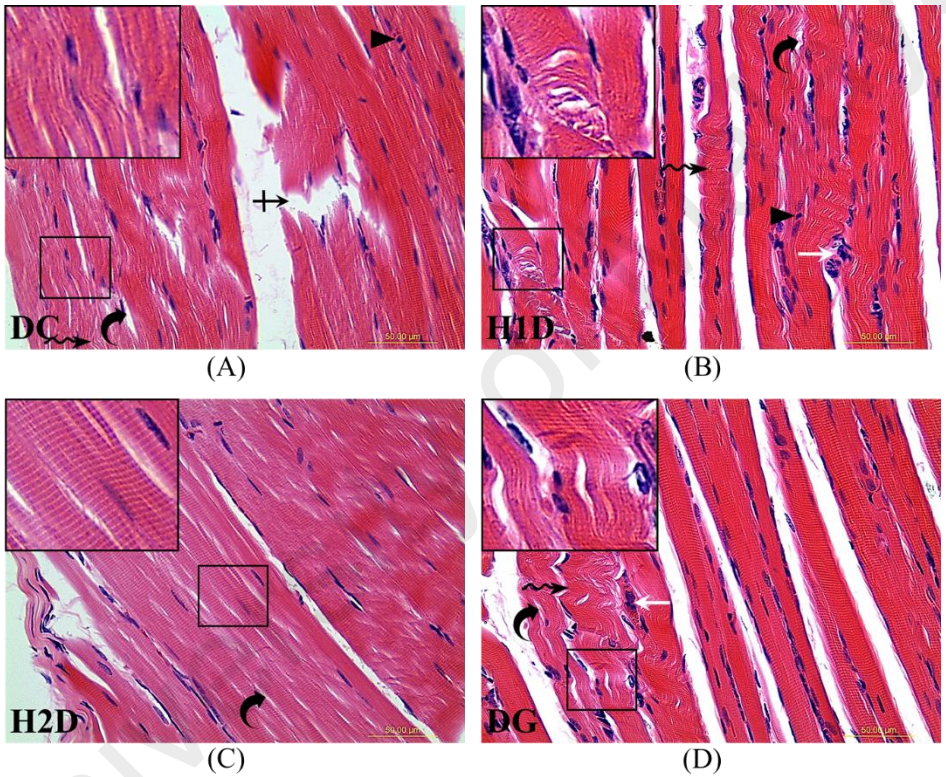

Figure 4.7: Effects of *Apis* bee honey on skeletal muscle architecture of diabetic rats (longitudinal sections). (A) DC group exhibited degenerated myofibers. In (B) H1D group, wavy course (enlarged image) of myofibers and myofiber splitting (curved arrow) were still clearly evident whereas only mild degeneration was observed in (C) H2D group. (D) DG group showed partially improved pathological changes where minimal myofiber splitting (curved arrow) were noticed. DC: non-treated diabetic rat; H1D: low-dose honey treated-diabetic rat; H2D: high-dose honey treated-diabetic rat; DG: glibenclamide-treated diabetic rat. Magnification x400. Scale Bar = 50 µm.

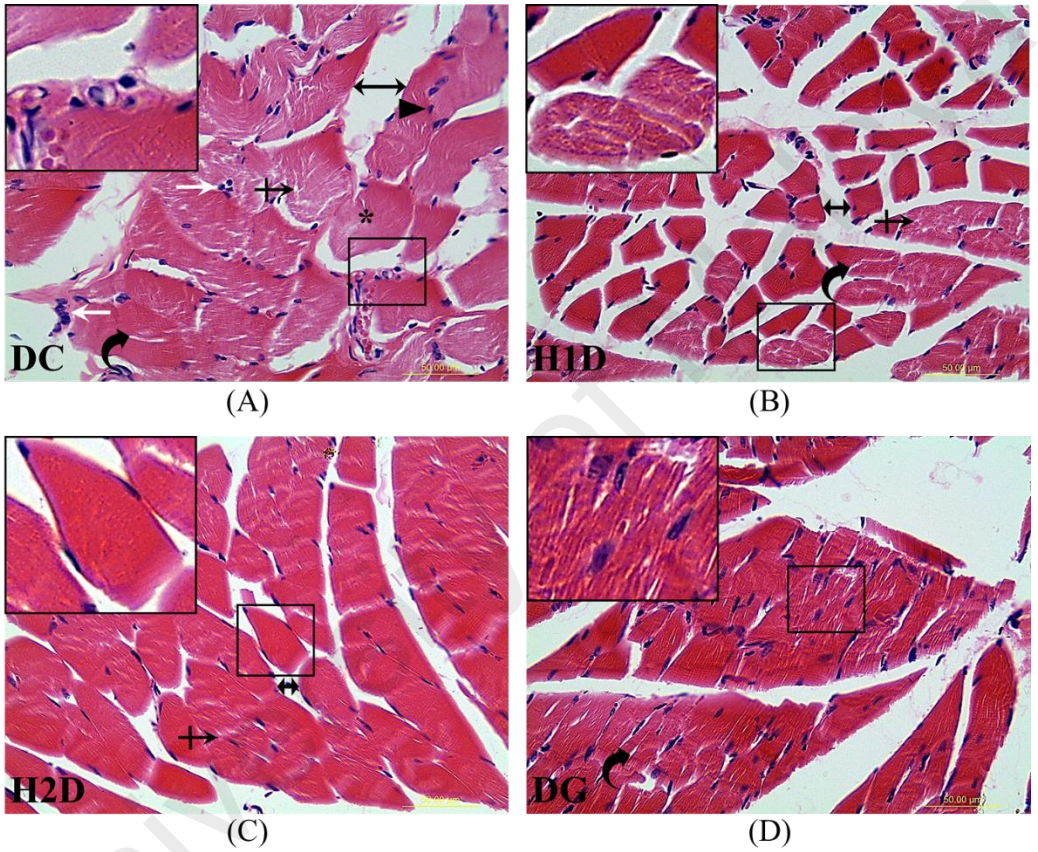

Figure 4.8: Effects of *Apis* bee honey on skeletal muscle architecture of diabetic rats (transverse sections). (A) DC group exhibited loss of polygonal shape of myofibers and widened gap (double headed arrow) between myofibers. (B) H1D, (C) H2D and (D) DG groups showed attenuated pathological damage where the polygonal shape of myofibers was improved with decreased gap (double headed arrow) between myofibers. DC: non-treated diabetic rat; H1D: low-dose honey treated-diabetic rat; H2D: high-dose honey treated-diabetic rat; DG: glibenclamide-treated diabetic rat. Magnification x400. Scale Bar = 50 µm.

Following 28 days of *Apis* bee honey treatment to diabetic rats, H&E-stained longitudinal section of H1D group portrayed attenuated muscular degeneration where relatively fewer abnormal wavy course and splitting of myofibers were observed (Figure 4.7B). Compared to H1D group, both H2D and DG groups depicted mild and moderate focal histological alterations. Between H2D and DG groups, the skeletal muscle architecture of H2D group was closely resembled that of NC group, for instance, the parallel myofibers with peripherally located nuclei and minimal myofiber disintegration were noted.

Transverse sections of H1D, H2D and DG groups (Figure 4.8B, C and D respectively) demonstrated a relatively lesser centrally located nuclei as compared to DC group (Figure 4.8A). Although low-dose honey and glibenclamide treatments decelerated the pathological changes in skeletal muscles of diabetic rats, degenerated myofibers and small areas of necrosis were occasionally detected. On the other hand, high-dose honey treatment resulted in mostly intact myofibers with minimal myofibers splitting or sarcoplasm fragmentation.

4.4 Effects of *Apis* bee honey on expression of insulin signalling pathway proteins in skeletal muscle

4.4.1 Effects of *Apis* bee honey on IRβ protein expression

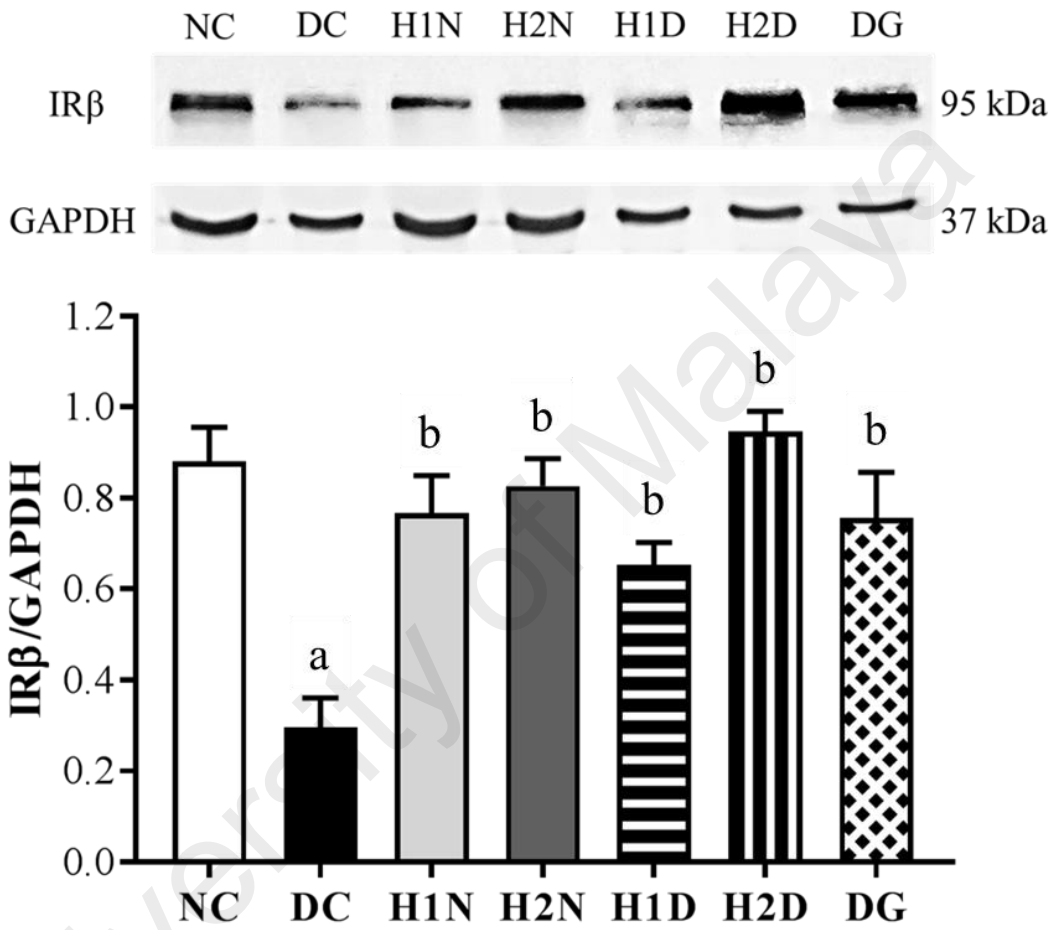

Figure 4.9: Effects of *Apis* bee honey on IRβ protein expression in skeletal muscle of diabetic rats. Mean values with different superscripts, for example (a) and (b), in a bar graph differ significantly from each other at $p < 0.05$. Results are presented as means ± SEM (n = 3 in each group).

The IRβ protein expression levels in non-diabetic rats were higher than in diabetic rats (Figure 4.9). DC group showed a significant decrease (2.93-fold, $p < 0.01$) in the IRβ protein expression level. In non-diabetic rats, *Apis* bee honey treatment did not cause significant changes to IRβ protein levels in skeletal muscle. However, in diabetic rats, IRβ protein levels markedly increased by 2.17- and 3.17-fold following low- and high-dose honey treatment respectively ($p < 0.01$). Also, treatment with glibenclamide resulted in an increased IRβ protein levels by 2.53-fold ($p < 0.01$), however it was not significantly different compared to low- and high-dose honey treatments.

4.4.2 Effects of *Apis* bee honey on IRβ protein distribution

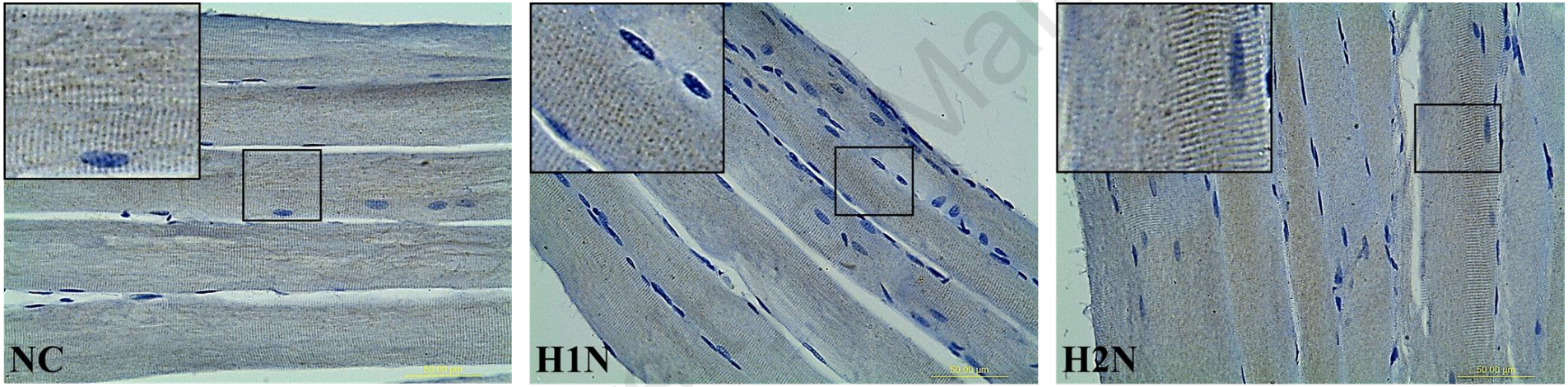

Figure 4.10: Effects of *Apis* bee honey on IRβ protein distribution in non-diabetic rats. (A) NC, (B) H1N and (C) H2N groups exhibited homogenous immunostaining and strong immunoreactivity for IRβ. Enlarged image in the left upper corner demonstrates the cross-striated myofiber positively-immunostained with IRβ antibody. The IRβ protein was visualized by peroxidase-DAB reaction (brown: positive IRβ immunostaining; blue: hematoxylin counterstaining). NC: non-diabetic rat; H1N: low-dose honey treated non-diabetic rat; H2N: high-dose honey treated non-diabetic rat. Magnification x400. Scale Bar = 50 µm.

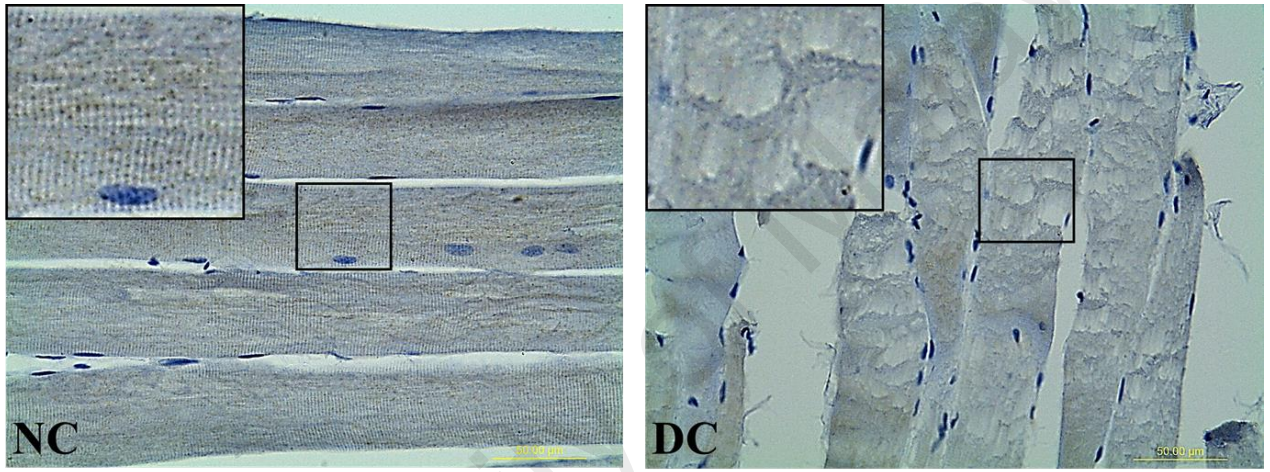

Figure 4.11: IRβ protein distribution in diabetic rats. (A) NC group demonstrated a relatively higher immunostaining for IRβ protein. However, degenerated myofibers in (B) DC group showed non-uniform immunostaining and weaker immunoreactivity for IRβ (enlarged image). IRβ protein was visualized by peroxidase-DAB reaction (brown: positive IRβ immunostaining; blue: hematoxylin counterstaining). NC: non-diabetic rat; DC: diabetic rat. Magnification x400. Scale Bar = 50 µm.

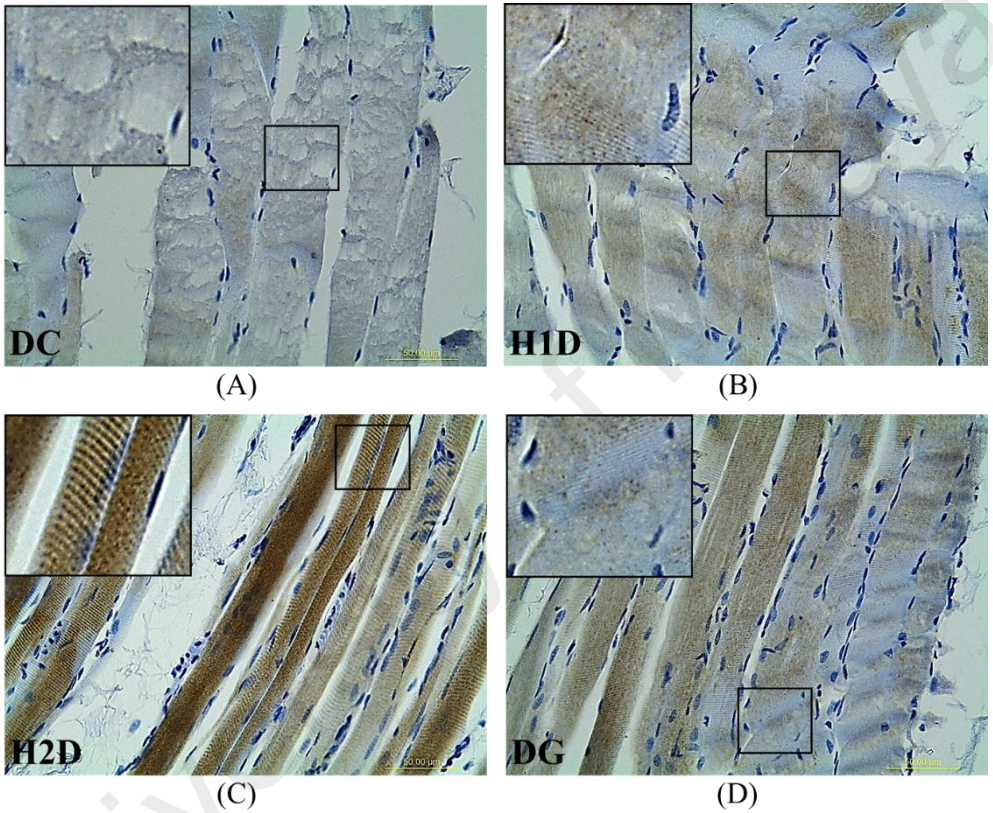

Figure 4.12: Effects of *Apis* bee honey on IRβ protein distribution in diabetic rats. Compared to (A) DC group, (B) H1D, (C) H2D and (D) DG groups exhibited moderate, strongest and strong immunoreactivity for IRβ. The remarkable variation in immunostaining intensities among all groups were clearly evident (enlarged image). The IRβ protein was visualized by peroxidase-DAB reaction (brown: positive IRβ immunostaining; blue: hematoxylin counterstaining). DC: non-treated diabetic rat; H1D: low-dose honey treated-diabetic rat; H2D: high-dose honey treated-diabetic rat; DG: glibenclamide-treated diabetic rat. Magnification x400. Scale Bar = 50 µm.

Immunohistochemical analysis revealed high distribution level of IRβ in the myofibers of non-diabetic rats. Treatment with *Apis* bee honey did not cause marked changes to the distribution of IRβ in the myofibers (Figure 4.10). On the other hand, IRβ protein distribution in the degenerative myofibers of diabetic rats were relatively reduced (Figure 4.11). *Apis* bee honey treatments to the diabetic rats intensified the IRβ immunoreactivity in the myofibers where high-dose honey treatment resulted in a relatively higher IRβ distribution levels as compared to low-dose honey. Glibenclamide treatment caused a relatively higher IRβ distribution in diabetic rat myofibers (Figure 4.11).

4.4.3 Effects of *Apis* bee honey on IRS1 protein expression

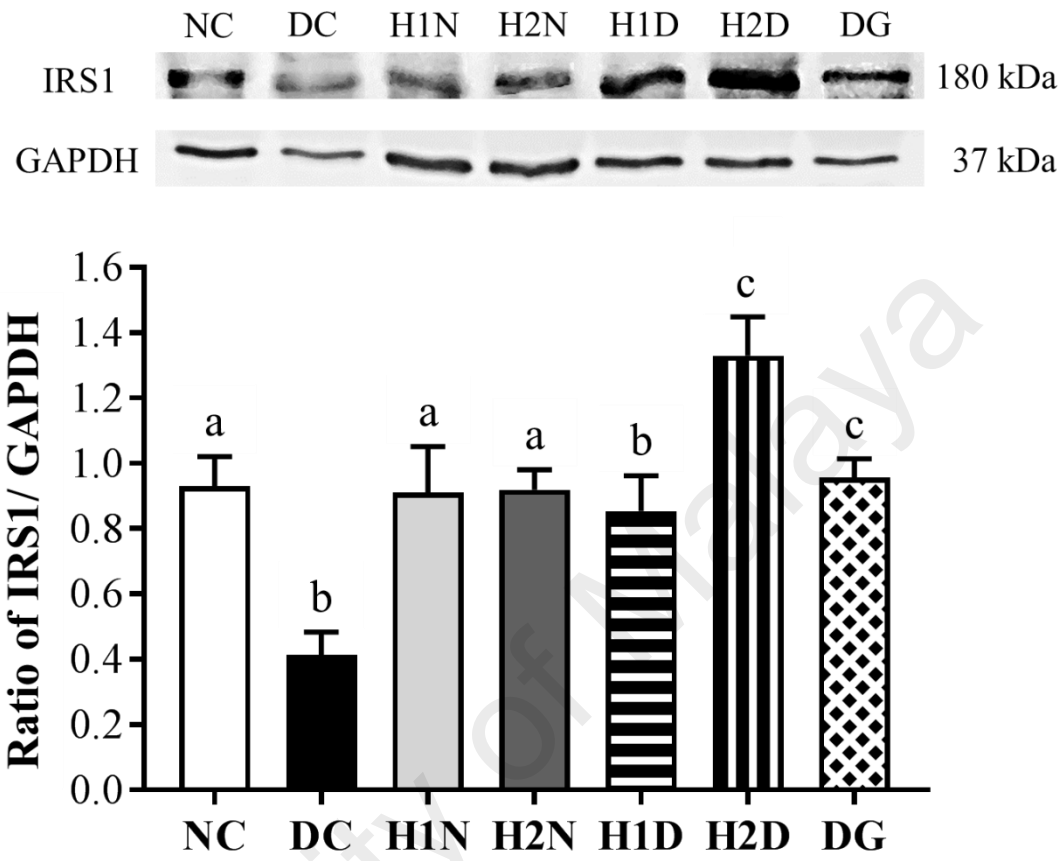

Figure 4.13: Effects of *Apis* bee honey on IRS1 protein expression in skeletal muscle of diabetic rats. Mean values with different superscripts, for example (a), (b) and (c), in a bar graph differ significantly from each other at $p < 0.05$. Results are presented as means ± SEM (n = 3 in each group).

NC, H1N and H2N groups showed similar IRS1 protein levels, suggesting that low- and high-dose honey treatments to non-diabetic rats did not alter the IRS1 protein levels significantly (Figure 4.13). In diabetic rat, IRS1 protein level was significantly reduced (2.27-fold, $p < 0.05$). Low-dose honey and glibenclamide treatments restored IRS1 protein levels in diabetic rats to near NC level. In comparison with DC group, significantly augmented IRS1 protein levels were observed in H2D (3.24-fold, $p < 0.01$) and DG groups (2.34-fold, $p < 0.05$) but not H1D group (2.07-fold).

4.4.4 Effects of *Apis* bee honey on IRS1 protein distribution

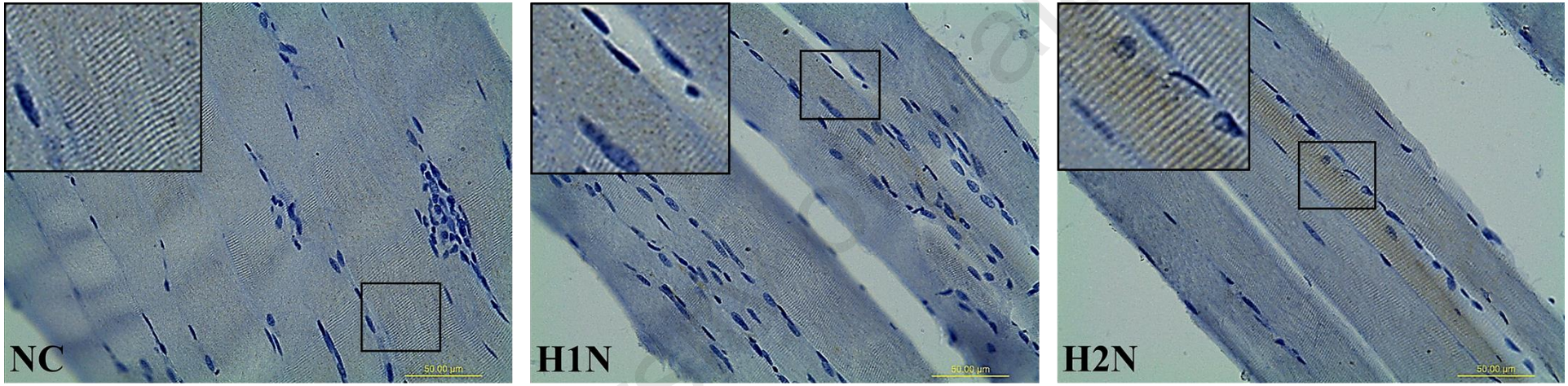

Figure 4.14: Effects of *Apis* bee honey on IRS1 protein distribution in non-diabetic rats. (A) NC, (B) H1N and (C) H2N groups exhibited moderate immunoreactivity for IRS1. The IRS1 protein was visualized by peroxidase-DAB reaction (brown: positive IRS1 immunostaining; blue: hematoxylin counterstaining). NC: non-diabetic rat; H1N: low-dose honey treated non-diabetic rat; H2N: high-dose honey treated non-diabetic rat. Magnification x400. Scale Bar = 50 µm.

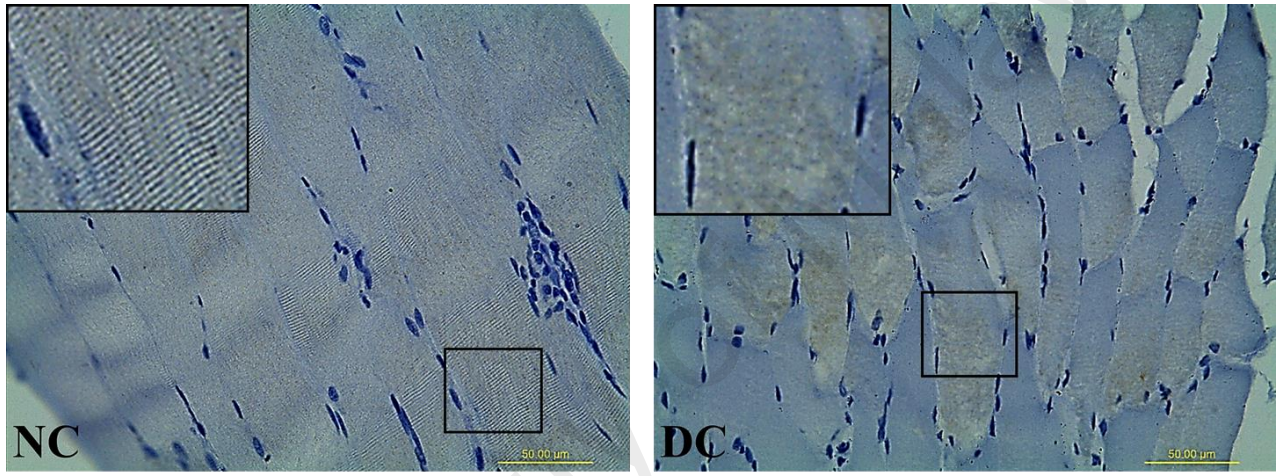

Figure 4.15: IRS1 protein distribution in diabetic rats. (A) NC group demonstrated a relatively homogenous staining for IRS1 compared to (B) DC group. IRS1 protein was visualized by peroxidase-DAB reaction (brown: positive IRS1 immunostaining; blue: hematoxylin counterstaining). NC: non-diabetic rat; DC: diabetic rat. Magnification x400. Scale Bar = 50 µm.

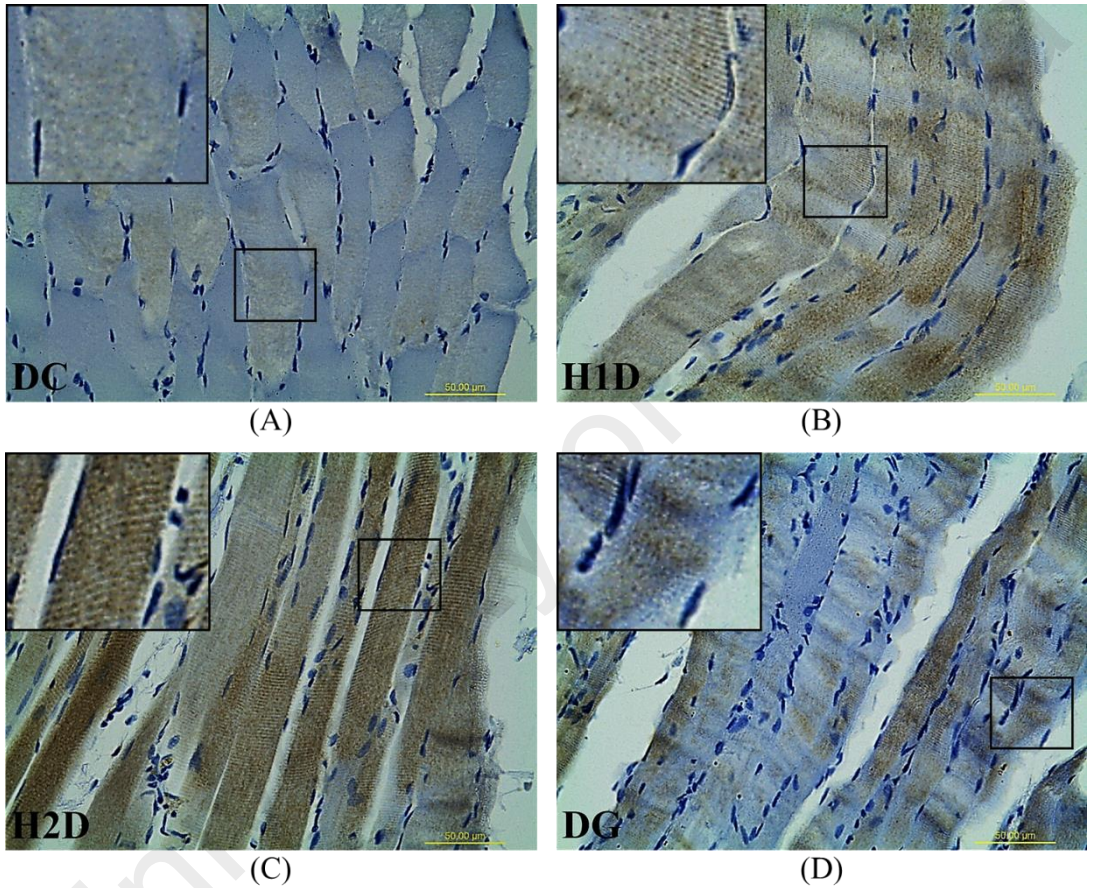

Figure 4.16: Effects of *Apis* bee honey on IRS1 protein distribution in diabetic rats. Compared to (A) DC group, (B) H1D, (C) H2D and (D) DG groups exhibited strong, strongest and moderate immunoreactivity respectively for IRS1. The IRS1 protein was visualized by peroxidase-DAB reaction (brown: positive IRS1 immunostaining; blue: hematoxylin counterstaining). DC: non-treated diabetic rat; H1D: low-dose honey treated-diabetic rat; H2D: high-dose honey treated-diabetic rat; DG: glibenclamide-treated diabetic rat. Magnification x400. Scale Bar = 50 µm.

NC, H1N and H2N groups showed similar levels of homogenous IRS1 protein distribution (Figure 4.14). On the contrary, DC group demonstrated lowest IRS1 protein distribution as reflected by its faint brown staining on sections (Figure 4.14). Following *Apis* bee honey treatments, the more intense brown staining on H2D than H1D section manifested that H2D group possessed greater IRS1 protein distribution. Besides, glibenclamide treatment also increased the IRS1 protein distribution in skeletal muscles of diabetic rats (Figure 4.15).

4.4.5 Effects of *Apis* bee honey on PI3K protein expression

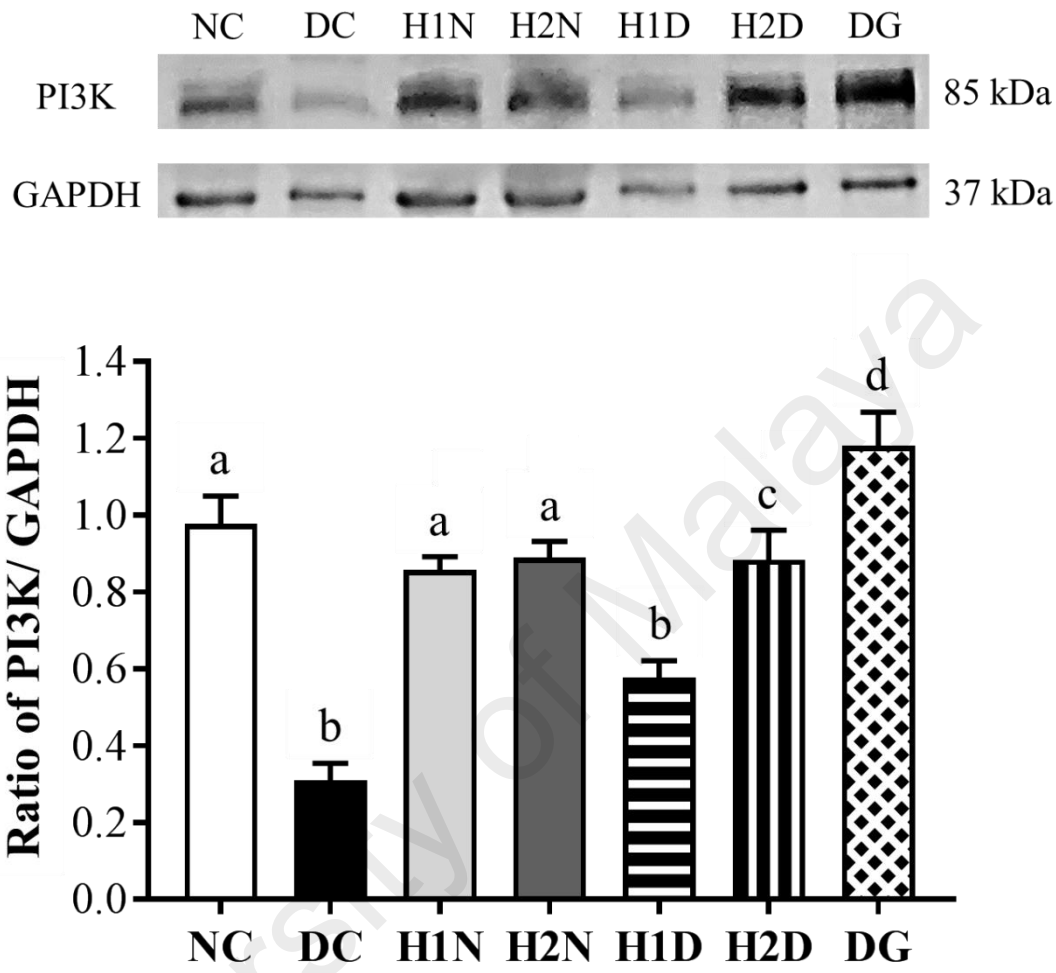

Figure 4.17: Effects of *Apis* bee honey on PI3K protein expression in skeletal muscle of diabetic rats. Mean values with different superscripts, for example (a), (b) and (c), in a bar graph differ significantly from each other at $p < 0.05$. Results are presented as means ± SEM (n = 3 in each group).

As shown in Figure 4.17, there was no significance difference in PI3K protein levels among NC, H1N and H2N groups. PI3K protein level was found to be reduced significantly in DC compared to NC groups (3.16-fold, $p < 0.01$). However, low- and high-dose honey as well as glibenclamide treatments induced higher PI3K protein levels in diabetic rats (1.87-, 2.84- and 3.81-fold respectively) wherein DG showed highest PI3K protein level then followed by H2D and H1D groups. Similarly to IRS1, significantly upregulated PI3K protein levels were observed in H2D and DG ($p < 0.01$) but not H1D group.

4.4.6 Effects of *Apis* bee honey on PI3K protein distribution

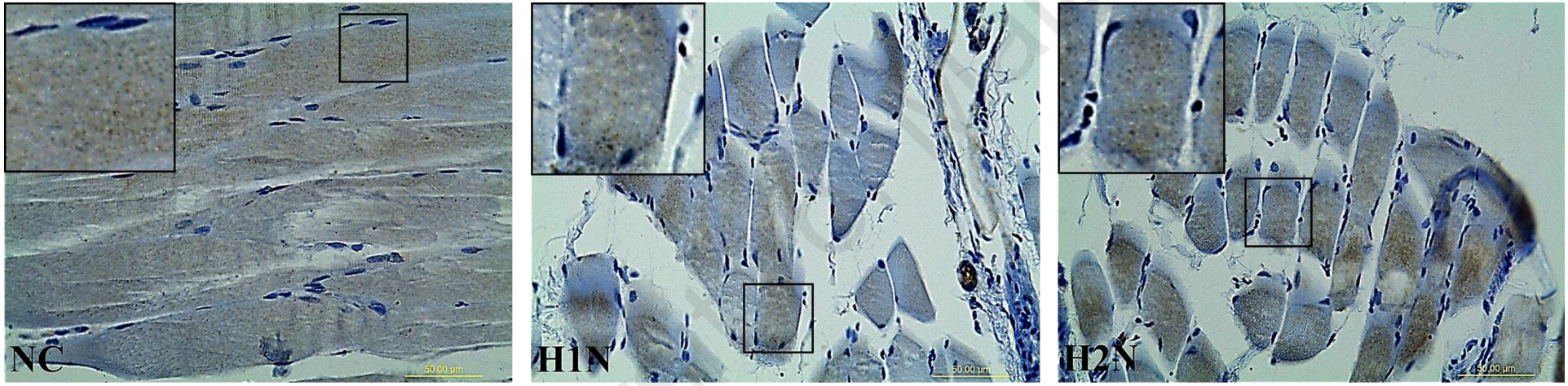

Figure 4.18: Effects of *Apis* bee honey on PI3K protein distribution in non-diabetic rats. (A) NC, (B) H1N and (C) H2N groups exhibited strong immunoreactivity for PI3K. The PI3K protein was visualized by peroxidase-DAB reaction (brown: positive PI3K immunostaining; blue: hematoxylin counterstaining). NC: non-diabetic rat; H1N: low-dose honey treated non-diabetic rat; H2N: high-dose honey treated non-diabetic rat. Magnification x400. Scale Bar = 50 µm.

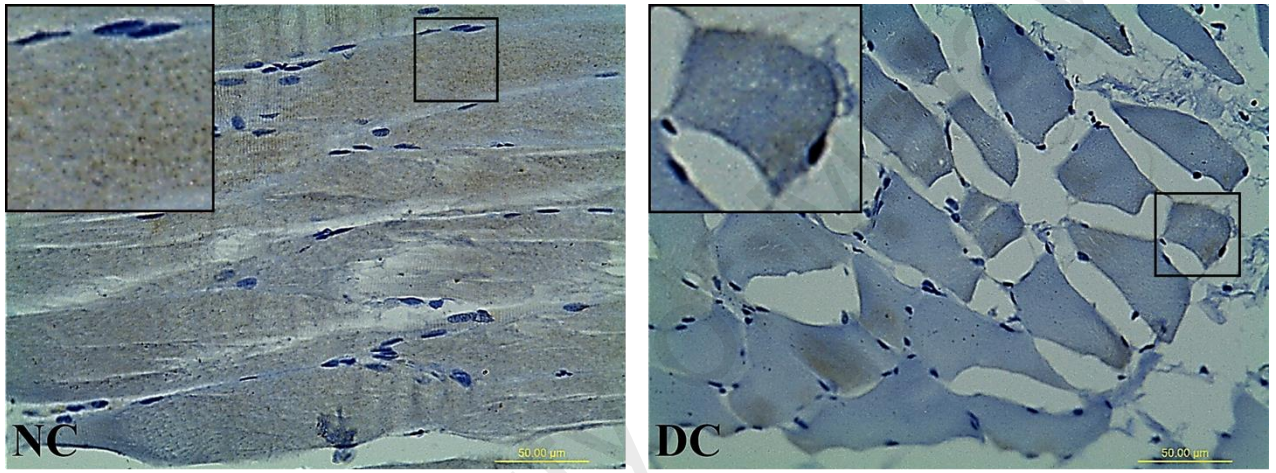

Figure 4.19: PI3K protein distribution in diabetic rats. (A) NC group demonstrated a relatively higher staining for PI3K than (B) DC group. Degenerated myofibers in DC group demonstrated non-uniform immunostaining and weaker immunoreactivity for PI3K. PI3K protein was visualized by peroxidase-DAB reaction (brown: positive PI3K immunostaining; blue: hematoxylin counterstaining). NC: non-diabetic rat; DC: diabetic rat. Magnification x400. Scale Bar = 50 µm.

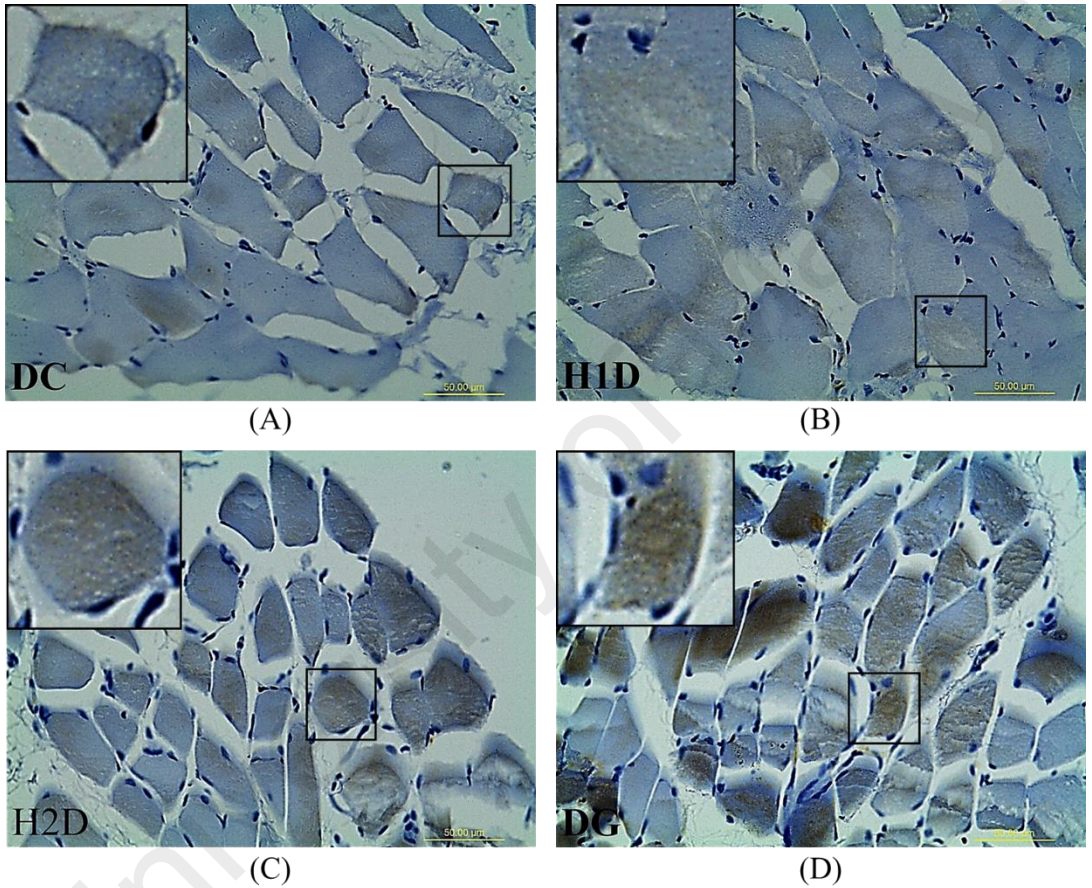

Figure 4.20: Effects of *Apis* bee honey on PI3K protein distribution in diabetic rats. Compared to (A) DC group, (B) H1D, (C) H2D and (D) DG groups exhibited moderate, strong and strongest immunoreactivity for PI3K. The PI3K protein was visualized by peroxidase-DAB reaction (brown: positive PI3K immunostaining; blue: hematoxylin counterstaining). DC: non-treated diabetic rat; H1D: low-dose honey treated-diabetic rat; H2D: high-dose honey treated-diabetic rat; DG: glibenclamide-treated diabetic rat. Magnification x400. Scale Bar = 50 µm.

As shown in Figure 4.18, uniform brown staining was observed in NC, H1N and H2N groups, mirroring a homogenous expression of PI3K proteins in skeletal muscle of non-diabetic rats without marked difference. PI3K protein distribution was found to be reduced drastically in DC compared to NC group (Figure 4.19). H1D group showed modest increase in PI3K protein where some myofibers did not show prominent immunoreactivity for PI3K. On the other hand, H2D and DG groups manifested more intense brown staining, signifying that myofibers in H2D and DG had higher level of PI3K proteins (Figure 4.20).

4.4.7 Effects of *Apis* bee honey on Akt protein expression

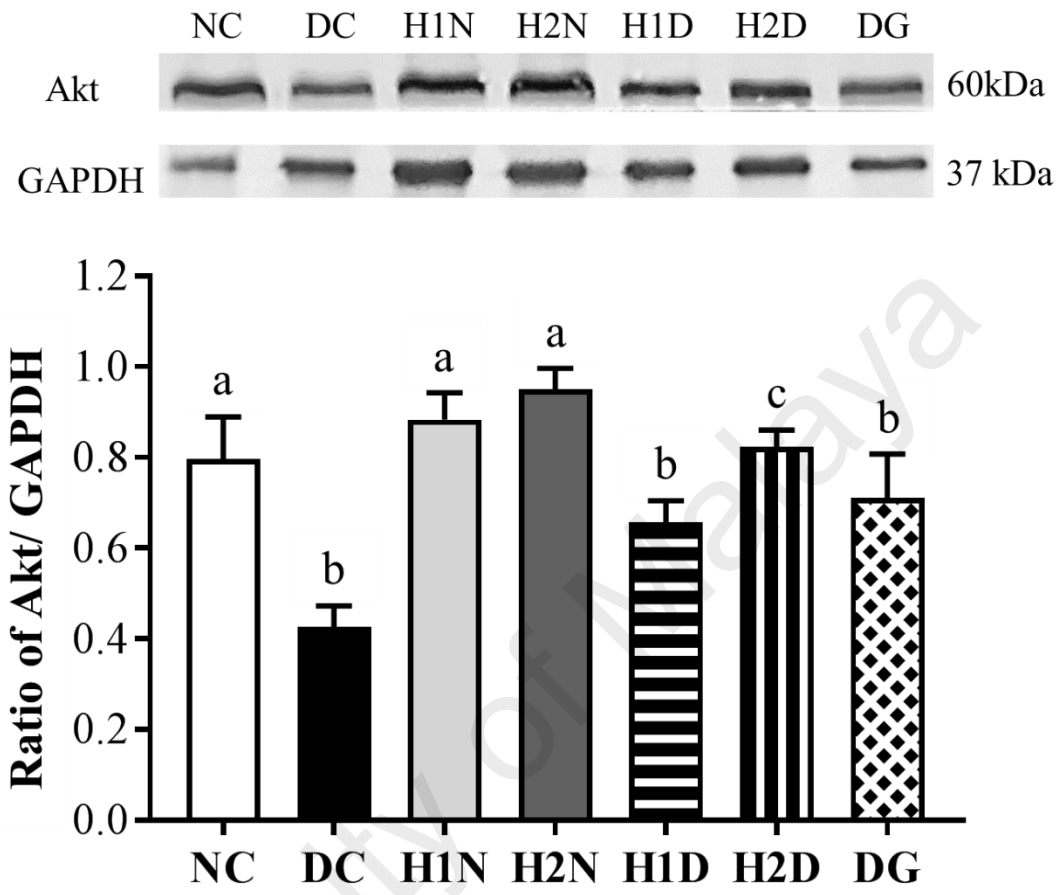

Figure 4.21: Effects of *Apis* bee honey on Akt protein expression in skeletal muscle of diabetic rats. Mean values with different superscripts, for example (a), (b) and (c), in a bar graph differ significantly from each other at $p < 0.05$. Results are presented as means ± SEM (n = 3 in each group).

In the present study, H1N and H2N rats demonstrated higher Akt protein levels compared to NC rats. Yet, no significance difference among NC, H1N and H2N groups was observed (Figure 4.21). In diabetic rat, Akt protein expression was significantly decreased (1.86-fold-, $p < 0.05$). Yet, low-dose honey and glibenclamide treatments reverted the Akt protein levels in diabetic rats to near NC level by increasing 1.53- and 1.65-fold respectively. High-dose honey maintained the Akt protein level in diabetic rats closest to NC level and this Akt protein level was significantly different from DC group ($p < 0.01$). These outcomes implied that *Apis* bee honey treatment, particularly at a high dose, had an enhancing effect on Akt protein expression in diabetic rats.

4.4.8 Effects of *Apis* bee honey on Akt protein distribution

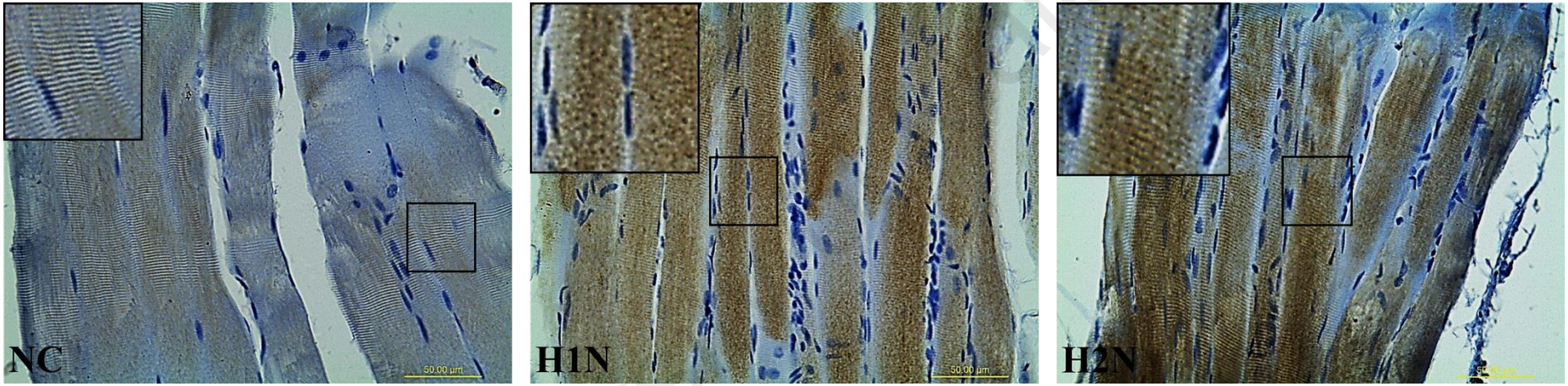

Figure 4.22: Effects of *Apis* bee honey on Akt protein distribution in non-diabetic rats. Compared to (A) NC group, (B) H1N and (C) H2N groups exhibited stronger immunoreactivity for Akt. The Akt protein was visualized by peroxidase-DAB reaction (brown: positive Akt immunostaining; blue: hematoxylin counterstaining). NC: non-diabetic rat; H1N: low-dose honey treated non-diabetic rat; H2N: high-dose honey treated non-diabetic rat. Magnification x400. Scale Bar = 50 µm.

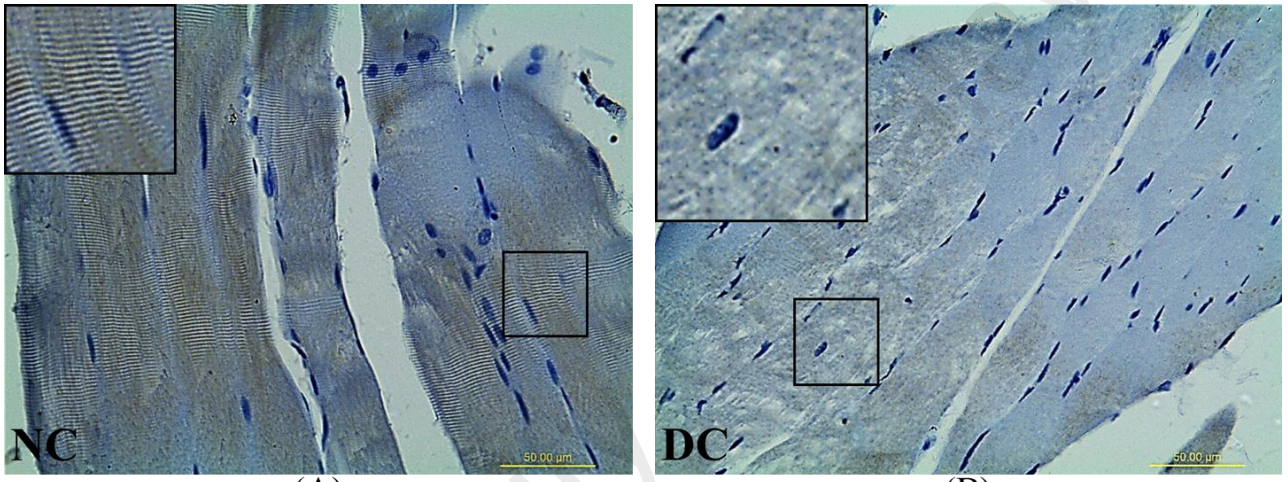

Figure 4.23: Akt protein distribution in diabetic rats. (A) NC group demonstrated a relatively higher immunostaining for Akt than (B) DC group. Akt protein was visualized by peroxidase-DAB reaction (brown: positive Akt immunostaining; blue: hematoxylin counterstaining). NC: non-diabetic rat; DC: diabetic rat. Magnification x400. Scale Bar = 50 µm.

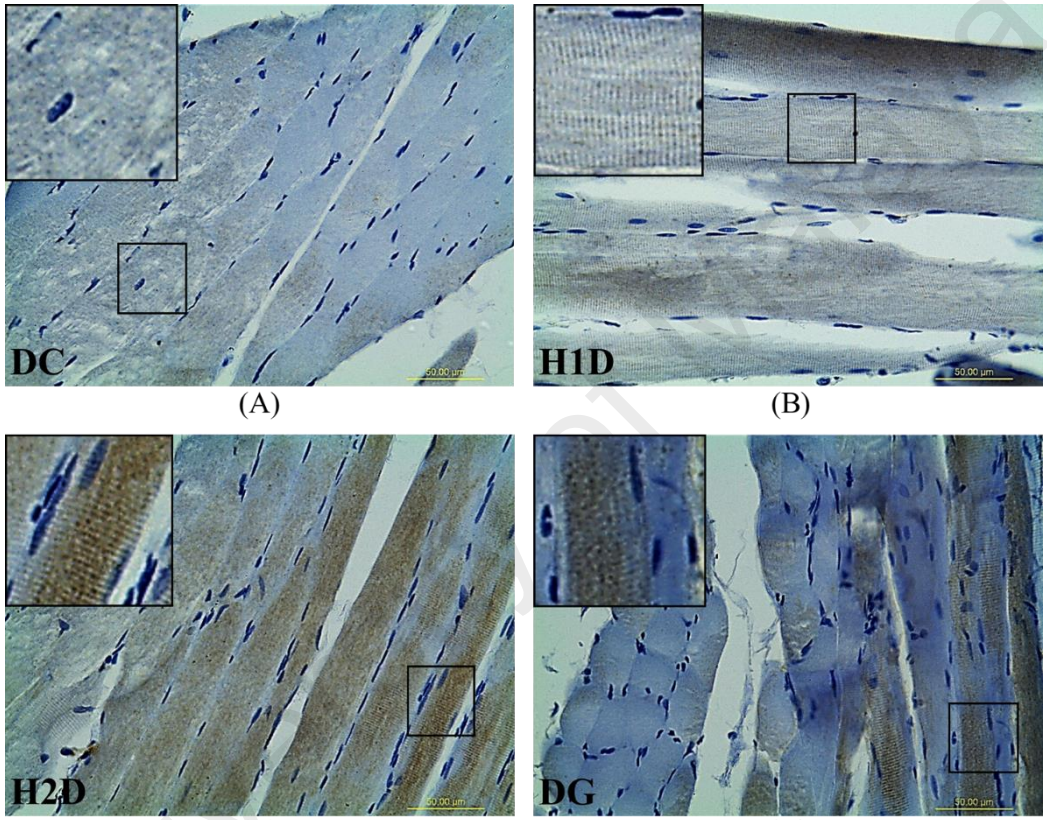

Figure 4.24: Effects of *Apis* bee honey on Akt protein distribution in diabetic rats. Compared to (A) DC group, (B) H1D and (C) H2D showed stronger and more homogenous immunostaining for Akt while (D) DG group exhibited relatively lower and non-homogenous immunostaining for Akt. The Akt protein was visualized by peroxidase-DAB reaction (brown: positive Akt immunostaining; blue: hematoxylin counterstaining). DC: non-treated diabetic rat; H1D: low-dose honey treated-diabetic rat; H2D: high-dose honey treated-diabetic rat; DG: glibenclamide-treated diabetic rat. Magnification x400. Scale Bar = 50 µm.

With reference to NC group, both H1N and H2N groups demonstrated relatively greater immunoreactivity for Akt proteins (Figure 4.22). DC group showed minimal level of Akt proteins (Figure 4.23) whereas the H1D and H2D groups showed distinguishable Akt protein distribution in myofibers. Besides, DG group demonstrated noticeable increase in Akt proteins but lower level than that in H2D group, as portrayed by its smaller area of brown staining (Figure 4.24).

4.4.9 Effects of *Apis* bee honey on GLUT4 protein expression

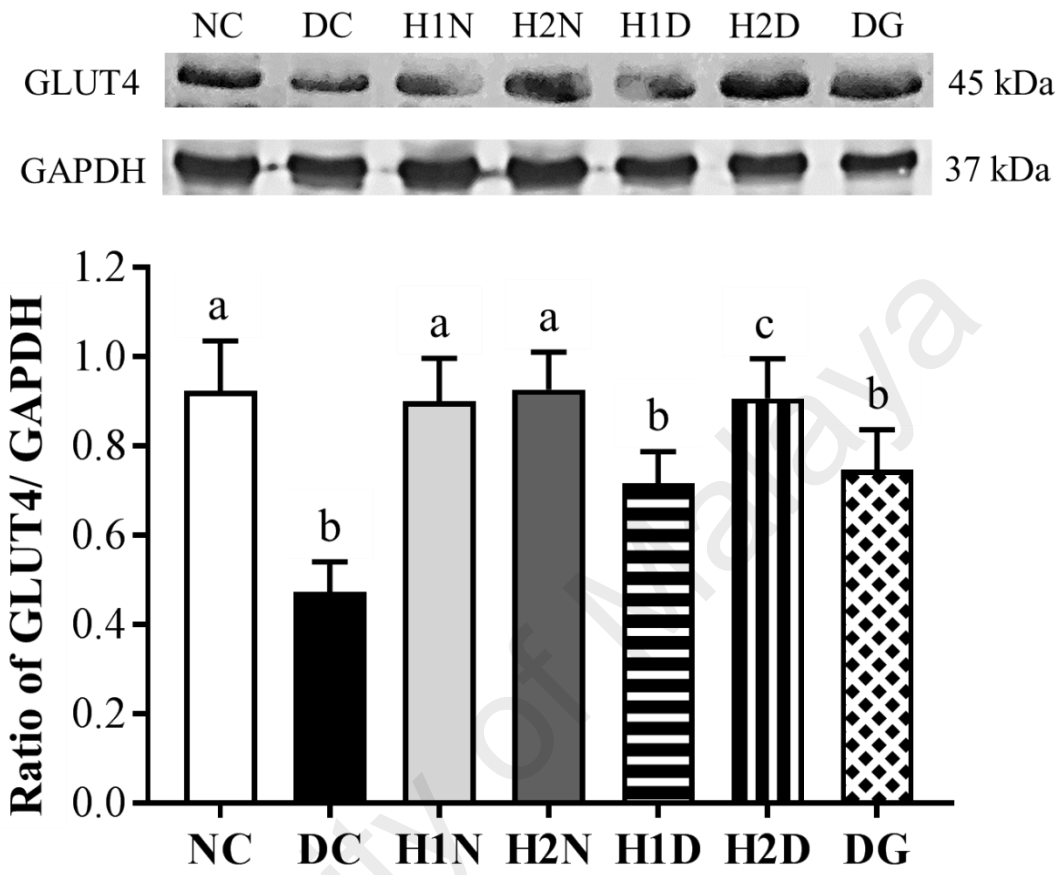

Figure 4.25: Effects of *Apis* bee honey on GLUT4 protein expression in skeletal muscle of diabetic rats. Mean values with different superscripts, for example (a), (b) and (c), in a bar graph differ significantly from each other at $p < 0.05$. Results are presented as means ± SEM (n = 3 in each group).

Augmented expression of key insulin signalling components, including IRβ, IRS1, PI3K and Akt, in the experiments prompted us to investigate its downstream player, GLUT4. As depicted in Figure 4.25, NC, H1N and H2N groups showed unaltered GLUT4 protein levels. GLUT4 protein level was significantly downregulated in diabetic rats (1.96-fold, $p < 0.05$), but low- and high-honey as well as glibenclamide treatments elevated the GLUT4 protein levels in diabetic rats (1.53-, 1.94- and 1.60-fold respectively). Among H1D, H2D and DG groups, only H2D group demonstrated significantly enhanced GLUT4 protein level ($p < 0.05$), suggesting that high-dose honey treatment to diabetic is effective in elevating GLUT4 protein level in diabetic rats.

4.4.10 Effects of *Apis* bee honey on GLUT4 protein distribution

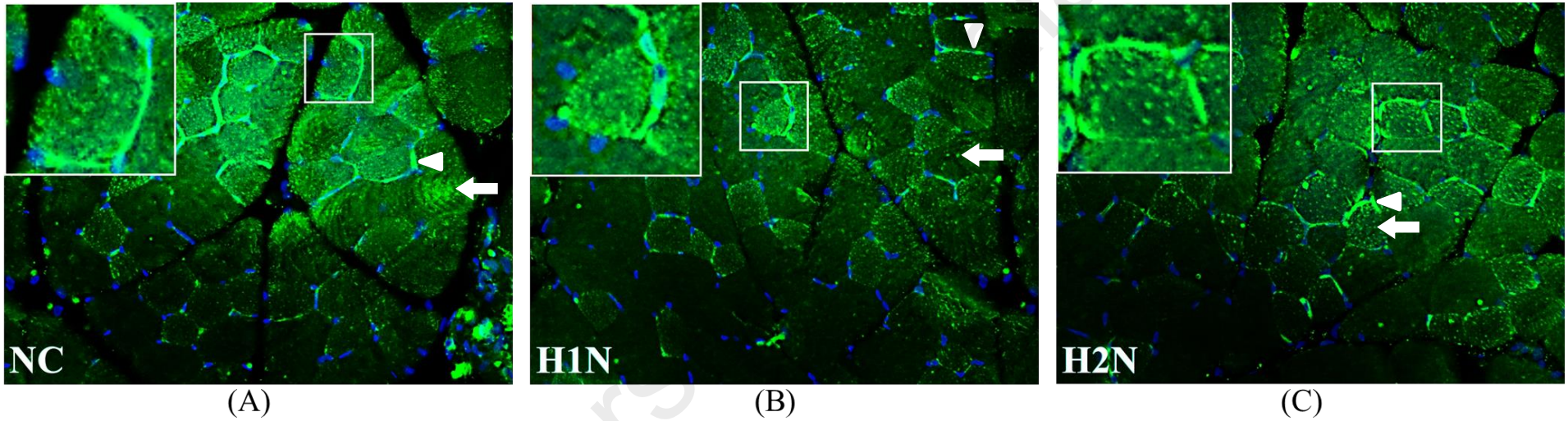

Figure 4.26: Effects of *Apis* bee honey on GLUT4 protein distribution in non-diabetic rats. Punctuate distribution of GLUT4 vesicles in cytosol (arrow) and localization of GLUT4 along the plasma membrane (arrow head) were clearly evident in (A) NC, (B) H1N and (C) H2N groups. The GLUT4 fluorescence intensities were comparable among these 3 groups. Insets show more detailed images of GLUT4 in cytosol and plasma membrane regions. GLUT4 was stained with FITC (green) while nuclei were stained with DAPI (blue). Magnification x400. Scale Bar = 50 µm.

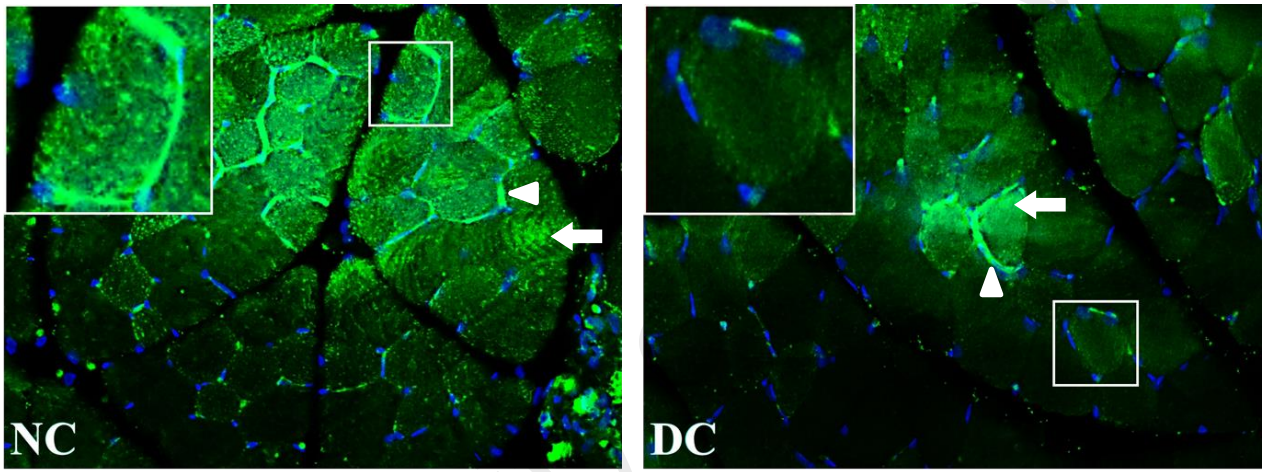

Figure 4.27: GLUT4 protein distribution in diabetic rats. (A) Normal myofibers exhibited GLUT4 expression on the cytosol (arrow) and plasma membrane (arrow head). However, (B) T2DM drastically diminished GLUT4 expression on the cytosol and plasma membrane as manifested by relatively weaker fluorescence intensity. GLUT4 was stained with FITC (green) while nuclei were stained with DAPI (blue). Magnification x400. Scale Bar = 50 µm.

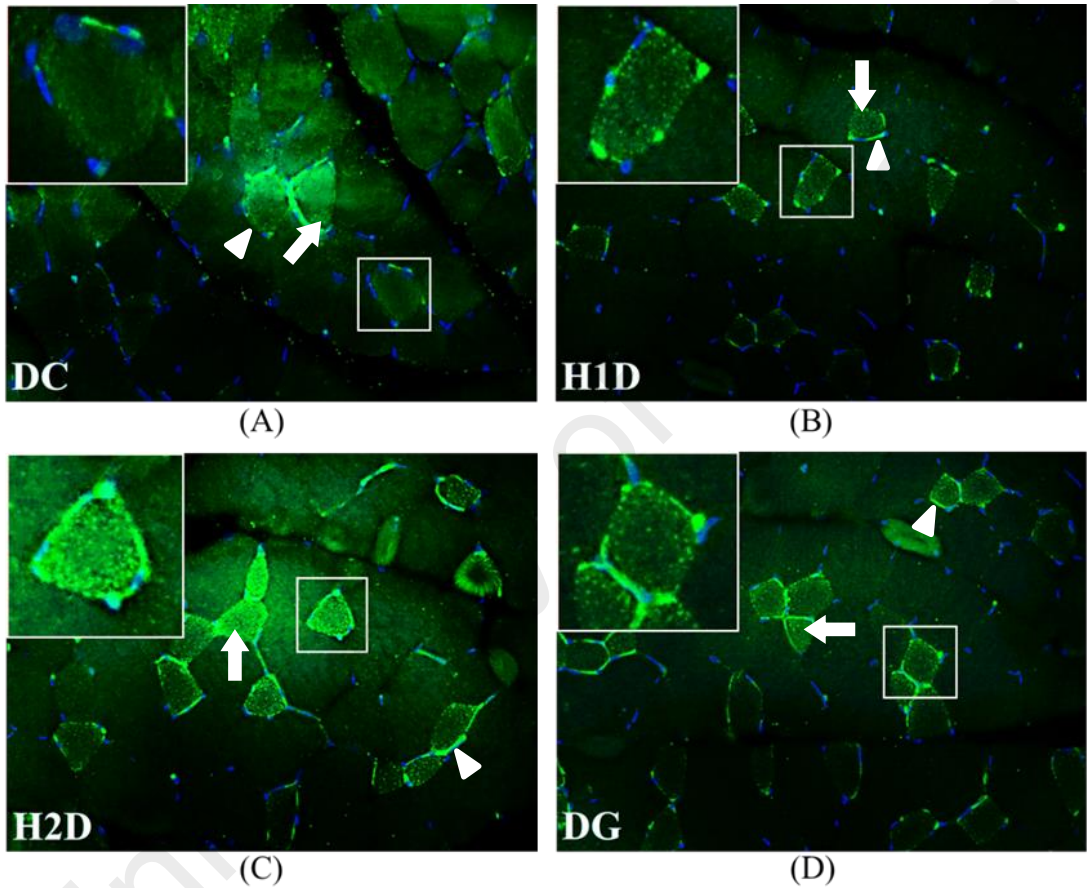

Figure 4.28: Effects of *Apis* bee honey on GLUT4 protein distribution in diabetic rats. In contrast to the minimal GLUT4 expression on plasma membrane (arrow head) in (A) DC group, (B) H1D, (C) H2D and (D) DG groups exhibited moderate, strongest and strong GLUT4 expression on cytosol (arrow) and plasma membrane of skeletal muscles. GLUT4 was stained with FITC (green) while nuclei were stained with DAPI (blue). Magnification x400. Scale Bar = 50 µm.

In order to examine the effect of *Apis* bee honey treatment on the GLUT4 translocation to the plasma membrane, immunofluorescence analyses for the GLUT4 proteins in muscle sections was performed. In non-diabetic rats, punctuate distribution of GLUT4 vesicles in cytosol and continuous distribution of GLUT4 along plasma membrane were clearly evident (Figure 4.26). Moreover, GLUT4 immunostaining is observed in large clusters and smaller punctuate structures which at a lower magnification appear as a diffuse background stain. In response to low- and high-dose of honey treatment, non-diabetic rats did not show prominent changes in GLUT4 immunofluorescence intensities where clusters of GLUT4 in intracellular regions and at the myofiber periphery remained clearly visible.

On the other hand, GLUT4 immunostaining in diabetic rats was relegated to noticeably lesser myofibers and no intracellularly scattered punctate staining was observed (Figure 4.27). *Apis* bee honey administration to diabetic rats augmented the cytosolic and plasma membrane expression of GLUT4 in myofiber sections of diabetic rats such that H2D group exhibited stronger GLUT4 expression than in DG and H1D groups (Figure 4.28).

CHAPTER 5: DISCUSSION

5.1 Choice of animal diabetic model

STZ-NA induced diabetic model has been widely adopted to emulate T2DM in human (Adam, Giribabu, Bakar, & Salleh, 2017; Aziz et al., 2017; Roslan, Giribabu, Karim, & Salleh, 2017). STZ selectively damages the pancreatic β-cells by triggering DNA strand break and synthesis of superoxide radical as well as inhibiting free radical scavenger enzymes (Damasceno et al., 2014). On the other hand, NA partially protects the pancreatic β-cells from cytotoxic action of STZ by scavenging free radical, limiting only minor damage to pancreatic β-cells, thereby establishing a state of partial insulin deficiency as manifested in T2DM (Ghasemi et al., 2014).

STZ-NA diabetic model resembles T2DM in several aspects including decreased pancreatic insulin, absence of the early phase of glucose-stimulated insulin secretion (Weng et al., 2014) and decreased insulin sensitivity (Birgani, Ahangarpour, Khorsandi, & Moghaddam, 2018), which jointly lead to hyperglycemia. In the present study, diabetic rats manifested a moderate and stable hyperglycemia (24.35 ± 1.63 mmol/L). Unlike T1DM, T2DM responds well to oral anti-diabetic drugs. Amelioration of hyperglycemia by administration of glibenclamide emphasizes that STZ-NA diabetic rats mimics T2DM (Ghasemi et al., 2014).

In concordance with other studies, induced diabetic milieu negatively impact the IRβ signalling and downstream proteins including IRS1, PI3K, Akt and GLUT4 (Chao et al., 2018; Shen et al., 2018). These findings support the notion that the STZ-NA-induced diabetic rats are characterised by impaired insulin signalling pathway, hence serve as an appropriate diabetic model for investigation on the anti-diabetic agent that potentially targets insulin signalling pathway (Morakinyo, Samuel, & Adekunbi, 2018; Shen et al., 2018).

5.2 Major components of *Apis* bee honey

In the present study, flavonoids (isorhamnetin 3-O-rutinoside and (epi)afzelechin-(epi)catechin conjugate) and phenolic acid (hydroxybenzoic acid-O-hexoside conjugate and syringic acid-hexose derivative conjugate) as well as monosaccharide derivative (gluconic acid) were present in highest abundance in the *Apis* bee honey. Other classes of bioactive compounds present in lower abundance are benzenoid, tannin and fatty acids.

The presence of flavonoids in honey could help to counter the high levels of oxidative stress in T2DM. In T2DM, high levels of oxidative stress are mainly due to exaggerated synthesis of ROS, which overwhelm the antioxidant defence of the tissues. Our postulation is based on the reports that flavonoid and phenolics in honey help to overcome tissue oxidative stress particularly in disease state as they are naturally occurring antioxidants (Ahmed et al., 2018; Aziz et al., 2017; Moniruzzaman et al., 2013).

In the meantime, (epi)afzelechin-(epi)catechin conjugate (Farag, Sakna, El-Fiky, Shabana, & Wessjohann, 2015; Sobeh et al., 2017) and isorhamnetin 3-O-rutinoside (Kawser Hossain et al., 2016; Rodriguez-Rodriguez et al., 2015) which are abundantly present in the honey sample have been shown to possess anti-diabetic activity from its ability to ameliorate hyperglycemia and insulin resistance in diabetic animal models.

Apigenin, one of the compounds detected in *Apis* bee honey, has been reported able to restore the pancreatic β-cells and insulin secretion (Osigwe, Akah, Nworu, & Okoye, 2017) as well as regulates the lipid peroxidation level and antioxidants defence enzymes in favour of improving T2DM as well as improves glucose homeostasis (Hossain, Ghosh, Satapathy, Dey, & Mukherjee, 2014). Taken together, we speculate that (epi)afzelechin-(epi)catechin conjugate, isorhamnetin 3-O-rutinoside and apigenin in *Apis* bee honey might be accountable for the anti-diabetic effect by alleviating hyperglycemia and oxidative stress in T2DM.

Additionally, proanthocyanidin has been shown to alleviate oxidative stress and ER stress in the skeletal muscle of diabetic rats. Among the proanthocyanidins, for instance dimers, trimers and oligomers, proanthocyanidin dimers such as (epi)afzelechin-(epi)catechin conjugate showed strong anti-inflammatory activities via reducing NO, and PGE2 as well as impedes the activation of NF-κB pathway that is involved in the inflammatory process (Tatsuno et al., 2012; Watson, 2017). Collectively, the (epi)afzelechin-(epi)catechin conjugate and isorhamnetin 3-O-rutinoside that is detected in *Apis* bee honey could potentially attenuate inflammation in the skeletal muscle in T2DM.

Besides, cinnamic acid in *Apis* bee honey was documented to exert anti-diabetic activity by enhancing insulin secretion (Hafizur et al., 2015) and glycogenesis as well as inhibiting gluconeogenesis (Huang & Shen, 2012). Importantly, cinnamic acid ameliorates diabetes-induced damage in the blood, liver and kidneys (Anlar et al., 2018). Hence, we postulate that cinnamic acid in *Apis* bee honey sample may plays extra-pancreatic role where it prevents T2DM-induced skeletal muscle damage.

Phenolic acids including hydroxybenzoic acid, protocatechuic acid, syringic acid and vanilic acid detected in *Apis* bee honey have been shown to actively involved in the carbohydrate digestion and absorption as well as insulin secretion in diabetic rats (Vinayagam, Jayachandran, & Xu, 2016). Linolenic acid and caprylic acid which were also present in *Apis* bee honey have been found to possess anti-diabetic properties. Linolenic acid involved in anti-hyperglycemic, anti-hyperlipdemic and organ protective activities against T2DM. Of note, linolenic acid improves insulin sensitivity by enhancing GLUT4 protein content in the sarcolemma of diabetic rats (Suanarunsawat, Anantasomboon, & Piewbang, 2016). On the other hand, caprylic acid targets insulin secretion and glycogen synthesis respectively (Bharti, Krishnan, Kumar, Kumar, & metabolism, 2018).

In agreement with other studies, octadecanoic acid was detected in *Apis* bee honey. In diabetic milieu, octadecanoic acid attenuates inflammation and prevents apoptosis via inhibition of NF-κB-mediated COX-2 expression and oxidative stress dependent BCL2-associated X protein/B-cell lymphoma 2/caspase-3 (BAX/Bcl-2/caspase-3) apoptotic pathway activation respectively (Neamatallah, El-Shitany, Abbas, Ali, & Eid, 2018). These findings imply that octadecanoic acid detected in this honey might have the potential to attenuate oxidative stress, inflammation and apoptosis in the skeletal muscle in T2DM.

It is reasonable to assume that the major compounds in *Apis* bee honey, including (epi)afzelechin-(epi)catechin conjugate and isorhamnetin 3-O-rutinoside, may protect against skeletal muscle damage, consequently improving the insulin sensitivity and ultimately blood glucose control in T2DM. Also, it is possible that synergistic effects among compounds exist in the antidiabetic activity of *Apis* bee honey. We speculate that the phenolic and flavonoid compounds are possibly the most important ones in inducing the synergistic antidiabetic effect and cytoprotective action.

5.3 Effect of *Apis* bee honey on FBG level

In agreement with *in vivo* animal studies (Hemmati, Karamian, Malekaneh, & Pharmacology, 2015; Rachmat, Yuslianti, Permatasari, & Koswara, 2017) and clinical studies (Enginyurt et al., 2017; Rajab, Takruri, Mishal, & Alkurd, 2017), administration of *Apis* bee honey decreased FBG levels and hence improves glycemic control. Compared to a dose of 5 mg/kg of *Apis* bee honey, 10 mg/kg of *Apis* bee honey presented more potential activity on glycemic control. It can be postulated that *Apis* bee honey ameliorates hyperglycemia by enhancing glucose uptake in peripheral tissues or improving glucose tolerance.

As aforementioned, we hypothesize that the phenolic compounds in *Apis* bee honey, including (epi)afzelechin-(epi)catechin conjugate, isorhamnetin 3-O-rutinoside, apigenin, cinnamic acid, linolenic acid and caprylic acid, might accountable for the anti-hyperglycemic effect of *Apis* bee honey in diabetic rats. It is well established that hyperglycemia is the predominant cause of diabetic complications, potentially via oxidative stress mechanism. Hence, improvement of glycemic control towards near-normoglycemia can impedes the development and progression of T2DM complications (Zheng et al., 2018).

5.4 Effect of *Apis* bee honey on skeletal muscle histology

To date, no studies have identified the effect of honey on the skeletal muscle, although several other studies have demonstrated that honey could exert protective effects on the pancreas, liver and kidney. Thus, this study could be the first to identify such effect. In concordance with other studies, skeletal muscle of STZ-NA-induced diabetic rats exhibited degenerative changes including abnormal wavy course of myofibers with splitting, fragmentation of the sarcoplasm, dense pyknotic nuclei and mononuclear cellular infiltration (Berridge et al., 2018; Guan et al., 2016). As reported by Samir et al., myofiber degeneration associated with inflammatory cell infiltration are prominent features of oxidative stress effect in the skeletal muscle (Samir, Abbas, Safwat, & Elserougy, 2018). Mononuclear cellular infiltration in myofibers might be initiated by the release of certain mediators during degeneration of myocytes which initiate the inflammatory reaction and attract inflammatory cells, resulting in apoptosis of the skeletal muscle (Hassani & Ibrahim, 2018).

Histopathological examination of skeletal muscles of *Apis* bee honey-treated diabetic rats showed lesser skeletal muscle deformation. Lesser deformity was observed with increasing dose of *Apis* bee honey treatment. It is likely that the phenolic compounds in *Apis* bee honey are accountable for its protective effect on skeletal muscle. Reportedly, proanthocyanidin effectively enhances antioxidant capacity and antagonizes oxidative damage by regulating the signalling pathway related with oxidative stress (Li et al., 2015). Importantly, proanthocyanidin inhibits oxidative and ER stress in skeletal muscle in T2DM, thereby ameliorates the muscular damage and dysfunction (Ding et al., 2013). On that account, we speculate that proanthocyanidin in *Apis* bee honey exerts protective effect on the skeletal muscle in diabetic rats.

5.5 Effect of *Apis* bee honey on expression of insulin signalling pathway proteins in skeletal muscle in T2DM

5.5.1 *Apis* bee honey prevents the decrease in IRβ protein expression

In concordance with other studies, IRβ protein expression was found to be reduced significantly in the skeletal muscle of diabetic rats. Decreased IRβ protein expression serves as the defect at the receptor level within the insulin signalling pathway, thus impairs the insulin signal transduction (Kubota, Kubota, & Kadowaki, 2017). Low-dose (5 mg/kg) and high-dose (10 mg/kg) *Apis* bee honey treatment was found to significantly enhance the IRβ protein expression in diabetic rats' skeletal muscle, comparable to the effect observed with glibenclamide treatment. These findings suggested *Apis* bee honey, even at low dose was sufficient to prevent the decrease in the IRβ level in diabetic rats' skeletal muscle as indicated by increased IRβ protein levels, thus enhancing skeletal muscle insulin signalling.

5.5.2 *Apis* bee honey prevents the decrease in IRS1 protein expression

In diabetic rats, it was found that low-dose honey treatment prevented the decrease in IRS1 protein level in diabetic rats' skeletal muscle. IRS1 serves as the first post-receptor molecule in the insulin signalling pathway (Kubota et al., 2017; Yousef et al., 2018). The effect of low-dose *Apis* bee honey was almost similar to the effect of glibenclamide in preventing the decrease in IRS1 protein level in the skeletal muscle. At higher dose of *Apis* bee honey treatment, much lesser decrease in IRS1 protein was observed in the skeletal muscle of diabetic rats, indicating that the *Apis* bee honey effect is dose-dependent. Together, the observed effects on IRS1 could help to enhance the signal transduction to the downstream molecule PI3K.

5.5.3 *Apis* bee honey prevents the decrease in PI3K protein expression

In insulin signalling pathway, signal transduction via IRS1/PI3K/Akt pathway plays a crucial regulatory role in insulin signalling transduction of the skeletal muscle (X. Huang, Liu, Guo, & Su, 2018). Studies have shown that impaired IRS1 and PI3K/Akt signalling suppresses glucose uptake and GLUT4 translocation. In other words, any defect in this signal transduction pathway can diminishes the sensitivity of skeletal muscles to insulin, resulting in impaired glucose uptake and utilisation and eventually increase in blood glucose level (Choi, Kim, Koh, & Lee, 2018; Long et al., 2018; Xing & Chen, 2018).

In current study, PI3K protein level was reduced in the skeletal muscle of diabetic rats. However, when *Apis* bee honey was co-administered at the day of T2DM development, lesser decrease in PI3K levels was observed in the skeletal muscle, particularly with high-dose of *Apis* bee honey. The effect of *Apis* bee honey was comparable to glibenclamide, hence it can be concluded that preserving high level of PI3K in the skeletal muscle would assist better insulin signal transduction, thus improving insulin sensitivity.

5.5.4 *Apis* bee honey prevents the decrease in Akt protein expression

In the present study, it was found that the level of Akt protein was significantly reduced in diabetic rats' skeletal muscle. This would likely impair the insulin signalling as Akt molecule is a downstream signalling molecule of the IRβ/IRS1/PI3K insulin transduction pathway. Studies have shown that defective Akt would result in impairment in insulin signalling in skeletal muscle (Ramachandran, Saravanan, & toxicology, 2015; Xing & Chen, 2018). *Apis* bee honey treatment to diabetic rats was found to significantly prevent the decrease in Akt protein levels and thereby would enhance the signalling upon insulin binding to IR. This would help to improve skeletal muscle insulin sensitivity.

5.5.5 *Apis* bee honey prevents the decrease in GLUT4 protein expression

It has been proven that GLUT4 is the main glucose transporter in the skeletal muscle as evidenced by a positive association between GLUT4 protein level and skeletal muscle glucose uptake (Wu, Cheng, Liu, Lv, & Liu, 2018; Zhou et al., 2016). In skeletal muscle, overexpression of GLUT4 enhanced basal (20 – 300%) and insulin-induced (60 – 200%) muscle glucose uptake. On the contrary, skeletal muscle-specific inactivation of GLUT4 reduces basal glucose uptake (70 – 80%) and completely prevents an insulin-induced glucose uptake (Tunduguru & Thurmond, 2017). Hence, GLUT4 plays a critical role in regulating skeletal muscle glucose uptake, and possibly, in response to stimulation by *Apis* bee honey.

As compared with non-diabetic rats, the level of GLUT4 was markedly reduced, implying that insulin signalling transduction of the skeletal muscle was impaired which would result in insulin resistance. When *Apis* bee honey was given to diabetic rats, significant decrease in GLUT4 levels were prevented, thus this would help to improve insulin sensitivity by enhancing glucose uptake in the skeletal muscle in T2DM.

Restoration of GLUT4 level at the cell surface, with relative increased in PI3K and Akt expression in skeletal muscle of diabetic rats would help to improve skeletal muscle glucose transport, thereby reducing hyperglycemia.

CHAPTER 6: CONCLUSION

6.1 Conclusion

In conclusion, our findings suggested that oral administration of *Apis* bee honey may improve insulin resistance in the skeletal muscle of diabetic rats. (Epi)afzelechin-(epi)catechin conjugate and isorhamnetin 3-O-rutinoside detected in *Apis* bee honey may were postulated to exert protective effect on the skeletal muscle of T2DM rats through its antioxidant, anti-inflammatory and anti-apoptotic actions. It is possible that the synergistic interactions among *Apis* bee honey constituents may contribute to the antidiabetic activity of *Apis* bee honey. Moreover, *Apis* bee honey may improve glucose uptake into skeletal muscle by enhancing insulin signalling molecules (IRβ, IRS1, PI3K and Akt) and plasma membrane GLUT4. Although the exact mechanism involved in the insulin action by *Apis* bee honey remains to be elucidated, as a natural and non-toxic product, is still a promising candidate for the treatment of T2DM. It can be concluded that *Apis* bee honey may play a significant role in the management of T2DM by improving the expression of insulin signalling molecules and GLUT4 protein to enhance glucose uptake in skeletal muscle.

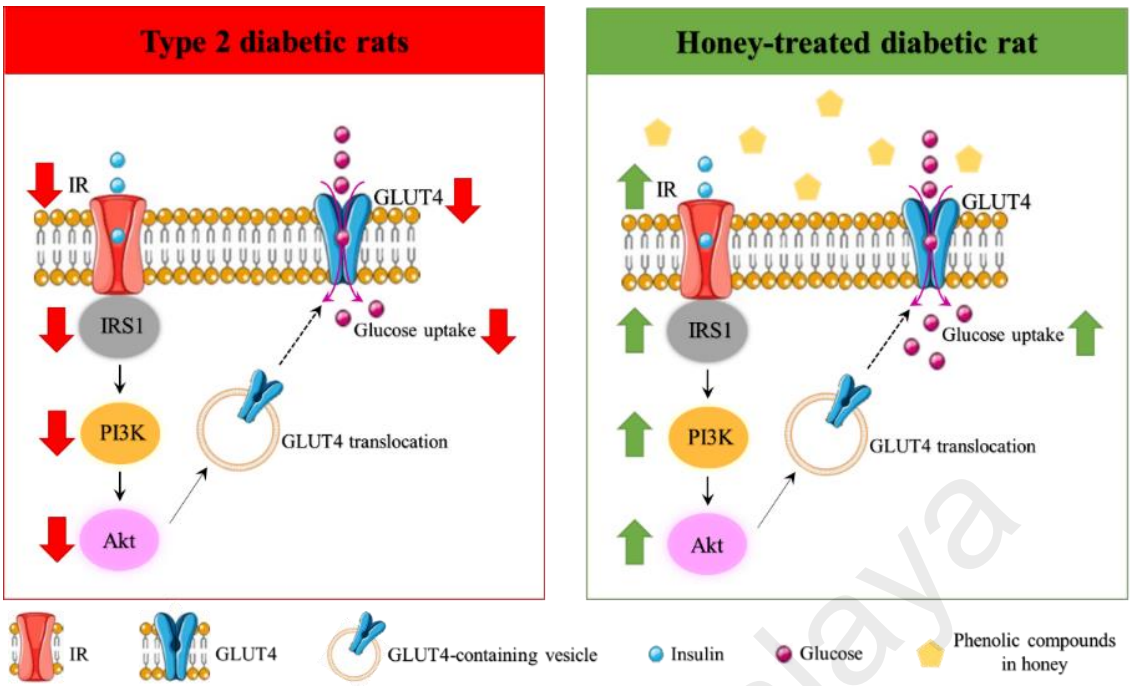

Figure 6.1: Honey improves insulin signalling pathway in the skeletal muscle of diabetic rats.

6.2 Limitation of the study

Apis bee honey exerts protective effects against hyperglycemia and skeletal muscle degeneration in diabetic rats. Yet, the precise mechanism has not been investigated, even if they are hypothetically attributed to antioxidant effect of the phenolic compounds in *Apis* bee honey. The antioxidant property of honeys is predominantly attributed to honey phenolics, hence future study can focus on determination of phenolic content and antioxidant activity of *Apis* bee honey as well as analysing the correlation between antioxidant activity and phenolic content of *Apis* bee honey.

In addition, an activation of each insulin signalling molecules at phosphorylation level was not studied. Therefore, *Apis* bee honey was found to increase the expression of insulin signalling molecules, presumably by enhancing the activation of each signalling molecules at phosphorylation level.

6.3 Future studies

STZ-NA-induced diabetic rats serve as an appropriate diabetes model for investigation on the anti-diabetic properties of *Apis* bee honey. Yet, fat-fed STZ-induced diabetes model could be a better model as it replicates the natural pathogenesis of human T2DM and renders the animals with insulin resistance, glucose intolerance and stable hyperglycemia that mimic the syndromes of human T2DM.

Besides, although *Apis* bee honey was found to enhance insulin signalling molecules and plasma membrane GLUT4, the exact mechanism involved in the insulin action by *Apis* bee honey remains to be fully elucidated.

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