ANTI-DIABETIC ACTIVITY OF *Leptospermum flavescens* LEAVES USING *IN VITRO* AND *IN VIVO* MODELS

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ANTI-DIABETIC ACTIVITY OF LEPTOSPERMUM FLAVESCENS LEAVES USING IN VITRO AND IN VIVO MODELS

ABSTRACT

Leptospermum flavescens Sm. (Myrtaceae) locally known as gelam bukit has been used traditionally to treat various ailments such as constipation, hypertension, diabetes, and cancer. To date, there is still limited scientific evidence on L. flavescens inducing anti-diabetic activity. Thus, the aim of the present study was to investigate the anti-diabetic effects of L. flavescens using in vitro and in vivo models. L. flavescens extraction yielded four extracts: hexane, ethyl acetate, methanol, and water extracts. The methanol extract of L. flavescens (MELF) revealed the highest biological activity such as exerting the greatest antioxidant activity, promoting the highest α-amylase and α-glucosidase inhibition, protecting INS-1 β cells against streptozotocin (STZ) induced apoptosis (with cell recovery up to 91.12 %) and increasing INS-1 β cells insulin secretion. Furthermore, MELF was found to inhibit apoptosis in STZ-induced INS-1 β cells according to Annexin V/PI, Hoechst 33342/PI, mitochondria membrane potential (MMP) and western blot assay. Besides, MELF inhibited autophagy and induced AKT and GSK-3β protein expression based on western blot assay. MELF was shown to regulate the redox potential by increasing intracellular catalase activity and inhibiting intracellular reactive oxygen species (ROS) production. The redox regulation was further corroborated by HO-1 and Nrf-2 protein expression towards STZ-induced INS-1 β cells. MELF treatment of 2 g/kg showed no signs of toxicity observed in Sprague Dawley (SD) rats for 14 days as confirmed by histopathological and biochemical analysis. MELF was found to reduce fasting blood glucose (FBG) as evidenced by oral glucose tolerance test (OGTT). Additionally, MELF also induced hypoglycemic effects in STZ-NA-induced SD rats. MELF also promoted insulin production in STZ-NA-induced SD rats based on immunohistochemistry assay. Notably, MELF stimulated its pancreas-protective effects
via inhibition of cleaved caspase 3 and LC3A/B proteins based on immunohistochemistry assay. Furthermore, MELF was shown to increase HDL and reduce LDL levels in STZ-NA induced SD rats. Therefore, based on the cumulative results, MELF might hold plausible anti-diabetic activity.

**Keywords**: apoptosis, autophagy, antioxidant, anti-hyperglycemic, INS-1 and diabetic rats
AKTIVITI ANTIDIABETIK DAUN LEPTOSPERMUM FLAVESCENS
MENGUNAKAN MODEL IN VITRO DAN IN VIVO

ABSTRAK

Leptospermum flavescens Sm. (Myrtaceae) lebih dikenali sebagai gelam bukit, digunakan sebagai rawatan tradisional untuk pelbagai jenis penyakit seperti sembelit, darah tinggi, diabetes dan kanser. Sehingga kini, hanya terdapat beberapa kajian saintifik mengenai *L. flavescens* yang digunakan sebagai rawatan diabetes. Oleh yang demikian, objektif utama kajian ini adalah untuk menyiasat keberkesanan *L. flavescens* sebagai rawatan diabetes melalui kaedah *in vitro* dan *in vivo*. Pelarut organik seperti heksana, etil asetat, metanol dan air digunakan untuk mengekstrak daun *L. flavescens*. Diantara semua ekstrak, *L. flavescens* metanol ekstrak (MELF) menunjukkan hasil biologi aktiviti tertinggi berbanding ekstrak yang lain. Contohnya, MELF mempunyai kandungan antioksidan tertinggi, merencat enzim α-amilase dan α-glukosidase, melindungi INS-1 β sel dari streptozotosin (STZ) (hampir 91.12 % pemulihan INS-1 β sel) dan membantu INS-1 β sel merembes insulin. MELF juga mampu untuk melindungi INS-1 β sel apoptosis dari STZ melalui kaedah Annexin/PI, Hoechst 33342/PI, MMP dan western blot asai. Tambah pula, MELF juga mampu untuk melindungi INS-1 β sel dari autophagi yang terjejas dan membantu meningkatkan AKT dan GSK-3β protein melalui kaedah western blot asai. Di samping itu juga, MELF meningkatkan antioksidan enzim seperti catalase dan melindungi dari spesis oksigen yang reaktif. Regulasi redox ini juga disebabkan oleh protein HO-1 dan Nrf-2 yang terdapat di dalam INS-1 β sel. MELF (2g/kg) diberikan kepada SD tikus dan keputusan menunjukkan tiada perubahan toksik dilihat selepas hari ke 14. Tambahan pula, ujian biokimia dan histologi membuktikan tiada perubahan toksik yang ketara berlaku kepada SD tikus. MELF dapat menurunkan gula darah terhadap SD tikus bukti dari eksperimen OGTT. Di samping itu juga, rawatan MELF kepada STZ-NA SD tikus mampu meningkatkan gula darah. MELF dapat

Kata kunci: apoptosis, autophagi, antioxidan, antihyperglicemik, INS-1 dan diabetes tikus.
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<td>AEU</td>
<td>Animal experimental unit</td>
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<tr>
<td>AIF</td>
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<td>cAMP response element-binding protein</td>
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<td>Diacylglycerol</td>
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<td>Dendritic cell</td>
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<td>Dichlorofluorescin diacetate</td>
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<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>ECM</td>
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<td>Focal adhesion kinase</td>
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<td>First apoptosis signal receptor</td>
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<td>Fas-ligand</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>Iron (III) chloride</td>
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<td>Free fatty acid</td>
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<td>FIP200</td>
<td>FAK family interacting protein of 200 kD</td>
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<td>Glucose transporter 4</td>
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<td>GPCR</td>
<td>G protein couple receptor</td>
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<td>GPx</td>
<td>Glutathione peroxidase</td>
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<td>Glutathione</td>
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<td>GSSG</td>
<td>Glutathione disulfide</td>
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<td>GSK-3</td>
<td>Glycogen synthase kinase 3</td>
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<td>GSIS</td>
<td>Glucose-stimulated insulin secretion</td>
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<td>H &amp; E</td>
<td>Hematoxylin and eosin</td>
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<td>H₂SO₄</td>
<td>Sulphuric acid</td>
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<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<td>Hb</td>
<td>Haemoglobin</td>
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<td>HbA₁C</td>
<td>Glycated haemoglobin</td>
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<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>HSC70</td>
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<td>High-density lipoprotein</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)piperazin-1-ethanesulfonic acid</td>
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<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>Heme oxygenase 1</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>Full Form</td>
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<tr>
<td>HtrA2</td>
<td>High temperature requirement protein A2</td>
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<tr>
<td>IACUC-FOM</td>
<td>Institutional Animal Care and Use Committee, Faculty of Medicine</td>
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<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
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<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired glucose tolerance</td>
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<td>IkB kinase</td>
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<td>Interleukin-1</td>
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<td>Interleukin-6</td>
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<tr>
<td>Ip</td>
<td>Intraperitoneal</td>
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<td>Inositol-requiring enzyme 1 alpha</td>
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<td>IRS</td>
<td>Insulin receptor substrate</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>K⁺</td>
<td>Potassium ion</td>
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<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt;</td>
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<td>Lys-Phe-Glu-Arg-Gln</td>
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<td>LAMP2A</td>
<td>Lysosomal-associated membrane protein 2A</td>
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<td>LCMS</td>
<td>Liquid chromatography mass spectrometry</td>
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<td>LC3A/B</td>
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<td>LD</td>
<td>Low dose</td>
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<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>Maf</td>
<td>Musculoaponeurotic fibrosarcoma oncogene homolog</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MCL</td>
<td>Myeloid cell leukemia</td>
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<td>Acronym</td>
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<td>Mdm2</td>
<td>Mouse double minute 2 homolog</td>
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<td>MELF</td>
<td>Methanol extract <em>L. flavescens</em></td>
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<td>MHC</td>
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<td>MLK3</td>
<td>Mixed-lineage protein kinase 3</td>
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<tr>
<td>MMP</td>
<td>Mitochondria membrane potential</td>
</tr>
<tr>
<td>Mn</td>
<td>Manganese</td>
</tr>
<tr>
<td>MOMP</td>
<td>Major outer membrane protein</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>NA</td>
<td>Nicotinamide</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
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<tr>
<td>Na₂CO₃</td>
<td>Sodium bicarbonate</td>
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<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
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<td>Neighbour of BRCA1 gene 1 protein</td>
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<td>Nitrotetrazolium blue chloride</td>
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<td>NIDDM</td>
<td>Non-insulin dependent diabetes mellitus</td>
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<td>NIX</td>
<td>BNIP3-like</td>
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<td>NADPH quinone oxidoreductase-1</td>
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<td>Nuclear factor-like 2</td>
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<td>O₂</td>
<td>Oxygen</td>
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<td>O₂⁻</td>
<td>Superoxide anion</td>
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<td>OECD-423</td>
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<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<tr>
<td>OPTN</td>
<td>Optineurin</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>p62</td>
<td>Nucleoporin p62</td>
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<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
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<tr>
<td>PAS</td>
<td>Phagophore assembly site</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PDK1</td>
<td>Pyruvate dehydrogenase lipamide kinase isozyme 1</td>
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<td>PDX1</td>
<td>Pancreas/duodenum homeobox protein 1</td>
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<td>PH</td>
<td>Pleckstrin homology</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-3,4,5-diphosphate</td>
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<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
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<td>PKA</td>
<td>Protein kinase A</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMP</td>
<td>Plasma membrane potential</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine methosulfate</td>
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<tr>
<td>PNPG</td>
<td>p-Nitrophenyl β-D-glucopyranoside</td>
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<td>PPA</td>
<td>Porcine pancreatic amylase</td>
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<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<td>QTOF</td>
<td>Quadrupole time of flight</td>
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<td>RAF</td>
<td>Rapidly accelerated fibrosarcoma</td>
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<td>RBP4</td>
<td>Retinol-binding protein 4</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RPM</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RS</td>
<td>Regenerated cellulose</td>
</tr>
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<td>Acronym</td>
<td>Full Name</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague Dawley</td>
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<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
</tr>
<tr>
<td>Ser-P</td>
<td>Phosphoserine</td>
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<tr>
<td>SGLT-2</td>
<td>Sodium/glucose transporter 2</td>
</tr>
<tr>
<td>Smac</td>
<td>Second mitochondria-derived activator of caspases</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
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<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TSC1</td>
<td>Tuberous sclerosis 1</td>
</tr>
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<td>TSC2</td>
<td>Tuberous sclerosis 2</td>
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<td>Thiazolidinedione</td>
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<td>Ubiquitin</td>
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<td>Unc-51 like autophagy activating kinase</td>
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<td>UPR</td>
<td>Unfolded protein response</td>
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<td>VDCC</td>
<td>Voltage-dependent calcium channel</td>
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<td>Vit C</td>
<td>Vitamin C</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>Vit E</td>
<td>Vitamin E</td>
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<tr>
<td>Vm</td>
<td>Action potential</td>
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<td>VPS15</td>
<td>Vacuolar protein sorting-associated protein 15</td>
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<tr>
<td>VPS34</td>
<td>Vacuolar protein sorting-associated protein 34</td>
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<td>WELF</td>
<td>Water extract <em>L. flavescens</em></td>
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<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>XBPI</td>
<td>X-box binding protein 1</td>
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<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes-associated protein 1</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
<tr>
<td>Z-VAD-FMK</td>
<td>N-Benzylxocarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone</td>
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Diabetes mellitus (DM) is one of the most common chronic diseases throughout all countries. According to the World Health Organization (2017), the number of people with diabetes mellitus have increased from 108 million since 1980 to 422 million in 2014. Without early detection, DM can bring many undesired complications such as hypertension, vision loss, kidney injury, weak muscle and gangrene (Olaokun et al., 2016). The most prevalent DM is type 2 diabetes (T2D), which accounts for 90-95% among all DM patients (Wang et al., 2016). There are several common medication used for treating T2D, such as insulin, α-glucosidase inhibitors, biguanides, dopamine agonist, DPP-4 inhibitors, glucagon-like peptide, meglitinides, SGLT2 inhibitors, sulfonylureas and thiazolidinediones (Nain et al., 2012). However, some of these chemical medications promote severe side effects to the human body such as liver or kidney toxicity. Therefore, the urge of developing new alternative treatment such as secondary metabolite from plants may help in prevent or cure DM (Arya et al., 2015).

One of the major factors that promote DM is the inability of pancreatic β cells to secrete insulin (Gerber et al., 2017). Pancreatic β cell are particularly sensitive to reactive oxygen species (ROS) because they have low antioxidant enzyme such as catalase and superoxide dismutase (Lenzen, 2017). Exposure to various cytotoxic matter such pro-inflammatory cytokines, high glucose or even ROS, can stimulate pancreatic β cells apoptosis and death (Tomita, 2016). In addition, the production of impaired autophagy has been associated to pancreatic β cell death (Marasco & Linnemann, 2018). Thus, deciphering the key mechanism in promoting the protection of pancreatic β cells is important to combat DM. Another therapeutic approach is to prevent the glucose production by inhibiting the α-amylase and α-glucosidase enzyme in the digestive system.
(Kalita et al., 2018). Diminishing of glucose in the small intestine result in lowering of postprandial hyperglycemia and therefore can be used as an effective means to fight DM.

*L. flavescens*, also known as *gelam bukit* has been used traditionally to treat cancer and diabetes. The plant grows in the mountains and is usually found in Australia, New Zealand and Malaysia. In South East Asia, *L. flavescens* has been used to treat constipation, lethargy, hypertension, diabetes and kidney pain (Demuner et al., 2011). The locals consume the leaves raw or as a concoction brewed from fresh plants. The water extract of *L. flavescens* was reported to reduce blood glucose in alloxan-induced diabetic rats (Asmawati et al., 2014). However, the underlying mechanisms for treating diabetes and improving β cells damage in *in vitro* and in *in vivo* remains a paradox. Therefore, the main objective is:

To investigate the anti-diabetic activity of the leaves extracts of *L. flavescens* using *in vitro* and *in vivo* models.

Specific objectives

1. To determine the antioxidant activity of the leaves extracts of *L. flavescens*.
2. To investigate the α-amylase and α-glucosidase inhibition of *L. flavescens* extracts.
3. To assess the hypoglycemic effect of *L. flavescens* extracts in STZ-induced diabetic rats.
4. To assess the effect of *L. flavescens* extracts on pancreatic β cell function through cell apoptosis and autophagy.
5. To investigate the protective underlying mechanisms of *L. flavescens* extracts in INS-1 β cells.
2.1 Diabetes mellitus (DM)

DM is a group of metabolic diseases characterized by high blood glucose or hyperglycemia in the body, mainly due to insulin resistance, inadequate insulin secretion, or excessive glucagon secretion (American Diabetes Association, 2010). DM consists of two types, namely type 1 diabetes (T1D) or type 2 diabetes (T2D) (Bahar et al., 2017). In general, there are two major hormones that control blood glucose homeostasis in our body, insulin, reduces blood glucose and glucagon, increases blood glucose (Henquin et al., 2017). Both hormones are synthesized in a cluster of cells inside the pancreas called the islet of Langerhans where insulin is secreted by β cells located in the middle of islet while glucagon is secreted by α cells which are located at the periphery of islet (Pedersen et al., 2017). Insulin control the blood glucose in the body by binding to the insulin receptor embedded in various insulin responsive tissues such as muscle cells or adipose tissue and activates vesicle containing glucose transporter to fuse into the plasma membrane (Cervone & Dyck, 2017). Activated glucose transporter such as GLUT1, GLUT2, GLUT3 or GLUT4 transport glucose from bloodstream into the cells where the glucose is used as a source of energy for most cellular mechanisms (Szablewski, 2017). GLUT 1 is found mostly in fetal tissues, erythrocytes, and endothelial cells of barrier tissues such as blood-brain barrier (Deng et al., 2014). Meanwhile, GLUT2 is usually found in renal tubular cells, liver cells, and pancreatic cells. In the liver cells, the uptake of glucose is used for glycolysis (breakdown of glucose for energy) and glycogenolysis (production of glycogen for energy storage) while the release of glucose is used during gluconeogenesis (Thorens, 2015). GLUT3 is found mostly in neurons and placenta and GLUT4 is in adipose tissues or striated muscle (Hresko et al., 2016). Glucagon does the opposite action of insulin which raises the concentration of glucose in the bloodstream.
Glucagon binds to the glucagon receptor located in the plasma membrane of liver cells and activates a process called glycogenolysis which causes the breakdown of glycogen into glucose and releases glucose into the bloodstream. As the amount of glucose decreases in the liver, glucagon stimulates liver cells to synthesize additional glucose through gluconeogenesis mechanism (Kim et al., 2017).

At basal glucose levels, pancreatic β cells are electrically inactive allowing the opening of ATP-sensitive K⁺ (K\text{ATP}) channels. Later, the plasma membrane potential (PMP) of pancreatic β cells are hyperpolarized which leads to inhibition of insulin secretion. In contrast, α cells are electrically charged which leads to the activation of a downstream mechanism such as closure of the K\text{ATP} channels, PMP depolarized and entry of Ca²⁺ through voltage-dependent Na⁺, Ca²⁺ (VDCC). These events eventually promote the release of glucagon into the bloodstream. When plasma glucose is increased in the bloodstream, the glucose diffuses into the pancreatic β cells through GLUT2 channels, where it is metabolized through glycolysis pathway. This pathway leads to the production of intracellular ATP/ADP ratio. Eventually, these signals induced the insulin released mechanisms of the pancreatic β cell such as the closure of K\text{ATP} channels, PMP depolarized and entry of Ca²⁺ through VDCC (Figure 2.1) (Gaisano et al., 2012).
Figure 2.1: Schematic diagram of glucose-dependent regulation of glucagon and insulin secretion inside α and β cells respectively. The figure was adapted from Müller et al. (2017).
2.2 Type 1 Diabetes (T1D)

T1D is a form of diabetes where the body does not produce enough insulin, resulting in increasing glucose levels in the bloodstream. The disease happens only 10% of all DM patients and usually fallen in early childhood. The cause of T1D is due to genetic abnormality of pancreatic β cells and immune response of dendrite cell (DC). When pancreatic β cells secrete antigens such as protein, the antigens are specifically targeted by immune attack (Rowe, 2017). The antigen also known as β-cell autoantigen is detected by dendrite cells and further cognate response towards CD8⁺ or CD4⁺ type of T cells (Roep & Tree, 2014). Activation of CD8⁺ T cells induced pancreatic β cells inflammation while CD4⁺ T cells induced pancreatic β cells apoptosis which leads to β cells damage and destruction (Figure 2.2) (Katsarou et al., 2017).

One set of genes involved in controlling the immune response is the human leukocyte antigen (HLA) system. The system is located at chromosome 6 and encoded the major histocompatibility complex (MHC) which is a type of proteins that help to recognize foreign antigen from our body as well as maintaining self-tolerance (Velthuis et al., 2010). For instance, if a healthy cell is infected with a virus, the system carries the virus protein fragments towards the plasma membrane. Later, the infected cells were detected and destroyed by the immune system. Most of the T1D patients display another type of HLA system such as HLA-DR3 and HLA-DR4. The presence of these specific defective HLA system gives out the genetic clue of how T1D is being developed (Kakleas et al., 2015). Although people with T1D are unable to produce insulin, they can still respond to insulin. Which means treatment involving lifelong insulin therapy can regulate their blood glucose. Thus, this disease is also called as insulin-dependent diabetes mellitus (IDDM) (Mbongue et al., 2017; McAuley et al., 2016).
2.3 Type 2 Diabetes (T2D)

T2D is the most prevalent form of DM, with approximately 90% of DM patients were diagnosed with T2D. The disease appears mostly in individuals over 30 years old. T2D disease is caused by environmental or behavioral factors such as living a sedentary lifestyle, excessive food intake, less physical activity or developing obesity (Selph et al., 2015). In early onset of T2D, β cells secrete normal amount of insulin towards increasing blood glucose. However, the insulin-responsive tissues or cell established resistance towards insulin is also known as insulin resistance (Leung, 2016). There are several factors that can promote insulin resistance such as lipid intermediates, proinflammatory cytokines, counter-regulatory hormones, mitochondria dysfunction or endoplasmic reticulum stress. These factors activate various serine/threonine protein kinases and further phosphorylate insulin receptor substrate (IRS) proteins as well as other components of the insulin signaling pathways. In doing so, the phosphorylated substrates exploit negative feedback towards the insulin receptor and thus terminate insulin signal transduction leading to the development of insulin-resistance state (Figure 2.2) (DeFronzo et al., 2015). Subsequently, insulin in the blood does not bind to insulin receptor which in turn prevents many proteins activation cascades such as translocation of GLUT4 transporter, glycogen synthesis, and glycolysis. Therefore, the cells did not receive much glucose, and the blood glucose levels start to increase (Haeusler et al., 2017).

Since tissues or cells didn’t respond to the normal level of insulin, pancreatic β cells hypersecreted insulin so that blood glucose level can return to normal (normoglycemia), where this condition is also known as β cell hyperplasia. The effect masks the presence of impaired glucose tolerance (IGT) for several years and β cells begin to increase in cell masses (Tajima et al., 2017). The over production of insulin makes the β cells work harder. In time, β cells are getting exhausted (loss of β cell mass) and the number of
insulin release started to decline. In addition, exposure of different cytotoxic matter such as pro-inflammatory cytokines, perforin, granzyme B, high glucose, ROS, fatty acid or amyloid polypeptides can also bring to the loss of β cell mass (Johnson et al., 2015). However, the fasting blood glucose levels are slightly above the normal range but still below the threshold for T2D (American Diabetes A., 2017). As β cell function starts to decline, a mild postprandial hyperglycemia develops, revealing the inability of the β cell to hypersecrete enough insulin to overcome insulin resistance (Ceriello & Genovese, 2016). The event of β cells dysfunction plus with insulin resistance is called as late stage T2D (Khodabandehloo et al., 2016). Notably, glucose level increased (hyperglycemia) which in turn induced apoptosis cell death towards β cells (Brereton et al., 2016). Unlike T1D, there is still several β cells able to secrete insulin for controlling blood glucose. Thus, treatments are not involved entirely to insulin therapy and this disease is non-insulin dependent diabetes mellitus (NIDDM) (Kalkman et al., 2017).
Figure 2.2: Pathogenesis of T1D. This disease is an immune-mediated disease. Dashed arrow indicates the potential interactions between B cell and CD8⁺ T cell and DCs. BCR: B cell receptor. TCR: T cell receptor. The figure was adapted from Katsarou et al. (2017).
Figure 2.3: Mechanism of insulin resistance inside a muscle or liver cells. This disease is caused either by environmental or behavioral. Arrow indicates the induction cascade between proteins while red line indicates the inhibition cascade between target proteins. IRS: Insulin receptor substrate. The figure was adapted from DeFronzo et al. (2015).
2.4 Other forms of diabetes

There are several subtypes of DM such as gestational diabetes mellitus (GDM) or drug-induced diabetes. In gestational diabetes, pregnant women at 3rd trimester have a spike in blood glucose due to pregnancy hormone interfere with insulin action towards insulin receptor. It represents the largest risk factor for future development of full T2D towards the mother (Hanna et al., 2017). The disease can cause considerable morbidity and long-term complications for both mother and child. For instance, GDM has shown to increase the risk of autism disorder, potentially schizophrenia, and behavioral and cognitive abnormalities towards the offspring (Money et al., 2017). However, the mother can prevent developing GDM by getting balance meal, regular light exercise, getting medical check-ups and always monitor fetal growth and well-being (Aune et al., 2016).

Besides that, some medications have side effects towards human body which can induce high blood glucose levels. In addition, the medications can also worsen pre-existing hyperglycemia wherein this case is called as drug-induced diabetes. The diabetogenic properties of these drugs, raise blood glucose through a variety of mechanisms such as decreased insulin biosynthesis or secretion, reducing tissue sensitivity to insulin, direct cytotoxic effects on pancreatic cells or increase in glucose production (Fathallah et al., 2015). For instance, of drug-induced diabetes are glucocorticoids, oral contraceptive pills, thiazide diuretics, non-selective β1-adrenoceptor antagonists, STZ, pentamide, cislosporin, diazoxide, β2-receptor agonists, growth hormone, protease inhibitor and antipsychotics (Ponte et al., 2016).
2.4.1 Streptozotocin (STZ)

STZ (2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose) is a naturally occurring diabetogenic compound (Figure 2.4) derived from *Streptomyces achromogenes* which is a type of soil bacteria (Eleazu *et al*., 2013). It is widely used for induction of mild and stable diabetes in experimental animals. STZ is selectively cytotoxic to the pancreatic β cells (Bathina *et al*., 2017). Its bind to a GLUT2 transporter protein of β cells and initiate a cell death downstream mechanism such as DNA and chromosomal damage and generate free radicals (Nahdi *et al*., 2017). In STZ experimental model, it does not induce insulin resistance; however, it can stun pancreatic β cells from releasing insulin, thus increase blood glucose (Liu *et al*., 2016). Under modified protocols, STZ can induce either T1D or T2D animal models. For instance, a single dose of STZ induced T2D while a multiple dose of STZ induced T1D (Adam *et al*., 2016; Al-Qattan *et al*., 2017). In addition, a single dose of nicotinamide (inhibit DNA methylation) together with STZ have shown to induce late stage of T2D where the pancreatic β cells secrete mild insulin levels (Arya *et al*., 2015).

![Molecular structure of STZ](image)

**Figure 2.4:** Molecular structure of STZ. Image was adapted from Chemspider
2.5 Diagnosis of DM

In general, the amount of blood glucose was measured in fasting patient (without the intake of food or sugary drink except for water) after more than 8 hours. The results of blood glucose levels between 110 - 125 mg/dL indicate prediabetes while more than 126 mg/dl indicate diabetes. A non-fasting or random glucose test can be measured with results of blood glucose more than 200 mg/mL indicate red flag for diabetes. Another test is oral glucose tolerance test, where the patient was given 2 mg/mL of glucose and the blood glucose levels were measured at time intervals for 2 hours. Blood glucose levels of 140 – 199 mg/dL indicate prediabetes, while more than 200 mg/dL indicate diabetes. When blood glucose increase, glucose binds to hemoglobin (Hb) of red blood cells, forming glycated hemoglobin (HbA1C). Thus, another set of tests for diagnosing DM is called HbA1C test. Percentage of HbA1C of 6 – 6.4 indicate prediabetes while HbA1C of more than 6.5 indicated diabetes (World Health Organization, 2006).

<table>
<thead>
<tr>
<th>Testing</th>
<th>Prediabetes</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting blood glucose</td>
<td>110 – 125 mg/dL</td>
<td>≥ 126 mg/dL</td>
</tr>
<tr>
<td>Non-fasting blood glucose</td>
<td>/</td>
<td>≥ 200 mg/dL</td>
</tr>
<tr>
<td>Oral glucose tolerance</td>
<td>140 – 199 mg/dL</td>
<td>≥ 200 mg/dL</td>
</tr>
<tr>
<td>HbA1C</td>
<td>6 – 6.4 %</td>
<td>≥ 6.5 %</td>
</tr>
</tbody>
</table>

Table 2.1: Summary of diagnostic criteria for prediabetes and diabetes.
2.6 Complication of DM

Clinical symptoms of uncontrolled DM involved polyphagia, glycosuria, polyuria, or polydipsia (Abbasi & Bradford, 2014). Without insulin, there is no uptake of glucose by the cells which makes the cells starved for energy. In response, adipose tissues start to break down fats called lipolysis and muscle tissues start to break down proteins. These catabolic states induce weight loss and the body starts craving for food (polyphagia). In the kidney, a high glucose concentration causes some of the glucose to spill into the urine (glycosuria) (Adinortey, 2017). Since glucose is hypertonic, water is excreted out which result in increased urea volume (polyuria). Thus, the body starts to dehydrate and increase in thirst (polydipsia) (Akhlaghi et al., 2017).

At times, uncontrol hyperglycemia can induce various impairment to the human body. In the blood vessels, hyperglycemia induces hyaline deposited around the wall of arteriole which makes it hard and inflexible (hyaline arteriolosclerosis). Subsequently, it further increased the risk of medium or large arterial wall damage leading to atherosclerosis and later can cause a heart attack or stroke (Fetterman et al., 2016). In the eyes, DM can lead to diabetic retinopathy, where the present of cotton wool spots or flame hemorrhages inside the eyes and leads to blindness (Lechner et al., 2017). Inside kidney, the afferent and efferent arteriole and glomerulus can get damaged causing nephrotic syndrome. Over time, the kidney loss ability to filter blood and ultimately leads to dialysis (Papadopoulou et al., 2017). DM also affects the function of nerves, which decrease sensation in toes and fingers called stocking-glove distribution. With poor blood supply and nerve damage leads to the formation of ulcer, typically in the feet which are not easily healing. Once the damage gets severe, the feet might get amputate (Feldman et al., 2017; Swaminathan et al., 2017).
Table 2.2: Characteristic of major currently available of each oral antidiabetic drugs. The table was adapted from DeFronzo et al. (2015).

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Glycaemic efficacy (HbA1c)</th>
<th>Durability</th>
<th>Mechanism of action</th>
<th>Body weight</th>
<th>Cardiovascular risk factors</th>
<th>Cardiovascular safety</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin</td>
<td>Decrease</td>
<td>No</td>
<td>Decrease hepatic glucose production</td>
<td>Slight decrease</td>
<td>Slight decrease</td>
<td>Possibly beneficial</td>
<td>Gastrointestinal and lactic acidosis</td>
</tr>
<tr>
<td>Sulfonylureas</td>
<td>Decrease</td>
<td>No</td>
<td>Increase insulin secretion</td>
<td>Slight increase</td>
<td>No</td>
<td>Possibly detrimental</td>
<td>Hypoglycaemia</td>
</tr>
<tr>
<td>TZDs (pioglitazone)</td>
<td>Decrease</td>
<td>Yes</td>
<td>Increase insulin sensitivity and β cell function</td>
<td>Increase</td>
<td>Decrease</td>
<td>Possibly beneficial</td>
<td>Fluid retention and bone fractures</td>
</tr>
<tr>
<td>DPP4 inhibitors</td>
<td>Mild decrease</td>
<td>No</td>
<td>Mild decrease glucagon secretion and Weak insulin secretion</td>
<td>Normal</td>
<td>No</td>
<td>Neutral</td>
<td>Unknown</td>
</tr>
<tr>
<td>SGLT2 inhibitors</td>
<td>Decrease</td>
<td>Not know</td>
<td>Decrease glucosuria and glucotoxicity</td>
<td>Decrease</td>
<td>Decrease</td>
<td>Unknown</td>
<td>Genital mycotic infections with volume-related</td>
</tr>
<tr>
<td>α-glucosidase inhibitors</td>
<td>Mild decrease</td>
<td>Not known</td>
<td>Mild decrease in carbohydrate absorption</td>
<td>Neutral</td>
<td>No</td>
<td>Possibly beneficial</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GLP1 receptor agonists</td>
<td>Decrease</td>
<td>Yes</td>
<td>Increase insulin secretion and decrease glucagon secretion</td>
<td>Decrease</td>
<td>Decrease</td>
<td>Unknown</td>
<td>Nausea and vomiting</td>
</tr>
</tbody>
</table>
2.7 Prevention and treatment

T2D is a preventable disease with proper consumption of healthy diet and daily physical exercise. Acquiring low-calorie foods, less trans-fat, less fried foods, less sugar foods or drinks, lean meat, more vegetables and fruits, high fiber food, high in vitamin and more drinking water can further reduce cholesterol level and lower blood sugar (Jannasch et al., 2017; Schwingshackl et al., 2017; Tuomilehto & Schwarz, 2016). Subsequently, T2D can be prevented by daily aerobic exercise due to physical activity that can naturally lower blood sugars (Hemmingsen et al., 2017). In some cases stated that 30 minutes of daily exercise can help in lowering risk of T2D development by 58% (Colberg et al., 2010). The association of healthy diet and exercise can further prevent others complications to the human body such as obesity (Moran et al., 2017), heart failure, blood pressure, kidney failure (Hall et al., 2014), cancer development (Trujillo et al., 2017) and skin aging (Couppe et al., 2017; Ekelund et al., 2016). Insulin therapy is essential for patients with T1D. The insulin usually administered under the skin by injections or by using an automatic insulin pump (Pickup et al., 2017). There are several types of insulin action which reacts at a different set of pharmacodynamics. For instance, the insulin can affect at a rapid, intermediate, or short time inside the body (Abiola et al., 2016; Pechenov et al., 2017). Patient with T2D may need exogenous insulin during acute stress (surgery or serious illness) or to supplement their oral medications for stronger blood glucose control. Most medications for T2D are oral drugs with different mechanism of action such as Metformin, Sulfonylureas, Meglitinides, Thiazolidinediones, DPP-4 inhibitors, SGLT-2 inhibitors, α-glucosidase inhibitors or GLP1 receptor agonists. The details of each oral drug in managing T2D are shown in Table 2.2.
2.8 Pancreatic β cell dysfunction

2.8.1 Pancreatic β cell apoptosis

Apoptosis or type 1 programmed cell death is an important mechanism for the regulation of homeostasis and tissues development inside the human body. It is a complex biological phenomenon which is characterized by cell shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies (Nagata & Tanaka, 2017). During birth, apoptosis is pivotal in remodeling the normal endocrine pancreas and the final growth of pancreatic β cell mass (Tomita, 2016). Furthermore, functionally β cell mass is regulated by a balance of β cell replication and apoptosis, islet hyperplasia and new islet formation from exocrine pancreatic ducts (Weir & Bonner, 2013). However, the present of pathological feature such as amylin (Park et al., 2017(b)), ROS (Bahar et al., 2017), fatty acid (Lee et al., 2017) or high glucose (Guo et al., 2017) affect the apoptotic regulatory machinery and induced apoptotic cell death towards β cells. Thus, increasing in apoptosis leads to β cell loss and further contribute either T1D or T2D (Liston et al., 2017; Storling & Pociot, 2017).

Apoptosis can be divided into two pathways namely, the extrinsic pathway (receptor-mediated) or the intrinsic pathway (mitochondria-mediated) (Sun et al., 2017). The extrinsic pathway is potentiated upon oligomerization between specific death ligands (Fas-L or TRAIL) and cell surface death receptors (Fas or TRAIL-R) (Thakor et al., 2017). Upon ligation, the proteins form a death-inducing signaling complex (DISC) containing the cytoplasmic Fas receptor, the adaptor protein Fas-associated death domain-containing protein (FADD) and procaspase 8 or 10 (Xue et al., 2017). Then, the complex proteins molecules activate caspase 8 or 10 and further induces activation of caspase 3 which lead to the events of apoptotic cell death (Derakhshan et al., 2017). In addition, caspase 8 or 10 can also induce the formation of tBid from Bid proteins which eventually trigger the mitochondria intrinsic signaling pathway (Vondálová et al., 2017).
Intrinsic pathway is activated through various signals such as hypoxia (Wu et al., 2017), cellular distress (Ahmadi et al., 2017), ROS (Pan et al., 2017), nutrient withdrawal (Villar et al., 2017), DNA damage (Rogers et al., 2017), ER stress (Glab et al., 2017) or cytotoxic drugs (Singh et al., 2017). These cytotoxic stimuli converge towards mitochondria to induce the regulation of B cell lymphoma 2 (Bcl-2) family proteins (Ashkenazi et al., 2017). Bcl-2 family proteins comprise of anti-apoptotic proteins (Bcl-2, Bcl-xl or MCL1) and proapoptotic proteins (Bax, Bad, Bim, Bak or Bid) (Birkinshaw & Czabotar, 2017). An excess of proapoptotic over antiapoptotic Bcl-2 members protein, induce the loss of mitochondria membrane potential (MMP) (Um, 2016). Thus, a set of proteins such as cytochrome c, Smac/Diablo, Omi/HtrA2, AIF or endonuclease G which are located inside the mitochondria were release through the mitochondria pores and into the cytosol (Renault et al., 2017). Consequently, cytochrome c accumulates with Apaf-1 forming a protein complex called as apoptosome, which induces cleavage of caspase 9 proteins and further activates caspase 3 or 7 proteins (White et al., 2017). In addition, Smac/Diablo interacts with XIAP to further prevent IAP inhibition of executioner caspase 3 activations for the induction of apoptosis (White et al., 2017). All apoptosis pathways can be shown in Figure 2.5.

During T1D, the induction of β cell apoptosis due to autoimmune has been studied extensively (Hosokawa et al., 2017; Marroqui et al., 2017). One key mechanism is the upregulation of cytokines from the β cell such as IL-1 which induce activation of Fas death receptor and eventually leads to apoptosis cell death (Park et al., 2017). Subsequently, perforin or granzyme B, which are released from CD8+T cells has been shown to induced β cell apoptosis and thus leads to the pathological of T1D (Newby et al., 2017; Yolcu et al., 2017). In T2D, there are several factors that induced β cell apoptosis such as endoplasmic reticulum (ER) stress (Abdulkarim et al., 2017), amyloid deposition (Templin et al., 2017), glucotoxicity (Zhang et al., 2016) or lipotoxicity.
(Cunha et al., 2016). Several studies have shown that prolong hyperglycemia in the blood induce β cell apoptosis through induction of Bcl-2 family proteins (Jadaun et al., 2017; Xiao et al., 2017). Due to a high demand for insulin, pancreatic β cells are easily susceptible to secretory stress pathway. Thus, increase in proteins flux, such as insulin through the ER and Golgi apparatus, induce the misfolding of proteins which later induce β cell apoptosis (Meyerovich et al., 2016; Paula et al., 2017). Apart from that, increase in fatty acids such as palmitate, induce β cell apoptosis via generation of ceramide and ROS (Fucho et al., 2017). Inhibition of apoptosis cell death is considered a therapeutic strategy for protection of pancreatic β cell. For example, the pharmaceutical anti-diabetic drug, metformin has been shown to inhibit caspase 3 formation thus ameliorates MIN6 pancreatic β cell apoptosis (Jiang et al., 2014).
Figure 2.5: Extrinsic and intrinsic apoptotic pathways. Figure was adapted from Ichim and Tait. (2016).
2.8.2 β cells impaired autophagy

Autophagy is an intracellular catabolic mechanism which is important for providing essential nutrient during cell deprivation or removing dysfunctional proteins or organelles during cellular stress (Kuwabara et al., 2017). The mechanism involves a long-lived proteins or organelles such as ER, mitochondria, peroxisomes, nuclease, or ribosomes to be degraded or “eaten” by the cell itself (Isaka et al., 2017). The collection of the unwanted organelles or proteins were then delivered to the lysosome for further degradation process. There are three types of mechanism involved in cellular degradation namely: macroautophagy, microautophagy or chaperone-mediated autophagy (Figure 2.6) (Kaur & Debnath, 2015).

There are four stages in macroautophagy process namely, induction, nucleation, elongation, and termination (Yang et al., 2017). Macroautophagy mechanism is initiated by the formation of phagophore assembly site (PAS) which is regulated by ULK1, ATG13, FIP200 and ATG101 proteins (Hurley & Young, 2017). The process continues by the formation of double-membrane (nucleation) and recruiting of unwanted cytosolic proteins or organelles which is mediated by Beclin-1, ATG14L, VPS15 or VPS34 proteins (Zhao et al., 2015). The double membrane phagophore expands and sequester the substrate forming a double membrane-bound vesicle which is known as autophagosomes. This process is mediated by ATG5, ATG12, ATG16L and LC3 proteins (Rogov et al., 2014). Then, these autophagosomes are fuses with the lysosome to form autolysosomes. In microautophagy, the substrates are directly engulfing through the lysosome membrane without the need of autophagosomes (Bellmore et al., 2017). Whereas in chaperone-mediated autophagy, the substrates that are selective recognize by KFERQ and HSC70 proteins are translocated into lysosomes lumen via the lysosomal-associated membrane protein 2A (LAMP2A) receptor (Tekirdag & Cuervo, 2017).
Eventually, the “cargos” are degraded inside via lysosomal hydrolases and used by the cell for nutrient (Jia et al., 2017). The mechanism of autophagy was shown in Figure 2.6.

Autophagy is also a type of programmed cell death, similar as apoptosis (Table 2.3) (Xiao et al., 2017). In response to cellular stress, autophagy promotes cell survival, however, once autophagy is overstimulated, cells can progress to autophagic cell death (Yang et al., 2017). The impairment of autophagy is due to either inhibition of fusion of lysosomes with autophagosome (Wang et al., 2017), inhibition of proteolytic degradation (Colacurcio et al., 2018) or inappropriate clearance of autophagy (Lim et al., 2016). These defects in autophagy mechanism can further induce pancreatic β cell dysfunction and death (Bartolomé et al., 2014). Several reports stated the impairment of autophagy are linked to the development of T2D (Lin et al., 2016; Su et al., 2013). For instance, accumulation of autophagosomes in the pancreatic β cell has been shown in a diabetic mouse model (Lo et al., 2015). In another example, pancreatic β cell with impaired autophagy has shown to reduce insulin secretion and promotes degeneration of cells (Kang et al., 2016). With constitutively activate autophagy, it can bring serious injurious effect on β cells which in turn can lead to autophagic cell death (Masini et al., 2017).
Figure 2.6: Autophagy mechanism. (a) represent macroautophagy, microautophagy, and chaperone-mediated autophagy. (b) represent mitophagy, aggrephagy, and pexophagy. Figure was adapted from Kaur and Debnath. (2015).

Table 2.3: Summarize features of apoptosis and autophagy programmed cell death.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Apoptosis (Type 1 programmed cell death)</th>
<th>Autophagy (Type 2 programmed cell death)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td>Chromatin condensation</td>
<td>Swollen organelles</td>
</tr>
<tr>
<td></td>
<td>DNA fragmentation</td>
<td>Autophagic bodies</td>
</tr>
<tr>
<td></td>
<td>Apoptotic bodies</td>
<td></td>
</tr>
<tr>
<td>Relative pathways</td>
<td>Death receptor pathway</td>
<td>AMPK pathway</td>
</tr>
<tr>
<td></td>
<td>Mitochondria pathway</td>
<td>AKT/mTOR pathway</td>
</tr>
<tr>
<td></td>
<td>ER stress pathway</td>
<td>MAPK pathway</td>
</tr>
<tr>
<td></td>
<td>Caspase-dependent pathway</td>
<td>ER stress pathway</td>
</tr>
<tr>
<td></td>
<td>Caspase-independent pathway</td>
<td></td>
</tr>
<tr>
<td>Proteins regulators</td>
<td>Caspases</td>
<td>Beclin-1</td>
</tr>
<tr>
<td></td>
<td>Bcl-2 family proteins</td>
<td>LC3</td>
</tr>
<tr>
<td></td>
<td>Cytochrome c</td>
<td>Atg family proteins</td>
</tr>
<tr>
<td></td>
<td>AIF, Calpain</td>
<td>ULK 1</td>
</tr>
</tbody>
</table>
2.9 Signaling pathway as protection in pancreatic β cells

2.9.1 PI3K/AKT and GSK-3β signaling pathway

The phosphoinositide 3-kinase (PI3K) pathway is an important signal transduction pathway that comprises many activators, effectors, inhibitors, and secondary messenger which promotes the survival and growth towards the pancreatic β cells (Gao et al., 2017; Kaneko et al., 2010). Activation of PI3K initiates when the physiological growth factors such as hormones, growth factors or component of the extracellular matrix (ECM) bind to the receptors tyrosine kinase (RTK) located in the plasma membrane (Manning & Toker, 2017). A major downstream mechanism of RTK involves G protein-coupled receptor (GPCRs), phospholipids, serine/threonine AKT and other effector pathways (Cattaneo et al., 2014). PI3Ks pathway can be divided into 3 class namely, class I (IA and IB), II and III (Fruman et al., 2017). In class IA PI3K, activation of RTK triggers a cross-phosphorylation of p85 adaptor subunit and p110 catalytic domains forming a heterodimer at the receptor site. The heterodimer complex phosphorylate phosphatidylinositol (3,4,5) diphosphate (PIP2) into phosphatidylinositol (3,4,5) triphosphate (PIP3) which induce PDK1 activation (Carnero & Paramio, 2014). In class IB PI3K, is a heterodimer consists of catalytic subunit p110γ and a regulatory subunit p101 (Thorpe et al., 2015). In class, II PI3K consist of a single catalytic subunit (Alliouachene et al., 2015). In class III PI3K, consists of single catalytic vacuolar protein-sorting defective 34 (Vps34) subunit. This subunit mediated signaling through mTOR and regulate autophagy mechanisms (Nemazanyy et al., 2015). The detail of each category of PI3K class is listed in Table 2.4.

AKT also was known as protein kinase B contains an N-terminal pleckstrin-homology (PH) domain, a central serine/threonine catalytic domain and a small C-terminal regulatory domain (Kriplani et al., 2015). AKT kinase activation is initiated when PDK1 or mTORC2 (the complex rictor/mTOR) phosphorylates AKT at the threonine 308
(T308) residue and serine 473 (S473) residue respectively (Dan et al., 2016; Lien et al., 2017). Eventually, activated AKT then phosphorylates its physiological substrates, which further promotes survival, migration, cell cycle progression, and metabolism of pancreatic β cells (Figure 2.7) (Hao et al., 2015; Vetere et al., 2014).

Activated AKT is important for the survival of pancreatic β cell from programmed cell death such as apoptosis (Ardestani et al., 2014) or autophagy (Fujimoto & Polonsky, 2009). The AKT involved in either inhibitory or stimulatory phosphorylation of different substrate proteins for survival or blocking of apoptosis. For inhibitory action, AKT phosphorylates YAP protein which inhibits p73-mediated apoptosis (Xiao et al., 2016); BAD and caspase 9 proteins which inhibit caspase cascade proteins (Dai et al., 2017); FoxO1, FoxO2, FoxO4 proteins which repress cell death genes (Zhang et al., 2011); and MLK3 and ASK1 which inhibit SAPK pathway (Ahn et al., 2013; Lee et al., 2014). For stimulatory action, AKT phosphorylates IKKα proteins for expression of pro-survival genes (Dan et al., 2014); Mdm2 proteins for inhibiting p53-mediated apoptosis (Daniele et al., 2015); and LC3-II proteins for promoting autophagosome (Noguchi et al., 2014).

GSK-3 protein is involved in various signaling pathways controlling metabolism, differentiation, and immunity, as well as cell death and survival (Maurer et al., 2014). There are two homologous isoforms of GSK-3 namely GSK-3α and GSK-3β (Hami et al., 2015). GSK-3 is a constitutively active enzyme and is inactivated by inhibitory phosphorylation in response to insulin, other growth factor or AKT protein (Beurel et al., 2015). GSK-3 is unique in its mode of substrate recognition and the regulation of its kinase activity, which is repressed by pro-survival PI3K–AKT signaling. In turn, GSK-3 exhibits pro-apoptotic functions when the PI3K–AKT pathway is inactive (Maurer et al., 2014). Moreover, GSK-3 has been proposed to be a possible target for β cell protective agents such as GSK-3 negatively (when its inactivate) affects β cell function by modulating the stability and subcellular localization of the β cell differentiation factor
Pdx1 (Mussmann et al., 2007). Thus GSK-3 plays a key role in the regulation of β cell mass and function.

Table 2.4: Categories of PI3K class.

<table>
<thead>
<tr>
<th>Class</th>
<th>Catalytic subunit</th>
<th>Adaptor subunit</th>
<th>Regulation</th>
<th>Lipid substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>p110α,β,δ</td>
<td>p50α, p85β, p55β</td>
<td>RTK, RAS and GPCR</td>
<td>PIP₂, PIP, PI</td>
</tr>
<tr>
<td>IB</td>
<td>p110γ</td>
<td>P101, p84</td>
<td>GPCR and RAS</td>
<td>PIP₂, PIP, PI</td>
</tr>
<tr>
<td>II</td>
<td>PI3K-C2α,β,γ</td>
<td>?</td>
<td>RTK, GPCR</td>
<td>PIP₂, PI</td>
</tr>
<tr>
<td>III</td>
<td>Vps34p analogue</td>
<td>P150</td>
<td>?</td>
<td>PI</td>
</tr>
</tbody>
</table>
2.10 Pancreatic β cells and oxidative stress

There are several factors that promote oxidative stress towards pancreatic β cells such as hyperglycemia (Fu et al., 2017), hyperlipidemia (Abebe et al., 2017), hypoxia (Gerber & Rutter, 2017), nutrient imbalance (Cappelli et al., 2018), toxin (Nahdi et al., 2017) or ER stress (Suganya et al., 2018). During T2D, increase demand for insulin render the β cell towards higher oxidative phosphorylation for the generation of ATP. With high consumption of oxygen, β cells are exposed to higher risk towards reactive oxygen species (ROS) production and oxidative stress. During chronic hyperglycemia, β cells are induced towards ROS-forming pathways such as glucose autoxidation, glycosylation or glucosamine pathways (Wang & Wang, 2017). Exposure to excessive lipid such as free
fatty acid (FFA) has been shown to activate β cell stress evidence by NADPH oxidase production and the modulation of the respiratory chain (Koulajian et al., 2013; Villa et al., 2018). During hypoxia, deprive of oxygen in pancreatic β cells has shown to induce ROS generation within complex I and III of the mitochondria electron transport chain (Gerber & Rutter, 2017). When the levels of misfolded proteins such as proinsulin exceed ER adaptive capabilities, hydrogen peroxide is generated as by-products. Thus, ER stress activates and release C/EBP homologous protein (CHOP) protein which induces the oxidative stress inside the pancreatic β cells (Chan et al., 2015; Marchetti et al., 2007).

Intracellular ROS such as superoxide anion (O$_2^-$) or hydrogen peroxide (H$_2$O$_2$) induces the oxidative stress towards pancreatic β cells (Lenzen, 2017; Schieber & Chandel, 2014). Hence, the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) eradicate ROS and shields the cells from oxidative stress (Wang et al., 2017). Superoxide anion is a reactive molecule which can be converted to hydrogen peroxide by superoxide enzyme. Meanwhile, hydrogen peroxide is broken down into water and oxygen by catalase and GPx enzymes (Figure 2.8) (Poprac et al., 2017). Thus, the amount of intracellular ROS during the oxidative phosphorylation is decrease where it converts to water and oxygen by these antioxidant enzymes. However, numerous reports stated that β cells have lower antioxidant enzymes to overcome ROS, making it more susceptible towards oxidative stress (Alejandro et al., 2015; Siniscalco et al., 2018). Moreover, the effectiveness of natural antioxidant in preventing T2D is remained unclear. Therefore, new strategies for controlling oxidative stress such as developing new mechanism-based antioxidant or bolstering the natural antioxidant can further prevent oxidative stress-related disorder such as diabetes mellitus.
Figure 2.8: Antioxidant defense in an organism. Figure was adapted from Lazo and Fernández (2013).

The regulation of antioxidant enzymes is much linked to the nuclear transcription factor erythroid 2p45-related factor (Nrf2) protein (Li et al., 2018). In an unstressed cell, Nrf2 is in an inactive form where its specific bind to cytoskeleton-associated cytosolic Kelch-like ECH-associated protein 1 (Keap1) within the cytosol. The complex promotes its rapid proteasome-mediated degradation via a Cul3 based E3 ubiquitin ligase complex. However, during oxidative stress, Nrf2 proteins dissociate from Keap1, translocate to the nucleus and binds to cis-element called as antioxidant response elements (ARE) to regulate gene expression (Suzuki & Yamamoto, 2015). Later the gene triggers the phase II (detoxifying and antioxidant) enzymes such as NAD(P)H quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), γ-gutamylcysteine synthetase, glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) which helps in cell survival and maintaining cellular redox homeostasis (Zhang et al., 2013). Thus, Nrf2 protein is a master regulator of cellular detoxification response and redox status and provides a protective action from various oxidative stresses and damages (Figure 2.9).
Research has shown that the expressions of Nrf2, induced anti-oxidative enzymes such as HO-1, NQO-1, and GPx-1 and were significantly increased in the diabetic kidney when treated with salvianolic acid A alone or in combination with metformin (Wu et al., 2016). In other research, Nrf2 activation has the potential to rescue pancreatic β cell against various oxidative insult (DiniĆ et al., 2016; Sireesh et al., 2017). Therefore, activation of Nrf2 proteins might hold the plausible protection of pancreatic β cell against oxidative stress damage and prevent cell dysfunction and death (David et al., 2017).

**Figure 2.9:** Overview of Nrf-2 pathway activation. Figure was adapted from Reis et al. (2016).
2.11 *Leptospermum flavescens*

*L. flavescens* is also known yellowish south sea myrtle or red tea tree. In others country the plant is also called as *gelam bukit, cina maki or serai kayu wangi* in Malaysia; *hurong* in Indonesian and *paripingain, malasulasi* or *tinikaran* in Philippines. It is a medium-sized shrub and grows mostly under the mountain in south-east Asia and New Zealand. The leaves are linear-lanceolate with many twiggy branches and the flowers are white color and growing out of the apex of the branch (Figure 2.10). In Malaysia, the leaves are boiled, and the tonic tea is used for the treatment of constipation, stimulate appetite, relieve stomach discomfort, and relieve of dysmenorrhea. Furthermore, the leaves are used for the treatment of lethargy, hypertension, diabetes, and kidney pains. The oil collected from the leaves could relieve bronchitis and rheumatism. It has been reported that *L. flavescens* induced its anti-tumor properties in lung cancer cells (Navanesan *et al.*, 2015) antidiabetic properties in alloxan-induced diabetic rats (Rahim *et al.*, 2014) and antimicrobial activities against gram-positive bacteria (Demuner *et al.*, 2011). The semi-pure fraction (LF1) which was precipitate during methanol extraction of *L. flavescens* induced the highest cytotoxic against human non-small cell lung carcinoma cell lines, A549 and NC1-H1299 with the lowest IC$_{50}$ at 7.12 ± 0.07 and 9.62 ± 0.50 respectively. The semi-pure fraction induced apoptosis cell death through activation of caspase-3 proteins and blocked cell cycle progression towards the lung cancer cells (Navanesan *et al.*, 2015). In another study, the water extracts of *L. flavescens* inhibit glycogen phosphorylase at 85% with IC$_{50}$ at 0.18 mg/mL, decrease significantly fasting plasma glucose levels by 61.9 % in alloxan-induced diabetic rats (T1D models), and decrease total cholesterol and triglycerides with an increase in HDL level in SD rats. In the same study, they found that the water extracts of *L. flavescens* present various bioactive compounds such as aromadendrin glucoside, kaempferol rhamnoside, quercetin rhamnoside and vindoline through LCMS/MS (Figure 2.11) (Rahim *et al.*, 2014). To date,
there is still limited scientific report of \textit{L. flavescens} in promoting antidiabetic properties. Thus, in this dissertation, \textit{L. flavescens} was chosen for investigation of its antidiabetic activity using \textit{in vitro} and \textit{in vivo} methods.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig210.png}
\caption{\textit{L. flavescens} plant.}
\end{figure}

\begin{table}
\centering
\caption{Scientific classification of \textit{L. flavescens}.}
\begin{tabular}{ll}
\hline
\textbf{Kingdom} & \textit{Plantae} \\
\textbf{Subkingdom} & \textit{Tracheobionta} \\
\textbf{Super division} & \textit{Spermatophyta} \\
\textbf{Division} & \textit{Magnoliophyta} \\
\textbf{Class} & \textit{Magnoliopsida} \\
\textbf{Sub class} & \textit{Rosidae} \\
\textbf{Ordo} & \textit{Myrtales} \\
\textbf{Family} & \textit{Myrtaceae} \\
\textbf{Genus} & \textit{Leptospermum} \\
\textbf{Species} & \textit{Leptospermum flavescens} \\
\hline
\end{tabular}
\end{table}
Figure 2.11: Some of the phytochemicals found in *L. flavescens*’s leaves. A is Kaempferol, B is Aromadendrin, C is Quercetin and D is Vindoline (all without its sugar constituent). The molecular structure was adapted from chemspider.
CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell line

INS-1 rat pancreatic β cell was purchased from AddexBio San Diego, CA, USA.

3.1.2 Chemicals and reagents

The following items were listed per manufacturer:

**Sigma:** Dimethyl sulfoxide (DMSO), 2-[4-(2-hydroxyethyl)piperazin-1-ethanesulfonic acid (HEPES), bisbenzimide H 33342 trihydrochloride (Hoechst 33342), propidium iodide (PI), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2',7'-Dichlorofluorescin diacetate (DCF-DA), iron (III) chloride (FeCl), sodium hydroxide (NaOH), nitrotetrazolium blue chloride (NBT), acarbose, porcine pancreatic amylase (PPA), starch from potato, α-glucosidase from *Saccharomyces cerevisiae*, p-Nitrophenyl β-D-glucopyranoside (PNPG), sodium bicarbonate (NaHCO₃), 3-methyladenine (3-MA), acridine orange hydrochloride hydrate (AO), nicotinamide, D-(+)-glucose, gallic acid, quercetin, L(+)-ascorbic acid, 3,5-dinitrosalicylic acid, potassium sodium tartrate, carboxymethylcellulose sodium (CMC), fetal bovine serum (FBS), sodium hydrogen carbonate, skim milk powder, sodium pyruvate, triciribine hydrate, SB 216763, magnesium sulfate hepta hydrate and calcium chloride- dihydrate.

**Biorad:** Ammonium persulfate, TEMED, resolving gel buffer, stacking gel buffer, clarity™ western ECL substrate, 10 x tris/glycine buffer, 10 x tris/SDS/glycine buffer, glycine, tris, SDS, 4 x laemmLi sample buffer, 30 % acrylamide/bis solution 29:1, precision plus protein™ kaleidoscope™, quick start™ Bradford 1x dye reagent, quick start™ bovine serum albumin (BSA), precision protein™ streptactin-HRP conjugate, phosphatase inhibitor cocktail 2 and phosphatase inhibitor cocktail 3,
Nacalai Tesque: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 0.5%-tryphan blue stain solution.

Merck: Streptozotocin (STZ), bismuth (III) nitrate, potassium iodide, glyburide, potassium chloride, potassium hydroxide, tri-sodium citrate dihydrate, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, β-mercaptoethanol, hexane, ethyl acetate, methanol, chloroform, xylene, absolute ethanol and 95 % ethanol.

Friendemann Schmidt: Hydrochloric acid 37% (HCl) and sodium chloride (NaCl).

Systerm: Sulphuric acid 95-98% (H₂SO₄), potassium sodium tartrate and 37 % formalin.

Santa Cruz Biotechnology: Phenazine methosulfate (PMS).

Bio Basic Canada INC: Nicotinamide adenine dinucleotide (NADH).

Thermo Scientific: 1-Step™ Transfer buffer, T-PER® tissue protein extraction reagent, pierce® BCA protein assay kit, pierce™ reversible protein stain kit, pierce® RIPA buffer, fast western antibody diluent and halt™ protease inhibitor cocktail (100x).

Gibco®: TrypLE™ express, anti-anti (100x) and RPMI medium 1640.

Ilium: Ketamil and ilium xylazil-100.

BD Pharmingen™: Z-VAD-FMK general caspase inhibitor.

Invitrogen: Seeblue® plus2 prestained standard (1x).

VWR amresco: Albumin bovine (BSA).
### 3.1.3 Antibodies

The following antibodies were listed in the Table 3.1 below:

**Table 3.1:** List of primary and secondary antibodies.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Cat: number</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved caspase-3 (Asp175)</td>
<td>19</td>
<td>R</td>
<td>1:1000</td>
<td>9661</td>
<td>Cell Signaligning</td>
</tr>
<tr>
<td>Cleaved caspase-9 (Asp353)</td>
<td>38</td>
<td>R</td>
<td>1:1000</td>
<td>9507</td>
<td>Cell Signaligning</td>
</tr>
<tr>
<td>Bcl-2 (D17C4)</td>
<td>26</td>
<td>R</td>
<td>1:1000</td>
<td>3498</td>
<td>Cell Signaligning</td>
</tr>
<tr>
<td>Bcl-xl (54H6)</td>
<td>30</td>
<td>R</td>
<td>1:1000</td>
<td>2764</td>
<td>Cell Signaligning</td>
</tr>
<tr>
<td>LC3A/B (D3U4C)</td>
<td>14,16</td>
<td>R</td>
<td>1:1000</td>
<td>12741</td>
<td>Cell Signaligning</td>
</tr>
<tr>
<td>Beclin-1 (D40C5)</td>
<td>60</td>
<td>R</td>
<td>1:1000</td>
<td>3495</td>
<td>Cell Signaligning</td>
</tr>
<tr>
<td>Phospho-AKT (Ser473) (D9E)</td>
<td>60</td>
<td>R</td>
<td>1:2000</td>
<td>4060</td>
<td>Cell Signaligning</td>
</tr>
<tr>
<td>Phospho-GSK-3β (Ser9) (D85E12)</td>
<td>46</td>
<td>R</td>
<td>1:1000</td>
<td>5558</td>
<td>Cell Signaligning</td>
</tr>
<tr>
<td>β-Actin (8H10D10)</td>
<td>45</td>
<td>M</td>
<td>1:1000</td>
<td>15452</td>
<td>MA5-Thermo Scientific</td>
</tr>
<tr>
<td>HMOX1 (HO-1)</td>
<td>32</td>
<td>M</td>
<td>1:1000</td>
<td>112</td>
<td>MA1-Thermo Scientific</td>
</tr>
</tbody>
</table>
Table 3.1, continued.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Cat: number</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>69</td>
<td>R</td>
<td>1:1000</td>
<td>PA5-19830</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Lamin B1 (X223)</td>
<td>68</td>
<td>M</td>
<td>1:50</td>
<td>MA1-8522</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Anti-rabbit IgG, HRP-linked</td>
<td>R</td>
<td>1:1000</td>
<td>7074</td>
<td></td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Insulin (C27C)</td>
<td>R</td>
<td>1:400</td>
<td>3014</td>
<td></td>
<td>Cell Signaling</td>
</tr>
</tbody>
</table>

3.1.4 Assay kits

The following kits were listed per manufacture:

**Enzo**: MITO-ID® Membrane potential detection kit.

**Merck**: Rat/Mouse Insulin ELISA l EZRMI-13K.

**BD Pharmingen™**: FITC Annexin V Apoptosis Detection Kit 1.

**Sigma**: Catalase assay kit.

**LONZA**: MycoZap™ 5 treatment kit and MycoAlert® mycoplasma detection kit.

**Thermo scientific**: NE-PER® nuclear and cytoplasmic extraction reagents.

**Nacalai Tesque**: Peroxidase stain DAB kit (brown stain).

3.1.5 Consumables

The following items were listed per manufacturer:

**Sartorius**: Minisart® syringe filter (CA, 0.2 µm, hydrophilic), sartolab-P 20 (CA, 0.2 µm, hydrophilic) and minisart RC 25 (RC, 0.2 µm, hydrophilic).
SPL life science: Cell culture flask (25 and 75 cm²), 96-well plate, cell culture dish and 6-well plate.

Biorad: Nitrocellulose membranes 0.45 µm and nitrocellulose membrane 0.22 µm.

BD Vacutainer®: K2 EDTA 10.8 mg (purple cap) and SST™ advance (yellow cap).

TUD®: Blood collection tube with clot activator (red cap).

Thermo scientific: Western blotting filter paper 7 cm x 8.4 cm.

Double rings: Filter paper qualitative (11 and 18 cm, circle).

Top gloves: Disposable latex medical examination gloves (size M).

Terumo®: Syringe without needle (20, 10, 5 and 1 cc/mL), syringe with needle (10 cc/mL) and needle (25 G 0.5 x 16 mm and 27 G 0.4 x 13 mm).

One touch: Ultrasoft lancet and glucose strip.

3.1.6 Instrument

The following instruments were listed per company:

Thermo scientific: Biosafety cabinet (1300 series A2), CO2 incubator (Forma stericycle) and pierce G2 fast blotter.

Biorad: Power pac™ basic.

Büchi: Rotavapor (R-200), heating bath (B-491), distillation chiller (B-741) and vacuum pump (V-700).

ASYS: Microplate ELISA reader (UV 340).

Eppendorf: Centrifuge 5804.

Accuri: Flow cytometry.

Leica: Inverted fluorescence microscope (DM6000B).

One touch: Blood glucose monitoring system (glucometer).

Agilent technologies: Q-TOF (LC/MS) 6550 iFunnel, 1290 binary pump (G4220A), 1290 auto sampler (G4226A) and 1290 TCC (G1316C).
3.2 Methods

3.2.1 Plant preparation

*L. flavescens*’ leaves were harvested at Genting Highland, Pahang, Malaysia, in August 2015. Two kilograms of leaves were collected. The plant was previously identified by Dr. Sugumaran Manickam, of Institute of Biological Science, University of Malaya, Malaysia with a voucher specimen number: KLU 47798. The leaves were air dried in 50 °C incubator chamber for 2 weeks. The leaves were crushed into powder using an electric blender and stored in 4 °C cold room.

3.2.2 Plant extraction

*L. flavescens* ’s leaves weight at 200 g were soaked with n-hexane (1 L) and macerated at room temperature for 72 h. Soluble n-hexane extract was filtered using filter paper and the insoluble hexane was air dried for 24 h. Once the leaves were dried in n-hexane solvent, the leaves were soaked with ethyl acetate (1 L) and macerated at room temperature for 72 h. The soluble ethyl acetate extract was filtered, and the residue was air dried for 24 h. Once the leaves were dried from ethyl acetate solvent, the leaves were added with methanol (1 L) and further macerated for 72 h. The soluble methanol extract was filtered, and the crude was air dried for 24 h for next extraction. Insoluble methanol crude was added to distilled water (1 L) and incubated for 24 h. Collected soluble n-hexane, ethyl acetate, and methanol extracts were evaporated using a rotary evaporator (Buchi) under reduced pressure at 40 °C to obtained n-hexane *L. flavescens* (HELF), ethyl acetate *L. flavescens* (EAELF) and methanol crude extract of *L. flavescens* (MELF). Water-soluble obtained was lyophilized using freeze dryer (Labconco) for 120 h to obtained water extract *L. flavescens* (WELF). All extracts were stored in 4° C cold room. All extraction procedure is illustrated as shown in Table 3.1.
3.2.3 Dissolving plant extracts

HELF, EAELF, and MELF were dissolved in DMSO, while WELF was dissolved in distilled water to a desired final concentration (1 or 40 mg/mL). All extracts were then filtered using sterile 0.22 µm filter (Minisart) and stored at -20°C for further use. For the biological assay, HELF, EAELF and MELF treatment was not exceeded 0.5% (v/v) of DMSO after diluting in PBS, tris-HCl or INS-1-medium.
Figure 3.1: Schematic flow diagram of *L. flavescens* extraction and fractionation.
3.2.4 Qualitative phytochemical analysis

HELF, EAELF, and MELF were dissolved in DMSO while WELF was dissolved in distilled water at a final concentration of 1 mg/mL. All qualitative phytochemical analysis was assessed as described by Deyab et al. (2016).

3.2.4.1 Test for alkaloids

Few drops of Dragendorff’s reagent was added into 1 mL each of HELF, EAELF, MELF, and WELF. The presence of green turbidity and white precipitation indicates the presence of alkaloids. Dragendorff’s reagent was prepared freshly by a combination of both solution A and B as shown.

Solution A: 0.25 g of bismuth nitrate added with 5 mL of distilled water and 5 mL of concentrated hydrochloric acid (HCl). Solution B: 2 g of potassium iodide added with 0.5 mL of distilled water.

3.2.4.2 Test for terpenoids

0.5 mL of chloroform and 0.5 mL of concentrated sulphuric acid (H₂SO₄) were added with 0.25 mL each of HELF, EAELF, MELF, and WELF. The presence of reddish brown color at the interface indicates the presence of terpenoids.

3.2.4.3 Test for steroids

0.5 mL of chloroform and 0.25 mL of concentrated sulphuric acid (H₂SO₄) were added into 0.25 mL each of HELF, EAELF, MELF, and WELF. The presence of reddish brown ring at the interface indicates the presence of steroids.

3.2.4.4 Test for tannins

1 mL of iron (III) chloride (5 % w/v FeCl₃) was added to 1 mL each of HELF, EAELF, MELF, and WELF. The formation of dark blue or greenish black color indicates the presence of tannins.
3.2.4.5 Test for saponins

1 mL of distilled water was added to 1 mL of each HELF, EAELF, MELF, and WELF. The mixture was shaken in test tube lengthwise for 15 min. The formation of 1 cm layer of foam indicates the presence of saponins.

3.2.4.6 Test for flavonoids

1 mL of 2 N sodium hydroxide (NaOH) was added to 2 mL each of HELF, EAELF, MELF, and WELF. The formation of yellow color indicates the presence of flavonoids.

3.2.4.7 Test for phenols

1 mL of distilled water was added to each 0.5 mL each of HELF, EAELF, MELF, and WELF. Later a few drops of 10 % w/v of iron (III) chloride was added. The formation of blue / green color indicates the presence of phenols.

3.2.4.8 Test for coumarins

1 mL of 10 % w/v of NaOH was added to 1 mL each of HELF, EAELF, MELF, and WELF. The formation of yellow color indicates the presence of coumarins.

3.2.4.9 Test for quinones

1 mL of concentrated sulphuric acid (H₂SO₄) was added into 1 mL each of HELF, EAELF, MELF, and WELF. The formation of red color indicates the presence of quinones.
3.2.5 Quantitative phytochemical analysis

3.2.5.1 Total phenolic content

The total phenolic content was conducted according to the methodology proposed by López et al. (2017) with slight modification. Briefly, all sample extracts (1 mg/mL) and gallic acid (0.03125 – 1 mg/mL) were dissolved in PBS. 20 µL of sample extracts and gallic acid were added with 100 µL of 1 % (v/v) of Folin-Ciocalteu reagent in 96-well plate and incubated for 5 min. 80 µL of sodium carbonate was added and incubated for 2 h in dark room. The absorbance reading was measured at 740 nm using a microplate reader. The standard graph of gallic acid was plotted. The amounts of total phenols in all extracts at 1 mg/mL was calculated based on the standard graph.

3.2.5.2 Total flavonoids content

The total flavonoids content was conducted according to Maalej et al. (2017) methodology with slight modification. Briefly, all sample extracts (1 mg/mL) and quercetin (1 – 0.03125 mg/mL) were dissolved in DMSO. 100 µL of sample extracts and quercetin were added with 15 µL of 5 % (w/v) of sodium nitrate and 15 µL of 10 % (w/v) of aluminum chloride in 96-well plate and incubated for 5 min. Next, the mixture was added with 100 µL of 1 M of sodium hydroxide and incubated for 2 min. The absorbance reading was measured at 510 nm using a microplate reader. The standard graph of quercetin was plotted. The amounts of total flavonoids in all extracts at 1 mg/mL was calculated based on the standard graph.
3.2.6 Antioxidant activity assay

3.2.6.1 DPPH free radical scavenging assay

The DPPH free radical scavenging assay was conducted according to Oliveira et al. (2017) methodology with slight modification. Briefly, 150 µL of all sample extracts (3.125 – 200 µg/mL) and ascorbic acid (3.125 – 200 µg/mL) were added with 50 µL of 50 µM of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) in 96-well plate and incubated for 30 min. Absorbance was measured at 517 nm using a microplate reader. Ascorbic acid was used as positive control while DMSO alone was used as negative control. The percentage of DPPH free scavenging activity was calculated based on equation below:

\[
\text{DPPH free radical scavenging activity (\%)} = \frac{\text{Negative control} - \text{(Sample or positive control)}}{\text{Negative control}} \times 100(\%)
\]

3.2.6.2 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power assay was conducted according to Oliveira et al. (2017) methodology with slight adjustment. Briefly, 20 µL of sample extracts (3.125 – 200 µg/mL) and ascorbic acid (3.125 – 200 µg/mL) were added with 200 µL of FRAP reagent (10 mM of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 40 mM of hydrochloric acid (HCl), 20 mM of iron (III) chloride (FeCl3) and 0.3 M of acetate buffer (pH 3.6)) in 96-well plate and incubated for 10 min. The absorbance was measured at 595 nm using a microplate reader. Ascorbic acid was used as positive control while DMSO alone was used as negative control. The percentage of Fe (II)-TPTZ complex formation was calculated based on equation below:

\[
\text{Formation of Fe(II)-TPTZ complex (\%)} = \frac{\text{Sample or Positive control} - \text{Negative control}}{\text{Negative control}} \times 100(\%)
\]
3.2.6.3 Superoxide radical scavenging assay (SORSA)

The superoxide radical scavenging assay was conducted according to the methodology proposed by Mandal et al. (2017) with slight modification. Briefly, 50 µL of sample extracts (25 – 1600 µg/mL) and gallic acid (25 – 1600 µg/mL) were added with 50 µL of 0.3 mM of nitrotetrazolium blue chloride (NBT) and 50 µL of 1.0 mM of nicotinamide adenine dinucleotide (NADH) in 96-well plate and incubated for 5 min. The mixture was added with 50 µL of 0.1 mM of phenazine methosulfate (PMS) and further incubated for 5 min. Absorbance was measured at 560 nm using a microplate reader. Gallic acid was used as positive control while DMSO alone was used as negative control. The percentage of superoxide radical scavenging activity was calculated based on equation below:

\[
\text{Superoxide scavenging activity (\%) = } \frac{\text{Negative control} - (\text{Sample or positive control})}{\text{Negative control}} \times 100(\%)
\]

3.2.7 Enzyme assay

All samples were diluted in PBS (pH 7.0) where the amount of DMSO does not exceed 0.5 % (v/v). \(\alpha\)-amylase and \(\alpha\)-glucosidase inhibition was measured by a spectrophotometric method according to Kazeem et al. (2013) with some modifications.

3.2.7.1 \(\alpha\)-amylase inhibition assay

Briefly, 200 µL of sample extracts (3.125 – 200 µg/mL) and acarbose (3.125 – 200 µg/mL) were added with 400 µL of 10 mU (50 µg/mL) of porcine pancreatic amylase (PPA) in test tube and incubated for 10 min at 37 °C. The mixture was added with 200 µL of 1 % (w/v) of starch from potato and incubated for 30 min at 37 °C. The reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid (DNSA) reagent and incubated for 5 min at 95 °C. 200 µL of the mixture was transferred in 96-well plated and the absorbance was measured at 540 nm using plate reader. Acarbose was used as positive control and PBS alone was used as negative control. DNSA reagent was prepared by
mixing the solution A and solution B as shown below and top up with distilled water till 40 mL.

Solution A: 12 g of sodium potassium tartrate dissolved with 20 mL of distilled water
Solution B: 0.4 g of 3,5-dinitrosalicyclic acid dissolved with 8 mL of 2 N sodium hydroxide (NaOH).

The IC$_{50}$ can be defined as the concentration of inhibitor that produces 50% inhibition of enzyme activity. The percentage of $\alpha$-amylase inhibition was calculated based on equation below:

$$\alpha$$-amylase inhibition (%) = $$\frac{\text{Negative control} - (\text{Sample or positive control})}{\text{Negative control}} \times 100(\%)$$

### 3.2.7.2 $\alpha$-glucosidase inhibition assays

Briefly, 50 µL of sample extracts (3.125 – 200 µg/mL) and acarbose (3.125 – 200 µg/mL) were added with 50 µL of 5 mM of PNPG in 96-well plate and incubated for 5 min at 37 °C. 100 µL of 10 µU of $\alpha$-glucosidase from *S. cerevisiae* was added to the mixture and incubated for 30 min at 37 °C. Next, 50 µL of 0.2 M sodium bicarbonate was added to stop the reaction. The absorbance was measured at 405 nm using a plate reader. Acarbose was used as positive control and PBS alone was used as negative control. The percentage of $\alpha$-glucosidase inhibition was calculated based on equation below:

$$\alpha$$-glucosidase inhibition (%) = $$\frac{\text{Negative control} - (\text{Sample or positive control})}{\text{Negative control}} \times 100(\%)$$

### 3.2.8 Cell culture

#### 3.2.8.1 Growth medium preparation

900 mL of sterile autoclaved distilled water was added to RPMI medium powder, 2 g of sodium bicarbonate, 0.526 g of HEPES, 10 µL of sodium pyruvate (10 mM), 10 µL of
anti-anti (100 U/mL of streptomycin, 100 µg/mL of penicillin and 0.25 µg/mL of amphotericin B), 3.5 µL of β-mercaptoethanol (0.05 µM) and 100 mL of heat-inactivated fetal bovine serum (10% v/v). The medium was filtered using P-20 nitrocellulose 0.22 µm sterile filter membrane. The medium was labelled as INS-1 medium and stored at 4 °C prior usage.

3.2.8.2 PBS preparation

900 mL of distilled water was added with 8 g of sodium chloride, 2 g of potassium chloride, 1.44 g of sodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate. The pH was adjusted to 7.0-7.4 and top up to 1 L using distilled water. The buffer was sterile autoclaved, stored at room temperature for few hours to cool down and filtered using P-20 nitrocellulose 0.22 µm sterile filter membrane. The buffer was labelled as PBS and stored at 4 °C.

3.2.8.3 Cell revival

Cryovial containing INS-1 cells were taken out from liquid nitrogen tank and gently thawed under running tap water. INS-1 β cells were pipetted into 15 mL falcon tube, added with 5 mL of medium and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 1 mL of medium. The cell suspension was transferred to 25 cm² of cell culture flask, added with 5 mL of medium and incubated inside 5 % humidified atmospheric CO2, 37 °C incubator chamber. The cells were observed daily under a light inverted microscope for cells adhered or cells contaminations.

3.2.8.4 Mycoplasma detection and treatment

Prior experiment, INS-1 β cell was initially run through mycoplasma detection using MycoAlert® mycoplasma detection kit and MycoZap™ 5 treatment kit respectively. The procedure was based on manufactured protocols.
For mycoplasma detection, MycoAlert™ reagent and MycoAlert™ substrate was added with MycoAlert™ assay buffer and incubated for 15 min. 100 µL of cell supernatant was transferred into 96-well plate and added with 100 µL MycoAlert™ reagent and incubated for 5 min. The luminescence was measured at 1 sec integrated using a microplate reader (reading A) (biotek). 100 µL of MycoAlert™ substrate was added to the mixture and the luminescence was measured using the same program (reading B). The analysis was based on the ratio of reading A over reading B (Table 3.2)

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.9</td>
<td>Negative for mycoplasma</td>
</tr>
<tr>
<td>0.9 – 1.2</td>
<td>Borderline: quarantine cells</td>
</tr>
<tr>
<td>&gt; 1.2</td>
<td>Mycoplasmas contamination</td>
</tr>
</tbody>
</table>

For mycoplasma treatment, 500 µL of MycoZap™ reagent 1 was added to 4.5 mL of culture medium containing INS-1 β cell in a 25 cm² culture flask and incubated for 3 days. Prior subculture, INS-1 β cells was added with 500 µL of MycoZap™ reagent 2 and further incubated for 3 days. The treatment was repeated twice, and the supernatant was collected for further mycoplasma detection.

3.2.8.5 Subculture cell

Once INS-1 β cells reached 70 – 80 % confluency, they were subculture into new cell culture flask. Briefly, the waste medium was discarded, and the cells were rinsed with PBS twice, added with 1 mL of tryplE and incubated in CO₂ incubator chamber for 5 min. The cells were observed under a microscope for floating and detached cells. Once INS-1 β cells were detached, the cells were added with medium and PBS and transferred into 15
mL falcon tube. The cells were centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the cell pellet obtained was added with 1 mL of medium and agitated thoroughly. 500 µL of cell suspension was transferred into new culture flask (new passage) while the remaining cells was transferred into the previous flask. INS-1 β cells were used between 20 ~ 30 passages.

3.2.8.6 Cell counting

Trypan blue exclusion assay was performed to count viable cells using hemocytometer. Briefly, the cells were detached, centrifuged, added with 1 mL of medium and agitated completely. 20 µL of cell suspension was mixed with 980 µL of trypan blue in a microcentrifuge tube and agitated thoroughly. The hemocytometer was cleaned with 70% ethanol, wiped with kimwipe and covered with a glass coverslip. 10 µL of trypan blue-treated cell suspension was filled both chambers underneath the coverslip, allowing the cell suspension to be drawn out by capillary action. The grid lines of hemocytometer were focused under a light inverted microscope, at 10 x objectives. There are 9 set of squares within the hemocytometer and shiny cells (viable cells) were counted at 5 squares located upper right and left, bottom right and left and middle only (Figure 3.2). The number of cells was calculated based on the following equation:

Number of cells: \[
\text{Number of cells} = \frac{\text{Cell count} \times \text{Cell density} \times (10^4) \times \text{dilution factor} (20)}{5}
\]

**Figure 3.2:** Hemocytometer glass slide. Cell located at grid number 1, 3, 5, 7, and 9 were counted.
3.2.8.7 Cell treatment

For cell culture treatments, INS-1 β cells were seeded for 48 h at various cell number depends on types of an assay performed. INS-1 β cells were treated with Z-VAD-FMK (20 µM), 3-Methyladenine (10 mM), or Triciribine hydrate (15 µM) and incubated for 1 h. Following pre-treatment with/without inhibitor, the inhibitor was removed and INS-1 β cells were induced with STZ (0.75 mM, 1.5 mM or 3.0 mM) and incubated for 30 min. Then, INS-1 β cells were treated with HELF, EAELF, MELF or WELF (3.125 ~ 100 µg/mL) and incubated for 4 h and 30 min. Later, INS-1 β cells were harvested and subjected to various assay. STZ was prepared freshly, protected from light, and dissolved in the medium prior added to INS-1 β cells.

3.2.8.8 Cell harvesting

At the end of incubation treatment, INS-1 β cells were harvested for further assay. Briefly, treatment medium was collected into falcon tube and INS-1 β cells were detached using 1 mL of trypLE. Floating cells were transferred into the same group as treatment medium and the plate was rinsed with ice-cold PBS to further collect remains cells. The cells were centrifuged at 1000 rpm for 5 min. The supernatants were removed, and the cell pellets were added with 1 mL of ice-cold PBS, agitated thoroughly, transferred into a microcentrifuge tube and centrifuged at 1000 rpm for 5 min (1st wash). The cell pellets were washed again (2nd wash) and ready for next assay.

3.2.9 MTT cytotoxic activity

INS-1 β cells were plated in 96 well plate at 20 000 cells per well. Once the cells were treated with inhibitor, STZ, and/or different extracts, the medium was removed and replaced with 200 µL of fresh medium containing 5 mg/mL of MTT. The cells were further incubated for 3 h at 37 °C. Later, MTT medium was removed and the formazan crystal formed was dissolved with 150 µL of DMSO. The absorbance reading was
measured at 570 and 650 nm as a background using a microplate reader. The percentage of the viable cell was calculated based on the following equation.

Cell viability (%) = (absorbance of treated cells/absorbance of control cells) x 100%

3.2.10 Glucose-stimulated insulin secretion (GSIS)

INS-1 β cells were plated in 96 well plate at 7 x 10⁴ cells per well. After treatment, the medium was replaced with Krebs-ringer bicarbonate buffer (5.5 mM or 20 mM glucose) and incubated for 30 min. The supernatant was collected for further GSIS assay. The amount of insulin in the supernatant was determined using rat/mouse insulin ELISA kit (Mercodia). The procedure was followed based on manufacture protocol.

3.2.10.1 Kreb-ringer buffer prep

11.9 mM of sodium chloride, 4.75 mM of potassium chloride, 2.54 mM of calcium chloride dihydrate, 1.2 mM of magnesium sulphate hepta hydrate, 1.2 mM of potassium dihydrogen phosphate, 5 mM of sodium bicarbonate, 20 mM of HEPES and 5.5 or 30 mM of D-(+)-glucose were mixed and added with distilled water.

3.2.10.2 Insulin assay

50 mL of 10 x wash buffer was diluted with 450 mL of de-ionized water. 300 µL of wash buffer was added into test well-plate and washed by inverting the plate and tapping onto the absorbent tissue. The plate was washed for three times. 10 µL of assay buffer was added into the well followed by 10 µL of samples, insulin standard (0.2, 0.5, 1, 2, 5 and 10 ng/mL) or quality control (QC1 and QC2). 80 µL of detection antibody was added and the plate was incubated for 2 h at room temperature. The mixture was removed, and the plate was washed for three times. 100 µL of enzyme solution was added and the plate was further incubated for 30 min at room temperature. The plate was washed with wash buffer three times. 100 µL of substrate solution was added and incubated for 5 min. 100 µL of stop solution was added and absorbance reading was measured at 450 nm using a
plate reader. Insulin standard graph was plotted. The amount of insulin release was calculated based on insulin standard graph.

3.2.11 Annexin /PI staining

The preliminary detection of apoptosis in STZ-induced INS-1 β cells was determined using FITC Annexin V apoptosis detection kit 1. Briefly, INS-1 β cells were seeded in 6 well dish at 1 x 10⁶ cells per well. INS-1 β cells were treated with STZ and post-treated with MELF. After incubation with MELF, INS-1 β cells were harvested and washed twice with ice-cold PBS. 1 x 10⁵ of cell suspension was collected and resuspended in 100 μL annexin binding buffer. 5 μL of FITC annexin and 5 μL of PI was added to the cells and incubated for 15 min in dark room. 400 μL of annexin binding buffer was added into the staining cells and examined using flow cytometry (Accuri). The fluorescence intensity was detected in FL1-A (x-axis) and FL2-A (y-axis) channel. The detection of early and late apoptotic cells and necrotic cells were qualitatively determined using a quadrant statistics analysis.

3.2.12 Mitochondria membrane potential (MMP) assay

The detection of MMP on STZ-induced INS-1 β cells were determined using MITO-ID® membrane potential detection kit. Briefly, INS-1 β cells were seeded in 6-well plate at 1 x 10⁶ cells per well, induced with STZ and post-treated with MELF. INS-1 β cells were harvested and washed twice with ice-cold PBS. 1 x 10⁵ of INS-1 β cells were collected and added with dual detection reagent (MITO-ID® MP detection reagent only) and stained for 15 min in dark room. The fluorescence intensity was analyzed using flow cytometry (Accuri) where the intensity of green fluorescent monomeric and orange fluorescent aggregated was detected at FL-1 and FL-2 channel respectively.
3.2.13 Hoechst 33342/PI double staining

Chromatin condensation or DNA fragmentation of STZ-induced INS-1 β cells can be distinguished through fluorescent morphological staining of the cells, such as dual staining with Hoechst 33342 and PI. Briefly, INS-1 β cells were seeded in 6-well plated at 1 x 10⁶ cells per well, induced with STZ followed by post-treated with MELF. INS-1 β cells were harvested washed twice with ice-cold PBS, stained with Hoechst 33342 (40µg/mL) and PI (50µg/mL) and incubated at a dark room for 30 min. The fluorescence intensity of the cells was captured under an inverted fluorescence microscope (Leica DM6000B).

3.2.14 Acridine orange (AO) staining

The detection of STZ-induced INS-1 β cellular vesicles was examined by staining with acridine orange. Briefly, INS-1 β cells were plated in 6 well plate at 1 x 10⁶ cells per well, incubated with/without 3-MA, followed by STZ and MELF. INS-1 β cells were harvested, washed twice with ice-cold PBS and stained with AO (1µg/mL) for 15 min at a dark room. After incubation, AO was removed, and the cells were washed again with ice-cold PBS three times and resuspended in RPMI medium. The fluorescence intensity of the cells was captured under an inverted fluorescence microscope (Leica DM6000B).

3.2.15 Animal experiment

Male or female Sprague Dawley (SD) rats aged 10 weeks, weighed 250-300 g were purchased from and housed at Animal Experimental Unit (AEU), Faculty of Medicine, University of Malaya, Malaysia. The standard environments were: temperature 22 ℃, 50 – 60 % humidity and 12:12 h light-dark cycle. Animals were given free access to commercial rat pellet diet and tap water *ad libitum*. Throughout the experiments, all animals were received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals,” which was prepared by the National Academy
of Sciences and published by the National Institute of Health. All experimental procedures were approved by the Institutional Animal Care and Use Committee, Faculty of Medicine (IACUC–FOM), University of Malaya, with ethics number: 2016-191103/IBS/R/AFAH.

3.2.16 Acute oral toxicity

Acute toxicity test was carried out according to the guidelines of the Organization for Economic Co-Operation and Development 423 (OECD-423). Female SD rats were fasted overnight for 16-18 h and oral forced to feed at a single dose of 1% CMC (control vehicle) and 2000 mg/kg of MELF (n=6). The animals were observed for any sign of toxic effects such as changes in skin, fur, eyes, the occurrence of secretions or excretions in the eyes or nose, response to handling, presence of abnormal movement such as repetitive circling or walking backward. The body weight was recorded daily for 14 days for any weight loss. At the end of the assay, the animals were euthanized by intraperitoneal (ip) overdose 50 mg/kg of ketamine and 5 mg/kg of xylazine. The blood was collected by cardiac puncture and stored inside vacutainer in 4 °C for further biochemical analysis. The animals were dissected to collect the organs such as liver, kidney, lung, heart, and pancreas and stored inside 10 % (v/v) formalin for further histopathological evaluation.
3.2.17 Tissues processing

For tissue processing, the tissue samples were followed through steps of dehydration, paraffin infiltration, tissue embedding and tissue sectioning. Once the organs were fixed in 10% formalin for 24 h, the tissues were transferred inside 70 % ethanol for 24 h. The tissue samples were then immersed in a series of alcohol solution with ascending concentration as indicated below (Figure 3.3).

![Dehydration and paraffin infiltration process](image)

**Figure 3.3:** Dehydration and paraffin infiltration process. Xylene-paraffin (1:1) mixture has been melted in the oven (60°C) enabling it to infiltrate the tissues. The tissues samples were infiltrated with paraffin as shown above. Infiltrated paraffin-tissues were placed in a small container and embedded with new paraffin. Once the paraffin solidified, the block-tissues were removed and ready for tissue sectioning. The block-tissues were sectioned using a microtome at 5 μM thickness and transferred onto poly-L-lysine coated slide. The slides were dried in an oven and ready for staining procedure.
3.2.18 Hematoxylin and eosin (H & E) staining

The slides were stained with H & E as shown in Figure 3.4. At the end of staining, the images of slides were observed and captured under a light inverted microscope (Leica).

**Figure 3.4:** Hematoxylin and eosin staining steps.
3.2.19 Oral glucose tolerance test (OGTT)

Healthy male SD rats were divided into 5 group (n = 6).

Group 1: Control,
Group 2: 125 mg/kg of MELF,
Group 3: 250 mg/kg of MELF,
Group 4: 500 mg/kg of MELF and
Group 5: 5 mg/kg of acarbose.

The animals have fasted overnight for 16-18 h. On the next day, the animals were fed with 1% CMC, 125, 250 and 500 mg/kg of MELF and 5 mg/kg of acarbose by oral gavage respectively for 30 min. 2 g/kg of glucose was fed to the animals and the blood glucose was measured starting at 0, 30, 60, 90 and 120 min. The blood was taken by pricking the tail of the animals with a lancet and the blood glucose was measured using a standard glucometer (Onetouch). At the end of the assay, the animals were euthanized by intraperitoneal (ip) overdose 50 mg/kg of ketamine and 5 mg/kg of xylazine.

3.2.20 STZ-induction in SD rats

Healthy male SD rats were divided into 5 group (n =6).

Group 1: Nondiabetic control,
Group 2: Diabetic control,
Group 3: Low dose (LD) treated (125 mg/kg of MELF),
Group 4: High dose (HD) treated (500 mg/kg of MELF),
Group 5: Positive control (5 mg/kg glyburide).

The animals were fasted overnight for 16-18 h. Fresh STZ was dissolved in citrate buffer at pH 4.5, cover form light, at 4 °C while nicotinamide (NA) was dissolved in saline. 80 mg/kg of nicotinamide was ip into the rats and left for 15 min. Subsequently, 60 mg/kg of STZ was ip into the rats and the water was changed to 5% (w/v) of glucose
for 24 h. Few drops of blood were collected by pricking the tail with a lancet and the blood glucose was measured using a glucometer. The blood glucose was measured at 0, 7, 14, 21 and 28 days. At the end of the experiment (at 28th days), the animals were euthanized by ip overdose 50 mg/kg of ketamine and 5 mg/kg of xylazine. The blood was collected by cardiac puncture and stored inside vacutainer at 4 °C for further biochemical analysis. The animals were dissected to collect the organs such as kidney, liver, and pancreas and stored inside 10 % (v/v) formalin for further immunohistochemistry analysis and -80 °C for further western blot analysis.

3.2.21 Western blot

3.2.21.1 INS-1 β cell protein lysate

Western blot protocol was adapted from Cell Signaling Technology® with slight modification. INS-1 β cells were plated in cell culture dish at 2 x 10⁶ of cell per dish. The cells were incubated with/without inhibitor followed by STZ and post-treated with MELF. Next, INS-1 β cells were harvested and washed twice with ice-cold PBS. The cell pellet was collected and added either with RIPA buffer (whole cell lysate) or NE-PER nuclear and cytoplasmic extraction buffer (nucleus and cytoplasmic lysate). RIPA, CER1 and NER1 buffer were added with protease and phosphatase inhibitor. The cell pellet containing RIPA buffer was incubated for 5 min and centrifuged at 20 000 x g for 15 min at 4 °C. The supernatant collected was stored at -20 °C. The nuclear and cytoplasmic proteins extraction was followed as manufactured protocol. Briefly, collected cell pellets were added with 100 μL of CER1, vortex for 15 min and incubated for 10 min. 5.5 μL of CER 2 was added to the cell pellet, vortex for 5 sec and incubated for 1 min. The cells were centrifuged at 16 000 x g for 5 min at 4 °C. The supernatant (cytoplasmic extract) was stored at -20 °C. The remaining insoluble cell pellet was added with 50 μL of NER 1 and vortex for 15 sec every 10 min for a total of 40 min. The cells lysate was further
centrifuged at 16000 x g for 5 min at 4 °C. The supernatant (nuclear extract) obtained was stored at -20 °C.

3.2.21.2 Bradford protein quantification

Protein lysate in RIPA buffer was quantified by using Bradford reagent. Briefly, 2 µL of protein lysates were added with 18 µL of distilled water. 5 µL of the diluted protein lysate and a standard BSA at 125, 250, 500, 750, 1000, 1500, and 2000 µg/mL were added to the 96-well plate (n=3). Distilled water was used as a blank. 250 µL of Bradford reagent was added to the protein lysates and standard BSA and the absorbance was measured at 595 nm using a plate reader. A standard graph of BSA was plotted. The amount of proteins was calculated based on BSA standard graph.

3.2.21.3 Bicinchoninic acid (BCA) protein quantification

Protein lysate in NE-PER buffer was quantified using BCA reagent. Briefly, 2 µL of protein lysates were added with 18 µL of distilled water. 10 µL of diluted protein lysate and a standard BSA at 125, 250, 500, 750, 1000, 1500, and 2000 µg/mL were added into the 96-well plate (n=2). Distilled water was used as blank. 200 µL of working reagent (prepared by mixing 50 parts of BCA reagent A and 1 part of BCA reagent B) was added into the protein lysates and standard BSA and incubated at 37 °C for 30 min. The absorbance was measured at 562 nm using a plate reader. Standard graph of BSA was plotted. The numbers of proteins were calculated based on BSA standard graph.

3.2.21.4 SDS-PAGE

10 or 15 % resolving and 2 % stacking gel was prepared as shown in Table 3.3. Polymerized gels were transferred into electrophoresis tank and added with running buffer (25 mM tris, 192 mM glycine and 0.1 % SDS, pH 8.3). 40 - 80 µg of proteins from cell or tissue lysates were added with ¼ part of loading buffer and incubated in 95 °C water bath for 5 min. Standard protein ladder was added into the gels followed by the
sample proteins lysates. The gels were run through electrophoresis at 150 v for 60 min. The proteins are now ready to be transferred into new membrane.

**Table 3.3:** Resolving and stacking gel preparation for 4 gels.

<table>
<thead>
<tr>
<th></th>
<th>10%</th>
<th>15%</th>
<th>2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>12.3 mL</td>
<td>7.2 mL</td>
<td>6.1 mL</td>
</tr>
<tr>
<td>Resolving buffer</td>
<td>7.5 mL</td>
<td>7.5 mL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.15 mL</td>
<td>0.15 mL</td>
<td>0.05 mL</td>
</tr>
<tr>
<td>Bis:Acrylamide (1:29)</td>
<td>9.9 mL</td>
<td>15.0 mL</td>
<td>1.34 mL</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.15 mL</td>
<td>0.15 mL</td>
<td>0.05 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.02 mL</td>
<td>0.02 mL</td>
<td>0.01 mL</td>
</tr>
</tbody>
</table>

**3.2.21.5 Wet and semi-dry transfer**

Once the proteins were finished running through electrophoresis, the gel was sandwiched together with blotting (0.2 or 0.45 µM pore size) and filter membrane and transferred either using wet or semi-dry transfer process. For wet transfer, the electrophoresis tank was filled with transfer buffer (25 mM tris, 192 mM glycine and 20 % methanol, pH 8.3) at 4°C and run through electrophoresis at 90 v for 80 min. For semi-dry transfer, the blotting and filter membrane was initially soaked in 1-step transfer buffer for 10 min. The gel, filter and blotting membrane was sandwiched accordingly and placed onto the cassette of G2 fast blotter. The transfer program was selected based on the molecular weight of target proteins. At the end of the transfer, the cassette was washed with hot water. The new blotting membranes are no ready for blocking.

**3.2.21.6 Blocking and antibody incubation**

The blotting membranes were blocked with either 5 % (w/v) BSA or 5 % (w/v) skim milk for 1 h. The blotting membranes were washed 3 times with tris-buffered saline in 1
% (v/v) tween-20 (TBST) for 5 min each. Next, the membranes were added with primary antibody (diluted in antibody diluent) and incubated overnight at 4 °C. The next day, the membranes were washed again with TBST 3 times for 5 min each. The membranes were added with secondary antibody (diluted in antibody diluent) and streptactin and incubated for 1 h. At the end of incubation, the membranes were washed again with TBST 3 times for 5 min each and ready for band detection.

### 3.2.21.7 Enhanced chemiluminescent band detection

Proteins band signals can be detected through enhance chemiluminescent (ECL) detection reagent. Briefly, 1 part of luminol enhancer solution was mixed with 1 part of peroxide solution. The membranes were soaked with the mixed ECL substrates and incubated for 10 sec. The bands were observed under gel documentation imaging system (Fusion – Fx7) and quantified using the Bio-1D software.

### 3.2.22 Immunohistochemistry staining

Immunohistochemistry staining was adapted from Cell Signaling Technology® with slight modification.

#### 3.2.22.1 Dewaxing

Tissue slides were incubated in xylene 1, xylene 2 and xylene 3 each for 3 min followed by incubated in absolute ethanol 1 and absolute ethanol 2 for 10 min each. The slides were then incubated in 95 % ethanol 1 and 95 % ethanol 2 for 10 min each followed by washed with PBS two times for 2 min each and mili-Q water two times for 2 min each. The slides are now ready for antigen retrieval.

#### 3.2.22.2 Antigen retrieval

2.94 g of tri-sodium citrate hydrate was added with 1 L of mili-Q water followed by 500 μL of tween-20 at pH 6.0 (citrate buffer was prepared freshly). The slides were placed into the slide holder and soaked with citrate buffer and boiled inside an oven for 40 min.
The slide holder was cooled for 20 min and washed in mili-Q water for 2 min. The slides were carefully wiped with tissue paper and 100 µL of 3 % (v/v) H₂O₂ was added to the slides and incubated for 30 min. The slides were washed with PBS 2 times for 5 min. The slides are now ready for blocking.

3.2.22.3 Blocking and antibody incubation

100 µL of 5 % (w/v) BSA was added to the slides and incubated for 1 h. Later, the slides were washed with BS two times for 5 min each. Later, 100 µL of selected primary antibody was added onto the slides and incubated overnight. On the next day, the slides were washed PBS 3 times for 5 min each and carefully cleaned with tissue paper. 100 µL of selected secondary antibody was added onto the slides and incubated for 1 h. The slides were washed again with mili-Q water three times for 5 min each. The slides are now ready for DAB staining.

3.2.22.4 DAB staining

DAB staining solution was prepared by adding a drop of DAB stock solution, buffer solution and substrates solution into 2 mL of mili-Q water. The preparation was based on manufacture protocols. 100 µL of DAB staining solution was added onto the slides and incubated for 5-10 min depending on the brown color formations. The reaction was stopped by washing with mili-Q water for 5 min.

3.2.22.5 Counterstain and dehydration

The slides were counterstain with hematoxylin for 20 sec and washed with distilled water 3 times. Next, the slides were washed in 95 % ethanol 1 and 95 % ethanol 2 for 10 sec each followed by absolute ethanol 1 and absolute ethanol 2 for 10 sec each. The slides were washed again with xylene 1, xylene 2 and xylene 3 for 10 sec each. At the end of dehydration, the slides were mounted with coverslip by DPX media and the image was observed and captured under a light inverted microscope (Leica DM6000B).
3.2.23 Biochemical parameter

Blood collected from the SD rats were analyzed for several biochemical parameters such as ALT, ALP, AST, total protein, creatinine, and urea. The samples were run by using a Clinical chemistry system ADVIA 2400 (SIEMENS).

3.2.24 Statistical analysis

All data were expressed as a mean ± standard error (S.E). One-way or Two-way analysis of variance (ANOVA) was used followed by Duncan’s or Dunnet’s multiple comparison tests to analyze the effect of extracts on all experiment group. Experiment differences were considered statistically significant if P < 0.05.
CHAPTER 4: RESULTS

4.1 Plant extraction yield

200 g of *L. flavescens*’s leaves yielded 7.01 mg (4.17 %) of HELF as oily crude, 80 mg (47.56 %) of EAELF as dark green powder, 60.3 mg (35.85 %) of MELF as a brownish powder and 20.9 mg (12.42 %) of WELF as a light brownish powder.

4.2 Qualitative phytochemical analysis of *L. flavescens*

A series of preliminary phytochemical analysis of *L. flavescens* was investigated based on observation through color formation, turbidity, and precipitation of sample extracts. All sample extracts were tested for the presence of alkaloids, terpenoids, steroids, tannins, saponin, flavonoids, coumarins and quinones. As shown in Table 4.1, MELF has shown to have an intensively presence of tannins, flavonoids, phenols, coumarins and quinones while normal presence of terpenoids and steroids. Meanwhile, WELF has an intensively presence of flavonoids while normal presence of terpenoids, steroids, coumarins and quinones. HELF and EAELF have an intensively presence of flavonoids while normal presence of coumarins. Therefore, these results showed that the polar extracts of *L. flavescens* (MELF and WELF) may have higher therapeutics compounds compared to non-polar (HELF) and semi-polar (EAELF) extracts.
Table 4.1: Qualitative phytochemical analysis of *L. flavescens* extracts.

<table>
<thead>
<tr>
<th>Phytochemical parameter</th>
<th>HELF</th>
<th>EAELF</th>
<th>MELF</th>
<th>WELF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Phenols</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Coumarins</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Quinones</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

The symbols of “-” for absent; “+” for present and “++” for intensively present
4.3 Quantitative phytochemical analysis of *L. flavescens*

To further investigate the quantitative phytochemicals analysis of *L. flavescens*, the total phenolic content (TPC) and total flavonoid content (TFC) were analyzed in all crude extracts. Gallic acid and quercetin were used as standard phenols and flavonoids respectively (Jiménez et al., 2018). As shown in Table 4.2, MELF has the highest amount of total phenolic and flavonoid at $333 \pm 5.66$ mg GA/mg and $903 \pm 17.63$ mg QE/mg of extracts respectively. These results showed that the polar extract of *L. flavescens* which is the methanol extract may have higher phytochemicals content compared to other non-polar and semi-polar extracts.

**Table 4.2:** The total phenolic content (TPC) and total flavonoid content (TFC) of *L. flavescens*.

<table>
<thead>
<tr>
<th></th>
<th>TPC (mg GA/g)</th>
<th>TFC (mg QE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HELF</td>
<td>$68.94 \pm 2.15^a$</td>
<td>$811.19 \pm 16.83^a$</td>
</tr>
<tr>
<td>EAELF</td>
<td>$70.29 \pm 1.56^a$</td>
<td>$727.86 \pm 10.49^b$</td>
</tr>
<tr>
<td>MELF</td>
<td>$332 \pm 5.66^b$</td>
<td>$903.10 \pm 17.63^c$</td>
</tr>
<tr>
<td>WELF</td>
<td>$138.81 \pm 4.26^c$</td>
<td>$332.50 \pm 9.29^d$</td>
</tr>
</tbody>
</table>

All data were express as means ± S.E. (n=6). Means with different lower-case letters (a, b & c) are significantly different values compare to other extracts ($P < 0.05$, using one-way ANOVA test followed by Duncan’s multiple comparison test).
4.4 Antioxidant activity of *L. flavescens*

The antioxidant activity of phytochemicals has been known to endorse antidiabetic, anticancer and anti-inflammation activities (Kocaadam & Şanlier, 2017). This is due to these phytochemicals have the ability to scavenge free radicals which presence inside the cells (Habashy *et al.*, 2018). Thus, to further investigate the antioxidant activity of *L. flavescens*, all sample extracts were evaluated through DPPH, FRAP and SORS assays.

4.4.1 MELF scavenged DPPH free radicals

DPPH organic compound has been used widely for antioxidant assay (Eseryin *et al.*, 2018). The compound has stable free radicals and used as an indicator for most scavenging activity. As a result, reduced forms of DPPH compound changes color from bright purple to pale yellow (Badejo *et al.*, 2014). As shown in Figure 4.1, MELF was found to exhibit the highest scavenging activity with lowest IC$_{50}$ values at 124.08 ± 2.99 µg/mL compared to other extracts. The IC$_{50}$ values of WELF is 257.66 ± 12.4 µg/mL, while HELF and EAELF is not detected. Furthermore, the antioxidant activity of MELF is only 1.82-fold less compared to positive control. Therefore, MELF has the highest antioxidant activity based on the DPPH free radical scavenging assay.
Figure 4.1: DPPH scavenging assay. Line graph represents the percentages of DPPH free radical scavenging effects of *L. flavescens*. All data were express as a mean ± standard error (n = 6). Asterisk (*) represent significant value compared to control alone (p < 0.05).
4.4.2 MELF FRAP assay

Ferric reducing antioxidant power (FRAP) assay is based on the ability of the antioxidant compound to reduce ferric iron to ferrous iron. As a result, the production of TPTZ-couple ferric iron expressed the number of ferric iron reduced (K et al., 2018). As shown in Figure 4.2, MELF was delineated to exhibit the higher reduction of ferric iron with the lowest EC$_{50}$ value at 14.15 ± 1.92 µg/mL compared to other extracts. The EC$_{50}$ value of WELF is 42.90 ± 2.56 µg/mL, while HELF and EAELF is not detected. The antioxidant activity of MELF is at 3.65-fold less compare to positive control. Therefore, MELF has the highest antioxidant activity compared to other extracts based on FRAP assay.
Figure 4.2: FRAP assay. Line graph represents the percentages of TPTZ-ferric ion formations in *L. flavescens*. All data were expressed as a mean ± standard error (n = 6). Asterisk (*) represent significant value compared to control alone (*p* < 0.05).
4.4.3 MELF scavenged superoxide free radicals

Superoxide anion has been known to promote the oxidative stress in pancreatic β cells (Shi et al., 2018). To further investigate the scavenging effects of superoxide anion, all crude extracts were analyzed through superoxide radical scavenging assay. As shown in Figure 4.3, MELF exhibit the highest superoxide anion scavenging activity with the lowest IC50 value at 248.12 ± 2.82 µg/mL compared to other extracts. The IC50 value of WELF is 288.89 ± 2.17 µg/mL while HEAF and EAELF is not detected. Moreover, MELF has shown the highest superoxide scavenging activity with 2.52-fold more compared to positive control. Thus, these results have shown that MELF has the highest antioxidant activity compared to other extracts and positive control according to the SORS scavenging assay.
Figure 4.3: SORS scavenging assay. Line graph represents the percentages of superoxide scavenging effects of *L. flavescens* and gallic acid. All data were express as a mean ± standard error (n = 6). Asterisk (*) represent significant value compared to control alone (p < 0.05).
Table 4.3: The antioxidant activity of *L. flavescens* in DPPH, FRAP and SORSA assay.

<table>
<thead>
<tr>
<th></th>
<th>DPPH (IC₅₀ µg/mL)</th>
<th>FRAP (EC₅₀ µg/mL)</th>
<th>SORSA (IC₅₀ µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HELF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EAELF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MELF</td>
<td>124.08 ± 2.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.15 ±1.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>248.12 ± 2.82&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WELF</td>
<td>257.66 ± 12.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.90 ±2.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>288.89 ± 2.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Positive</td>
<td>68.24 ± 2.65</td>
<td>3.87 ± 0.12</td>
<td>625.32 ± 21.2</td>
</tr>
</tbody>
</table>

All data were expressed as means ± S.E. (n=6). The means with different lower-case letters (a & b) are significantly different value of MELF vs WELF (*P* < 0.05, using one-way ANOVA test followed by Duncan’s multiple comparison test). ND (not detected)
4.5 Enzyme inhibition

4.5.1 MELF inhibit α-amylase enzyme

Inhibition of pancreatic α-amylase has been shown to promotes the hypoglycemic activity of postprandial blood glucose (Ayyash et al., 2018). α-Amylase enzyme action involves hydrolysis of α (1-4) glycosidic bonds of starch or polysaccharides to yields maltose or glucose (Dhital et al., 2017). Thus, inhibition of α-amylase activity reduced the glucose absorption inside the small intestine (Taher et al., 2016). According to Figure 4.4, MELF exhibited compelling inhibition towards α-amylase enzyme, with IC$_{50}$ value at 37.40 ± 1.07 µg/mL. Meanwhile, the IC$_{50}$ value of HELF, EAELF and WELF is not detected due to the α-amylase inhibition activity is too low or not presence. MELF displayed a 6.09-fold less of enzymatic inhibition activity compared to positive control. Thus, MELF has the potential antidiabetic activity compared to other extracts according to the inhibition of α-amylase enzyme activity.
Figure 4.4: α-amylase inhibition assay. Line graph represents the percentage of α-amylase inhibition by *L. flavescens* and acarbose. All data were expressed as a mean ± standard error (n = 6). Asterisk (*) represent significant value compared to untreated ($p < 0.05$).
4.5.2 MELF inhibit α-glucosidase enzyme

Pancreatic α-glucosidase enzyme hydrolyzed starch and disaccharides into glucose, where the inhibition of this enzyme can leads to hypoglycemic effects inside the blood. (Rasheed et al., 2018). As shown in Figure 4.5, MELF exhibited the highest inhibition towards α-glucosidase enzyme with the lowest IC₅₀ value at 5.90 ± 0.04 µg/mL, compared to others extracts. The IC₅₀ value of WELF is 21.72 ± 0.15 µg/mL while HELF and EAELF is not detected do the inhibition activity is too low or not presence. Accordingly, MELF was also shown to induce higher α-glucosidase inhibitory activity compared to the positive drug, acarbose with 33.90-folds more. Therefore, these results presented that MELF may hold possible hypoglycemic effects through inhibiting α-glucosidase enzyme, which in turn decreased the postprandial blood glucose.
Figure 4.5: α-glucosidase inhibition assay. Line graph represents the percentage of α-glucosidase inhibition by *L. flavescens* and acarbose. All data were express as mean ± standard error (n = 6). Asterisk (*) represent significant value compared to control alone ($p < 0.05$).
4.6 Anti-diabetic activity of MELF in STZ-induced INS-1 β cells

4.6.1 MELF protects INS-1 β cell against STZ toxicity

STZ has been shown to selectively inhibit β cells viability, which resulted in inhibition of insulin secretion and induction of high blood glucose. Thus, inhibition of STZ activity against β cells may prevent diabetes mellitus (Bollard et al., 2018). To further investigate the protective effects of *L. flavescens* against STZ-induced INS-1 β cells, the cells were first evaluated through MTT cell viability assay. In viable cells, MTT is reduced to formazan crystal due to the presence of NADH cellular dehydrogenase contain in mitochondria. However, if the cells are damaged or disrupted, the amount of NADH dehydrogenase decrease and the MTT is not reduced (Waseem et al., 2015). INS-1 β cells were exposed to STZ and later were post-treated with *L. flavescens*’s extracts for 5 h. As shown in Figure 4.6 (A), STZ treated alone decreased INS-1 β cells viability into 54.45 ± 0.84 %. However, upon treatment with all the extracts, MELF has shown to increase the highest INS-1 β cells viability with cell recovery up to 91.71 ± 0.61 % compared to other extracts. The cell recovery of INS-1 β cells viability for HEAL, EAELF and WELF is 71.47 ± 2.13 %, 49.75 ± 5.35 % and 45.67 ± 2.05 %, respectively. In addition, MELF has higher protection activity in INS-1 β cells compared to positive control with 1.28-folds more. Based on Figure 4.6 (B) there was no sign of cytotoxicity at 100 µg/mL of MELF in INS-1 β cells for 5 h. These results revealed the first insight of MELF in protecting INS-1 β cells from deleterious STZ compounds. Therefore, MELF was further investigated in INS-1 β cells protection against STZ-induced cell death.
Figure 4.6: INS-1 β cells viability against STZ-induced cell death. (A) Bar chart represents INS-1 β cells viability induced by STZ and treated with HELF, EAELF, MELF, WELF and glyburide at a concentration ranging from 3.125 to 100 µg/mL. (B) Bar chart represents treatment of MELF ranging concentration from 3.125 to 100 µg/mL in STZ-induced INS-1 β cells. All data were expressed as a mean ± standard error (n=6). Small letters “a” and “b” represent significant values compared to control and STZ induction alone (p < 0.05).
Figure 4.6, continued.
4.6.2 MELF induce insulin secretion in INS-1 β cell.

Phytochemical agents that can improve insulin secretion are considered key to prevent T2D (Saha et al., 2018). Based on MTT assay, MELF was shown to induce the highest INS-1 β cells recovery against STZ damage. Thus, the ability of MELF to induce INS-1 β cells insulin secretion in STZ condition was investigated through glucose stimulated insulin secretion (GSIS) assay. The insulin secretion of INS-1 β cells was detected through rat/mouse insulin ELISA kit. As shown in Figure 4.7, STZ induction decrease insulin secretion by 0.277 ± 0.09 and 0.711 ± 0.03 µg/mL at 5 and 20 mM glucose, respectively. However, when post-treated with MELF (100 µg/mL), INS-1 β cells markedly increased the insulin secretion to 0.982 ± 0.14 and 1.307 ± 0.12 µg/mL at 5 and 20 mM of glucose, respectively. These results showed a dose-dependent increase in insulin secretion in STZ-induced INS-1 β cells when treated with MELF. Therefore, MELF has the potential to induce insulin secretion in STZ-induced INS-1 β cells based on the glucose stimulated insulin secretion assay.
Figure 4.7: Bar chart represents the effects of MELF on GSIS. INS-1 β cells were induced with STZ and treated with MELF for 5 h. Later, the cells were incubated at 5 mM and 20 mM of glucose for 30 min and the insulin release were collected for analysis. All data were expressed as a mean ± standard error (n=6). Small letters “a” and “b” represent significant value compared to control alone and STZ treated alone (p < 0.05).
4.6.3 MELF decrease phosphatidylserine externalization in INS-1 β cell

The induction of INS-1 β cell by STZ has been known to decrease cells viability and insulin secretion. The reduction of cell viability is likely caused by a type of programmed cell death such as apoptosis. One of the hallmarks of early events of apoptosis cell death is the translocation of phosphatidylserine from the inner side to the outer side of the plasma membrane (Seong & Lee, 2018). Thus, to further investigate the translocation of phosphatidylserine in STZ-induced INS-1 β cells, the cells were stained with Annexin V/PI and analyzed through flow cytometry. Based on the flow cytometry quadratic analysis, the lower left, lower right, upper left and upper right quadrant represent viable cells, early apoptosis, necrotic cell and late apoptosis, respectively (Pietkiewicz et al., 2015). As shown in Figure 4.8, STZ-induction alone increased INS-1 β cells in early and late apoptosis (annexin positive cells). However, in post-treatment with MELF, the early and late apoptosis cells markedly decreased in a dose-dependent manner. Pretreatment with the apoptosis inhibitor, Z-VAD-FMK was used to further investigate the anti-apoptotic effects of MELF in STZ-induced INS-1 β cells. The inhibitor was preincubated for 1 h, prior to STZ followed by posttreatment by MELF. Based on the results, pretreatment of Z-VAD-FMK attenuated the annexin positive cells of INS-1 β cells compared to STZ induction alone. However, pretreatment with Z-VAD-FMK and MELF further inhibited INS-1 β cells annexin positive cells compared to MELF treatment. Therefore, these results suggested that MELF might promote the anti-apoptotic activity against STZ-induced INS-1 β cells.
Figure 4.8: Detection of phosphatidylserine externalization in INS-1 β cells. (A) Flow cytometric analysis represents INS-1 β cells stimulated with/without Z-VAD-FMK, followed by STZ and treatment of MELF (25, 50 and 100 µg/mL). The lower right, lower left, upper right and upper left quadrants represent viable, early apoptotic, late apoptotic, and necrotic cells, respectively. (B) Bar chart shows the fold change of annexin positive cells (early and late apoptosis) in STZ-induced INS-1 β cells. All data were expressed as a mean ± standard error (n=3). Small letters “a”, “b” and “c” represent significant value compared to control, STZ induction and MELF treatment, respectively (p < 0.05).
**Figure 4.8**, continued.
4.6.4 MELF suppress INS-1 β cell mitochondria membrane potential (MMP)

The anti-apoptotic activity of MELF as evidenced by the translocation of phosphatidylserine was further verified by the dissipation of MMP assay. The protection against disruption of MMP has been shown to protect β cell from apoptosis cell death (Agarwal et al., 2018). Therefore, the protective activity of MELF in STZ-induced INS-1 β cells was investigated by MMP assay where the cells were stained with Mito-ID dye and analyzed through flow cytometry. Mito-ID is a dual-emission dye displayed as orange fluorescence J-aggregates (mitochondria) or green fluorescence monomer (cytosol) in the cells. Based on the flow cytometric data, the upper gate represents viable cells while the lower gate represents apoptotic cells (Ko et al., 2016; Park et al., 2017). As shown in Figure 4.9, STZ induction alone increased INS-1 β cells dissipation of MMP. However, in treatment with MELF, the amount of MMP, significantly decreased in a dose-dependent manner. Pretreatment with Z-VAD-FMK can further verify the anti-apoptotic activity of MELF in STZ-induced INS-1 β cells. Based on the data, incubation with the inhibitor, further decreased INS-1 β cells dissipation of MMP compared to STZ. However, incubation with inhibitor and MELF decreased the loss of MMP in INS-1 β cells when compared to MELF treated alone. Therefore, these findings suggested the involvement of anti-apoptotic effects of MELF in STZ-induced INS-1 β cells.
Figure 4.9: Detection of dissipation of MMP in INS-1 β cells. (A) Flow cytometric analysis represents INS-1 β cells treated with/without Z-VAD-FMK, followed by STZ incubation and treatment with various concentrations of MELF (25, 50 and 100 µg/mL). The lower and upper gate represent viable and apoptotic cells, respectively. (B) Bar chart represents the total green fluorescence apoptotic cells (%) in STZ-induced INS-1 β cells. All data were expressed as a mean ± standard error (n=3). Small letters “a”, “b” and “c” represent significant values compared to control, STZ treated alone and MELF with STZ treated alone, respectively (p < 0.05).
Figure 4.9, continued.
4.6.5 MELF inhibit nuclear alteration in INS-1 β cell

MELF was shown to inhibit STZ-induced apoptosis in INS-1 β cells by decreasing PS translocation and dissipation of MMP. Likewise, dual staining with Hoechst 33342 and PI can verify the morphological anti-apoptotic effects of MELF in STZ-induced INS-1 β cells. Hoechst 33342 has smaller molecular size compared to PI and can easily pass through the plasma membrane and stained chromatin or DNA fragmentation inside the cells. Light blue, bright blue and purple fluorescence cells represent viable, early and late apoptosis cells, respectively (Rendošová et al., 2017). As shown in Figure 4.10, STZ-induced INS-1 β cells exhibited a typical morphological characteristic of early and late apoptotic cells with intense blue and purple fluorescence. However, treatment with MELF decreased the formation of chromatin condensation or DNA fragmentation in STZ-induced INS-1 β cells. These observations suggested that MELF exerted anti-apoptotic effects in STZ-induced INS-1 β cells.
Figure 4.10: The morphological analysis of STZ-induced INS-1 β cells apoptosis. The fluorescence images of treated MELF in STZ-induced INS-1 β cells stained with Hoechst 33342 and PI. Magnification at x 400.
4.6.6 Effects of MELF in INS-1 β cells apoptotic protein expression

Based on previous flow cytometry and morphological apoptotic assays, MELF has been shown to inhibit apoptosis in STZ-induced INS-1 β cells. Thus, the induction of MELF in modulating the apoptosis proteins such as Bcl-2 family and caspase protein expression were investigated in STZ-induced INS-1 β cells through western blot assay. MELF was found to inhibit the dissipation of MMP in which trigger the anti-apoptotic Bcl-2 family protein. As shown in Figure 4.11 (A) and (B), STZ attenuated the production of anti-apoptotic Bcl-2 and Bcl-xl while induced cleavage of caspase 3 and 9 protein expressions in INS-1 β cells. However, after treatment with MELF, the anti-apoptotic Bcl-2 and Bcl-xl protein expressions significantly increased. Concurrently, MELF significantly decreased cleaved caspase 3 and 9 protein expression in STZ-induced INS-1 β cells. The investigation of anti-apoptotic effects of MELF was further clarified by pretreatment of STZ-induced INS-1 β cells with the universal caspase inhibitor, Z-VAD-FMK. Based on the results, pretreatment with the inhibitor inhibited cleaved caspase 3 and 9 protein expression compared to STZ induction alone. Subsequently, pretreatment with the inhibitor together with MELF further attenuated cleaved caspase 3 and 9 protein expression compared to MELF treatment alone (Figure 4.11 (C) and (D)). Therefore, the current findings provide a possible anti-apoptotic effect of MELF in STZ-induced INS-1 β cells.
Figure 4.11: Western blot analysis of STZ-induced INS-1 β cells apoptosis. (A) Protein blot images of cleaved caspase 3, 9, Bcl-2 Bcl-xl and β-actin protein expression of MELF treatment in STZ-induced INS-1 β cells. (B) Bar chart represents cleaved caspase 3, 9, Bcl-2 and Bcl-xl relative protein expression. (C) Protein blot images of cleaved caspase 3, 9 and β-actin protein expression of MELF treatment in INS-1 β cells with pretreated Z-VAD-FMK. (D) Bar chart represents cleaved caspase 3 and 9 relative protein expression. β-actin was used for loading control for all protein expression. All data were expressed as a mean ± standard error (n=3). Small letters “a”, “b” and “c” represent significant values compared to control, STZ induction and MELF treatment, respectively (p < 0.05).
### Figure 4.11, continued.

**Figure 4.11.** Continued.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cleaved Caspase 9 (37 kDa)</th>
<th>Cleaved Caspase 3 (19 kDa)</th>
<th>β-actin (45 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MELF (100 µg/mL)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>STZ (1.5 mM)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Z-VAD-FMK (20 µM)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Figure 4.11 Continued.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cleaved caspase 3</th>
<th>Cleaved caspase 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>MELF (100 µg/mL)</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>STZ (1.5 mM)</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>Z-VAD-FMK (20 µM)</td>
<td>-</td>
<td>a</td>
</tr>
</tbody>
</table>

Legend:
- a, b, c indicate significant differences at p < 0.05.
4.6.7 Inhibition of INS-1 β cell autophagosomes by MELF

The induction by STZ in INS-1 β cells can also induce another type of programmed cell death, such as impairment of autophagy. The formation of impaired autophagy in pancreatic β cells have been reported to promote beta cell death (Lim et al., 2016). Thus, to investigate the induction of autophagosome by STZ, INS-1 beta cells were stained with acridine orange and observed under fluorescence microscope. Acridine orange dye was used to detect the morphological late stage of autophagy where it stained acidic autophagosomes inside the cells and presented as orange dotted images (Won et al., 2018). As shown in Figure 4.12, STZ induction alone caused the formation of autophagosome inside INS-1 beta cells. However, treatment with MELF significantly inhibited the formation of autophagosomes in a dose-dependent manner. Pretreatment with a known autophagy inhibitor, 3-MA can further prove the involvement of autophagy in STZ-induced INS-1 β cells. Based on the results, pretreatment with 3-MA in STZ-induced INS-1 β cells showed a decrease in autophagosome compared to STZ-induction. In addition, pretreatment with inhibitor and MELF has shown to inhibit the formation of autophagosomes, with no differences in the image compared to treatment with MELF. Therefore, these results present the first insight of MELF in inhibiting autophagy in STZ-induced INS-1 β cells.
Figure 4.12: The morphological analysis of INS-1 β cells autophagy. STZ-induced INS-1 β cells were pretreated with/without 3-MA (10 mM) followed by treatment with MELF at different concentration (25, 50 and 100 µg/mL). At the end of the assay, the cells were stained with acridine orange dye and observed under a fluorescence microscope. Magnification at 400 x.
4.6.8 Inhibition of autophagy protein expression in MELF-induced INS-1 β cell

According to the acridine orange assay, MELF was shown to inhibit the formation of autophagosomes inside STZ-induced INS-1 β cells. Therefore, to further corroborate the attenuation of autophagy, the detection of Beclin-1 and LC3A/B protein expression was investigated via western blot analysis. As shown in Figure 4.13 (A) and (B), STZ induction alone increased LC3A/B and Beclin-1 protein expression when compared to control. However, after treatment with MELF, the LC3A/B and Beclin-1 protein expression significantly decreased in a dose-dependent manner. In addition, 3-MA inhibitor was incubated with INS-1 β cells to further investigate the dependency of autophagy with MELF. As shown in Figure 4.13 (C) and (D), treatment with 3-MA in INS-1 β cells further decreased LC3A/B and Beclin-1 proteins expression. However, in cells treated with 3-MA and MELF further attenuated LC3A/B and Beclin-1 proteins expression when compared to MELF treatment alone. Therefore, these findings suggested the protective effects of MELF against STZ-induced autophagy in INS-1 β cells.
**Figure 4.13:** Induction of autophagy in STZ-induced INS-1 β cells. INS-1 β cells were pre-treated with/without 3-MA followed by STZ and MELF at a ranging concentration 25, 50 and 100 µg/mL. (A) Protein blot images of LC3A/B, Beclin-1 and β-actin protein expression. (B) Bar chart represents LC3A and Beclin-1 relative protein expression. (C) Protein blot images of LC3A/B, Beclin-1 and β-actin protein expressions of pretreated 3-MA in INS-1 beta cells. (D) Bar chart represents LC3A/B and Beclin-1 relative protein expressions of pre-treated 3-MA in INS-1 beta cells. β-actin was used for loading control for all protein expression. All data were expressed as a mean ± standard error (n=3). Small letters “a”, “b” and “c” represent significant values compared to control alone, STZ treated alone and MELF and STZ treated alone, respectively (p < 0.05).
Figure 4.13, continued.
4.6.9 Modulation of AKT and GSK-3β protein expression in MELF-induced INS-1 β cell

The activation of AKT and inactivation of GSK-3β proteins play an important role in the regulation of β cells survival and proliferation (Mackenzie & Elliott, 2014). Therefore, the modulation of phospho AKT and phospho GSK-3β protein expression by MELF in STZ-induced INS-1 β cells was investigated via western blot analysis. As shown in Figure 4.14 (A) and (B), phospho AKT (ser) protein expression was inhibited in STZ-induced INS-1 β cell. Notably, when phospho AKT is inhibited, the GSK-3β proteins remain activated. Thus, according to the results, phospho GSK-3β protein expression remained inhibited in STZ-induced INS-1 β cells. However, in treatment of MELF, phospho AKT (ser) and phospho GSK-3β proteins expression significantly increased dose-dependently in STZ-induced INS-1 β cells. Therefore, these data presented the potential of MELF in inducing AKT activation and inactivation of GSK-3β protein in INS-1 β cells. Activation of AKT protein further induced downstream substrates such as inhibition of apoptosis and autophagy. Therefore, INS-1 β cells were pretreated with AKT known inhibitor, API, and the modulation of cleaved caspase 3, LC3A/B and GSK-3β protein expression were investigated through western blot analysis. Based on Figure 4.14 (C) and (D), treatment with API and STZ, further inhibited phospho AKT (ser) with increased cleaved caspase 3 and LC3A/B protein expression compared to STZ alone. However, treatment with API and MELF increased phospho AKT (ser) protein expression compared to API and STZ induction. In addition, treatment with API and MELF was found to further down-regulated cleaved caspase 3 and LC3A/B proteins expression when compared to API and STZ alone. These data suggested the involvement of MELF mediated AKT in anti-apoptotic and protection against autophagy. Pretreatment with API has shown to further inhibited phospho GSK-3β protein expression compared to STZ-induction alone. However, treatment with API and MELF was found to increased phospho GSK-3β
protein expression compared to API and STZ treatment. Therefore, the data suggested, the activation of AKT by MELF is dependent for inactivation of GSK-3β proteins.

Figure 4.14: Induction of AKT and GSK-3β by MELF in INS-1 β cells. (A) Protein blot images of phospho AKT (ser), phospho GSK-3β and β-actin proteins expression of MELF in STZ-induced INS-1 beta cells. (B) Bar chart represents phospho AKT (ser) and phospho GSK-3β relative protein expression. (C) Protein blot images of phospho AKT (ser), cleaved caspase 3, LC3A/B, phospho GSK-3β and β-actin protein expression of API and MELF treatment in STZ-induced INS-1 β cells. (D) Bar chart represents phospho AKT (ser), cleaved caspase 3, LC3A/B and phospho GSK-3β relative protein expression. β-actin was used for loading control for all protein expressions. All data were expressed as a mean ± standard error (n=3). Small letters “a”, “b” and “c” represent significant values compared to control alone, STZ treated alone and MELF and STZ treated alone, respectively (p < 0.05).
Figure 4.14, continued.
4.6.10 Inhibition of intracellular reactive oxygen species (ROS) by MELF in INS-1 β cells

Induction of STZ has been known to selectively induce oxidative stress towards pancreatic β cells (Fernandez et al., 2016). Therefore, the amount of ROS in INS-1 β cells when treatment with MELF was investigated via flow cytometry analysis. As shown in Figure 4.15, STZ induced higher level of intracellular ROS in INS-1 β cells compared to control. However, following treatment with MELF the amount of ROS in STZ-induced INS-1 β cells has significantly decreased in a dose-dependent manner. Thus, these results suggested the protective effects of MELF in inhibiting intracellular ROS production from deleterious STZ.

4.6.11 MELF induced HO-1 and Nrf-2 proteins expression in INS-1 β cells

STZ has been shown to increase ROS in INS-1 β cells, which leads to the inhibition of Nrf-2 and HO-1 antioxidant proteins expression. (Pi et al., 2010). Therefore, the induction of Nrf-2 and HO-1 proteins expression can protect β cells from oxidative stress (Lee et al., 2011). The regulation of Nrf-2 and HO-1 protein expression in MELF-stimulated INS-1 β cells was investigated via western blot assay. Based on Figure 4.16, STZ attenuated Nrf-2 protein translocation from cytoplasm to the nucleus in INS-1 β cells. Furthermore, STZ has also shown to inhibit downstream HO-1 protein expression in INS-1 β cells. However, following treatment with MELF, Nrf-2 protein translocation and HO-1 protein expression were found to significantly increase in dose-dependently in STZ-induced INS-1 β cells. Therefore, MELF has shown to induce antioxidant protein expression in INS-1 β cells based on the Nrf-2 protein translocation and HO-1 protein expression.

4.6.12 MELF increased catalase activity in INS-1 β cells

Translocation of Nrf-2 proteins from the cytoplasm into nucleus promotes several antioxidant enzymes such as superoxide dismutase, catalase or glutathione (Xie et al.,
Based on the western blot assay, MELF was shown to induce Nrf-2 protein translocation in STZ-induced INS-1 β cells. Therefore, the catalase activity was investigated in STZ-induced INS-1 β cells. Based on Figure 4.17, STZ decreased catalase activity in INS-1 β cells. However, when treatment with MELF, catalase activity was found to significantly increase in a dose dependent manner inside STZ-induced INS-1 β cells. Therefore, these results have showed the ability of MELF in promoting catalase activity in INS-1 β cells.
Figure 4.15: The intracellular ROS activity in INS-1 β cells. (A) Flow cytometry represents intracellular ROS of MELF treatment in STZ-induced INS-1 β cells. (B) Bar chart represents the intracellular ROS fold change of MELF treatment in STZ-induced INS-1 β cells. All data were expressed as a mean ± standard error (n=6). Small letters “a” and “b” represent significant values compared to control alone and STZ treated alone, respectively (p < 0.05).
Figure 4.16: Induction of Nrf-2 and HO-1 protein expression in INS-1 β cells. (A) Protein blot images of HO-1, Nrf-2 and β-actin protein expressions of post-treated MELF in STZ-induced INS-1 β cells. (B) Bar chart represents HO-1 and Nrf-2 relative protein expression of post-treated MELF in STZ-induced INS-1 β cells. β-actin was used for loading control for all protein expression. All data were expressed as a mean ± standard error (n=3). Small letters “a” and “b” represent significant values compared to control alone and STZ treated alone, respectively (p < 0.05).
Figure 4.17: Catalase activity in STZ-induced INS-1 β cells. Bar chart represents the catalase activity in STZ-induced INS-1 β cells after treatment with MELF. All data were expressed as a mean ± standard error (n=6). Small letters “a” and “b” represent significant values compared to control alone and STZ treated alone, respectively (p < 0.05).
4.7 Acute toxicity in SD rats

The acute toxicity test was based on OECD 423 guideline (Padgaonkar et al., 2018). The bodyweight, biochemical analysis, and histopathological detection data of vehicle and MELF group rats were collected to verify the toxic nature of MELF.

### 4.7.1 Effects of MELF in SD rats body weight

The animals were monitored for 14 consecutive days after dosing of MELF. The result was shown that there is no sign of death was observed for all SD rats after 14 days. The general appearance, grooming, posture, behavior, physiological changes, and all observational parameter were normal during the study periods. The body weight analysis shows that both control and treated MELF were increased towards every day. Furthermore, there is no significant variation in weight gain between the control and treated MELF groups throughout the study period (Figure 4.18). Therefore, MELF shows no toxicity effects on SD rats based on the gaining of body weight.

![Figure 4.18](image)

**Figure 4.18**: The acute toxicity test of MELF towards SD rats. The bar chart shows the effects of SD rat’s body weight on treatment with MELF (2 g/kg of rat body weight) and control (1% CMC). All data were expressed as a mean ± standard error (n=6).
4.7.2 The effect of MELF on blood biochemical parameters

The liver and kidney function parameters namely ALP, AST, ALT, creatinine, urea, and total proteins were detected in control and MELF treated groups of SD rats. Accordingly, there was no significant difference observed between control and MELF treated rats for all biochemical parameters except for ALP and urea levels (Figure 4.19). For control, the value for parameter AST, ALT, ALP, creatinine, total protein and urea is 88.5 ± 3.18 U/L, 109.67 ± 5.06 U/L, 38.67 ± 3.00 U/L, 43.00 ± 3.28 µmol/L, 66.33 ± 2.78 g/L and 7.78 ± 0.26 mmol/L, respectively. Meanwhile for MELF treatment, the value for parameter AST, ALT, ALP, creatinine, total protein and urea is 110.16 ± 7.42 U/L, 111.83 ± 10.09 U/L, 39.50 ± 3.67 U/L, 38.33 ± 2.17 µmol/L, 69.67 ± 2.62 g/L and 5.00 ± 0.05 mmol/L, respectively. Therefore, these results indicated that MELF showed no toxicity towards SD rats based on the liver and kidney function test.
Figure 4.19: The biochemical parameter of liver and kidney function test. SD rats were treated either with MELF (2 kg/kg of rat body weight) or control (1% CMC). (A) Bar chart shows ALP levels. (B) Bar chart shows AST levels. (C) Bar chart shows ALT levels. (D) Bar chart shows creatinine levels. (E) Bar chart shows urea levels. (F) Bar chart shows total protein levels. All data were expressed as mean ± standard error (n=6).
4.7.3 The effects of MELF on liver and kidney histopathological condition

To further corroborate the effects of MELF on liver and kidney function, the histopathological analysis of the organs was investigated. As shown in Figure 4.20, treatment of MELF showed no significant alterations of the tissue organs compared to control. The physiological aspect of liver tissue such as hepatocyte strings, centrilobular vein or sinusoid capillaries shows no significant difference. In addition, the physiological aspect of kidney tissues such as glomerulus, renal and proximal tubules also showed no significant difference between control and treated MELF. Therefore, based on the biochemical and histopathological assays, MELF was found no toxic effects in SD rats.

Figure 4.20: Photomicrography of liver and kidney section. Female SD rats were treated with 1% CMC (control) and MELF (2 g/kg of rat body weight). The tissues were stained with H and E and captured under a light inverted microscope. Magnification at x 10.
4.8 MELF induced hypoglycemia in non-diabetic SD rats

Based on previous *in vitro* assays, MELF was shown to inhibit α-amylase and α-glucosidase enzymes in a dose-dependent manner. The inhibition of these enzymes decreased glucose absorption and postprandial blood glucose (Chiu *et al.*, 2018). Therefore, the hypoglycemic effects of MELF in a non-diabetic rat was investigated based on OGTT assay. According to Figure 4.21, the fasting blood glucose was found to increase after 30 minutes of oral consumption of glucose. However, in pre-treatment with MELF, the fasting blood glucose was found to decrease after 30, and 60 minutes of glucose consumption. Accordingly, treatment with anti-diabetic drug acarbose caused the fasting blood glucose to decrease after 30 and 60 minutes of glucose consumption compared to control alone. At 90 minutes, the fasting blood glucose of all groups was returned to normal. Therefore, these data suggested that MELF induced hypoglycemia activity in non-diabetic SD rats.
Figure 4.21: The hypoglycemic activity of MELF in non-diabetic rats. Bar chart represent the fasting blood glucose of control, MELF treatment (125, 250 and 500 mg/kg) and acarbose treatment marked at 0, 30, 60, 90 and 120 minutes. All data were expressed as a mean ± standard error (n=6). Small letters “a” represent significant values compared to control alone (p < 0.05).
4.9 MELF induced hypoglycemic activity in diabetic-induced SD rats

The body weight and fasting blood glucose results for non-diabetic, diabetic control, treated MELF and glyburide were collected every week for 4 weeks

4.9.1 Effects of MELF in STZ-NA-induced SD rats body weight

According to Figure 4.22, non-diabetic rats was found to increase in body weight while diabetic control was decreased in body weight from week 0 until week 4. However, the body weight of MELF and glyburide treatment was found to increase from week 0 until week 4 in diabetic rats. Therefore, these data suggested the effects of MELF in increased body weight of diabetic rats.

Figure 4.22: Effects of MELF on body weight in diabetic rats. Bar chart represents the body weight of vehicle, diabetic control, treatment MELF (125 and 500 g/kg) and glyburide in STZ-NA-induced diabetic rats marked from 0 week to 4th week. All data were expressed as a mean ± standard error (n=6). Small letters “a” represent significant values compared to week 0 ($p < 0.05$).
4.9.2 MELF exerted anti-hyperglycemic activity in STZ-NA-induced SD rats

The anti-hyperglycemic effect of MELF was investigated by monitoring the fasting blood glucose of diabetic rats for 4 weeks. As shown in Figure 4.23, the fasting blood glucose of diabetic control group was found to elevated compared to control group. However, in treatment of MELF, the fasting blood glucose levels of diabetic rats was found to decrease at the ends of week 4. Furthermore, MELF was shown to decrease fasting blood glucose by 21.67 and 31.67 % for 125 and 500 mg/kg of MELF treatment, respectively. Treatment with the anti-diabetic drug glyburide caused a similar reduction of fasting blood glucose by 23.38 %. These results showed that MELF has higher antihyperglycaemic activity compared to glyburide at 0.7 folds more. These results presented the anti-hyperglycemic effects of MELF in STZ-NA-induced diabetic SD rats.
Figure 4.23: Effects of MELF on FBG in diabetic rats. Bar chart represents the fasting blood glucose of vehicle, diabetic control, MELF (125 and 500 mg/kg) and glyburide in STZ-NA-induced diabetic rats marked from 0 week to 4th week. All data were expressed as a mean ± standard error (n=6). Small letters “a” represent significant values compared to week 0 (p < 0.05).
4.9.3 MELF induced pancreatic insulin protein expression

Insulin hormone plays an imperative role in reducing the fasting blood glucose. Therefore, the pancreatic islets were collected and investigated for insulin protein expression through immunohistostaining assay. As shown in Figure 4.24, the amount of insulin protein expression was found to decrease in diabetic control compared to vehicle. However, in post-treatment with MELF, the amount of insulin protein expression was increased significantly in a dose-dependent manner. Therefore, based on immunohistostaining data, MELF was shown to promotes the insulin secretion in STZ-NA-induced diabetic SD rats.

4.9.4 MELF inhibited cleaved caspase 3 protein expression in SD rats

The anti-apoptosis activity of MELF in protecting pancreatic islet was investigated by immunohistostaining. As shown in Figure 4.24, the amount of cleaved caspase 3 protein expression was increased in diabetic control rats compared to vehicle. However, in post-treatment with MELF, the amount of cleaved caspase 3 was decreased compared to diabetic control pancreas. These results showed that MELF inhibited pancreatic β cells apoptosis in STZ-NA-induced SD rats.

4.9.5 MELF decrease LC3A/B protein expression in SD rats

According to previous in vitro assay, MELF was found to inhibit the impairment of autophagy in INS-1 β cells. Therefore, the inhibition of pancreatic autophagy in diabetic rats was investigated through immunohistostaining. Based on Figure 4.24, the amount of LC3A/B protein expression was increased in diabetic control rats compared to control. However, in post-treated MELF, the amount of LC3A/B was decreased compared to diabetic control rats. Therefore, these data indicate inhibition of autophagy by MELF in pancreatic β cells of STZ-NA-induced SD rats.
Figure 4.24: Immunohistostaining of pancreatic insulin, cleaved caspase-3 and LC3A/B protein in SD rats. (A) Immunohistochemical images of insulin, cleaved caspase-3 and LC3A/B of representative islets from each group. Magnification 400 x. (B) Bar chart showed the relative fold change of insulin protein. (C) Bar chart exhibited the relative fold change of cleaved caspase 3. (D) Bar chart exhibited the relative fold change of LC3A/B. All data are presented as means ± SE (n=6). Small letters “a” and “b” represent significant values compared to control and STZ-NA group alone respectively (p < 0.05).
Figure 4.24, continued.
4.9.6 MELF induced anti-hyperlipidemia in SD rats

The anti-hyperlipidemic activity of MELF was further investigated in STZ-NA-induced diabetic rats. Based on Figure 4.25, the total cholesterol and LDL levels were increased in diabetic control rats. However, in treatment of MELF, the total cholesterol and LDL levels were decreased by 9.46 % and 47.22 %, respectively. Furthermore, MELF was found to increase HDL levels by 10.59 % in diabetic rats. Thus, MELF present the anti-hyperlipidemic activity according to the assay.

Figure 4.25: The lipid profiling treated MELF in diabetic SD rats. (A) Bar chart shows the total cholesterol level. (B) Bar chart shows the HDL cholesterol level. (C) Bar chart shows the LDL cholesterol level. All data were expressed as a mean ± standard error (n=6). Small letters “a” and “b” represent significant values compared to control and DC respectively (p < 0.05).
T2D is a metabolic disorder characterized by hyperglycemia due to insulin resistance and decreased secretion of insulin (Wang et al., 2018). Since pancreatic β cells promote the normoglycemia by producing insulin, thus it is important to protect pancreatic β cells from any deleterious factors. During early phase of T2D, the tissues develop resistance towards insulin, known as insulin resistance and induced hyperglycemia (Kasangana et al., 2018). This situation induced mass production of insulin to compensate the increase blood glucose, subsequently caused an increase in pancreatic β cells mass. In addition, the early phase of T2D masks the glucose intolerance for several years, and the body can naturally inhibits the insulin resistance (Chiu et al., 2018). However, without early diagnosis, the pancreatic β cells are susceptible to decreasing cell mass due to prolonged hyperglycemia and other factors (Lima et al., 2018). In the late phase of T2D, the pancreatic β cells slowly undergo cell death, but the cells can still secrete insulin in response to glucose which is different case from T1D where no insulin is presence in the blood (Liston et al., 2017). Therefore, induction of minimal pancreatic β cells death mimics the pathological conditions of T2D. STZ is a β cell specific toxin where it can induce diabetes by selectively destroying insulin producing cells of the pancreas (Gheibi et al., 2017). However, β cells can regain its functionality through treatment with antidiabetic drugs such as sulfurylase, plant extracts or pure compounds.

Natural products have been shown to naturally cure many complications including diabetes, and possess several other advantages compared to pharmaceutical drugs, such as lower cost, less side effects, and multifactorial targets (Kooti et al., 2016). Therefore, in this study, *L. flavescens* was investigated for its antidiabetic properties. *L. flavescens* has been reported to induce antidiabetic in alloxan-induced diabetic rats (T1D inducer) but the mechanism action of antidiabetic remains unclear particularly in preventing T2D
L. flavescens leaves were extracted by using solvents of increasing polarity starting from hexane, ethyl acetate, methanol, and water to extract the bioactive compounds according to its polarity. Each extract was analyzed for antidiabetic activities using different in vitro assays.

Treatment of INS-1 β cells with a low dose of STZ (1.5 mM) for 5 h mimicked the T2D pathological conditions and has resulted in cell death up to 45.55 ± 0.84 % compared to control. Following the induction of INS-1 β cell death, the cells were treated with all the extracts to investigate the survivability of STZ-induced INS-1 β cells. The MTT cell viability assay has shown that MELF exerts the highest cell viability when compared to other extracts. Since MELF increased the cell viability of STZ-induced INS-1 β cells, the insulin secretion of the INS-1 β cells was further investigated. According to the glucose stimulate insulin secretin assay, post-treatment with MELF has increased insulin secretion by the β cells. These results are consistent with dapnetin and coffee silverskin extracts in increasing cell viability and insulin secretion in STZ-induced INS-1 β cells (Fernandez et al., 2016; Vinayagam & Su., 2017).

Exposure of pancreatic β cells to cytotoxic factors can lead to a type of programmed cell death such as apoptosis and induce either T1D or T2D. The involvement of β cells apoptosis further deteriorates pancreatic islet mass and depletes insulin secretions (Rashid & Sil., 2015). Therefore, the identification of the key regulators of β cell apoptosis proposed a new therapeutic target for treating T2D (Lee et al., 2017). Apoptosis cell death consist two pathways namely, the extrinsic pathway (involves activation of cell death receptor) or intrinsic pathway (involves mitochondrial stress) (Lim et al., 2018). Several studies reported that exposure of pancreatic β cells to STZ induced apoptosis (Ahn et al., 2015). To further investigate the preliminary involvement of INS-1 β cells apoptosis, the externalization of phosphatidylserine was stained with Annexin V/PI and analyzed through flow cytometry. Based on the results, STZ induced phosphatidylserine
translocation to outer leaflet of plasma membrane and treatment with MELF prevented the phosphatidylserine translocation of INS-1 β cells. Furthermore, STZ-induced INS-1 β cells were double stained with Hoechst 33342 and PI for the morphological apoptosis confirmation. The dual dye has been used to differentiate between early and late apoptotic events. In early apoptosis, the cells exhibited bright blue indicating cellular DNA damage, while in late apoptosis, the cells turned purple indicating cellular DNA damage and degradation of plasma membrane. The results have shown that the number of purple and bright blue cells was decreased in INS-1 β cells when treatment with MELF compared to STZ-induced cells. The current findings indicated the anti-apoptotic effects of MELF towards STZ-induced INS-1 β cells through series of biochemical and morphological apoptosis detection.

The occurrence of downstream intrinsic pathways is propagated through activation of Bcl-2 family proteins and caspase cascade pathways. Bcl-2 family proteins comprised of pro- or anti-apoptosis proteins such as Bax, Bak, Bcl-2 or Bxl-xl. Imbalance ratio of these proteins prompts the opening of mitochondria pores and releases a set of apoptogenic factors into the cytosol. Thus, by activating the anti-apoptotic proteins (such as Bcl-2 or Bcl-xl), can induce the normal permeability of mitochondria and prevent activation of pro-apoptotic factors (Johnson et al., 2015). The apoptogenic factors then bind together with active caspase 9 (activator caspase) forming an apoptosome and eventually initiates caspase 3 (executioner caspase). Activation of caspase 3 leads to apoptosis cell death (Janikiewicz, et al., 2015). To further substantiate the intrinsic pathways in STZ-induced INS-1 β cells, the cells were stained with Mito-ID dye and analyzed through flow cytometry. Mito-ID dye is a cationic dye which exhibits high amount of J-aggregates inside healthy mitochondria (yields orange fluorescence) and low amount of monomer in cytoplasm (yields green fluorescence). Thus, the staining of MMP can differentiate between viable and apoptosis cell death. Upon exposure to STZ, the orange fluorescence
decreased in INS-1 β cells. However, post-treatment with MELF markedly increased the amount of orange fluorescence in STZ-induced INS-1 β cells. This assay reflects the ability of STZ to induce mitochondria-mediated apoptosis cell death, followed by MELF ability to induce anti-apoptotic effects in preventing MMP loss. Furthermore, based on the results, treatment of MELF markedly increased Bcl-2 and Bcl-xl anti-apoptotic proteins in STZ-induced INS-1 β cell. In addition, treatment with MELF able to inhibit caspase 9 and 3 proteins in a dose dependent manner which protects INS-1 β cells from STZ-induced apoptosis cell death. Therefore, these results indicate the possible mechanisms of anti-apoptotic effects of MELF through modulation of Bcl-2, Bcl-xl, caspase-9 and caspase-3 proteins expression in STZ-induced INS-1 β cells.

To further clarify the involvement of anti-apoptotic effects of MELF, INS-1 β cells were pretreated with apoptosis inhibitor, Z-VAD-FMK for 1 hour. Based on the results, pretreatment with the inhibitor together with MELF further inhibited phosphatidylserine translocation and attenuated MMP loss when compared to cells post-treated with MELF in INS-1 β cells. These results suggested that MELF exerted its anti-apoptotic effects through phosphatidylserine translocation and dissipation of MMP in STZ-induced INS-1 β cells. The effect of apoptosis inhibitor and MELF was further investigated for the modulation of apoptosis protein expression. Intriguingly, caspase 9 and 3 protein expressions were further inhibited in STZ-induced INS-1 β cells when pretreated with Z-VAD-FMK compared to post-treatment with MELF alone. Therefore, the results showed the activation of MELF-mediated anti-apoptosis activity in STZ-induced INS-1 β cells. Several studies have reported the anti-apoptotic effects of certain compounds by regulating the Bcl-2 and caspase cascade proteins and protects β cell damage. For instance, sesamin was able to induce Bcl-2 protein expression in STZ-induced INS-1 β cells (Zheng et al., 2015) and, hexarelin inhibited caspase 9 and 3 in STZ-induced MIN6 β cells (Zhao et al., 2016).
Apart from apoptotic induced cell death, several studies have reported that dysregulation of autophagy can induce cell death (Bartolomé et al., 2014). Autophagy is a dynamic process involving the rearrangement of subcellular membranes (referred as an autophagosome) to sequester cytoplasm and organelles for delivery to lysosomes, where the sequestered material is degraded and recycled. Autophagy is basically cell-protective; however, it may also promote cell death (impairment of autophagy) through excessive degradation of cellular constituents, depending on the cellular and environmental context (Quan et al., 2012). Therefore, by attenuating the autophagy protein expression such as Beclin-1 or LC3A/B, β cell death can be prevented. For instance, Exendin-4 has been shown to inhibit tacrolimus-induced autophagy in INS-1 β cells by inhibiting LC3B protein expression (Lim et al., 2016). Based on the present study, the formation of autophagosomes induced by STZ was markedly decreased after treatment with MELF based on morphological acridine orange staining. Furthermore, the expression of autophagy-related proteins such as Beclin-1 and LC3A/B was dose-dependently downregulated after treatment with MELF in STZ-induced INS-1 β cells. INS-1 β cells were further pretreated with autophagy inhibitor, 3-MA to verify the involvement of inhibition in impairment of autophagy by MELF. From the results, Beclin-1 and LC3A/B protein expressions were further inhibited in 3-MA-pretreated with MELF compared to MELF treatment alone. These collective findings suggested the involvement of MELF to counter impaired autophagy induced by STZ in INS-1 β cells. By targeting the autophagy pathways, MELF can further protect β cells from cell death and thus prevent onset of T2D.

Activation of survival protein expression such as in PI3K/AKT pathways has been reported to increase β cell mass, proliferation, growth, and cell size (Li et al., 2015; Rashid & Sil., 2015). In addition, activated AKT (phosphorylated isoform) has a primary role in insulin metabolism by inducing GLUT4 translocation resulting in increased
glucose uptake. When AKT (Ser21) protein expression is activated (phosphorylated), it further inactivates GSK-3β (phosphorylated) and inhibits glycogen synthase, where the inhibition of GSK-3 protein expression promotes β cell replication and survival (Mussmann et al., 2007). As shown in our results, treated MELF in STZ-induced INS-1 β cells markedly increased AKT protein expression followed by inactivated GSK-3β proteins expression. These results were similarly reported for curcumin inducing phosphorylation of AKT and GSK-3β of pancreatic tissue and pancreatic islets in STZ-induced diabetic rats (Rashid & Sil., 2015). Furthermore, the induction of AKT-mediated apoptosis and autophagy was evaluated by pretreatment with a known AKT inhibitor, triciribine hydrate (API) in STZ-induced INS-1 β cells. Based on the western blot analysis, treatment with MELF together with the inhibitor markedly increased activated caspase 3 and LC3A/B protein expression compared to treatment alone. These results suggested the role of activated AKT in inducing anti-apoptosis and reducing impaired autophagy in MELF-treated INS-1 β cells. Subsequently, treatment with the inhibitor and MELF further activated GSK-3β (dephosphorylated) compared to MELF treatment alone. These findings proposed the AKT-mediated inhibition of glycogen synthesis which leads to INS-1 β cell survival after treatment with MELF.

The production of endogenous cellular antioxidants or exogenous dietary antioxidants can protect the body from deleterious effects of reactive oxygen species. Several reactive oxygen species such as superoxide anion, hydroxyl radical, singlet oxygen or hydrogen peroxide can cause lipid peroxidation or protein oxidation and lead to serious cellular damage. Any disparity or imbalance between the creation of free radicals and antioxidants inside the living cells can lead to the production of “intracellular oxidative stress”. Several reports stated the production of oxidative stress inside the pancreatic β cells, leading to the onset of diabetes mellitus. This is due to the β cells containing low levels of antioxidant enzymes, such as catalase, superoxide dismutase or glutathione to overcome
these intracellular free radicals. STZ has been shown to selectively induce oxidative stress in pancreatic β cells by binding to GLUT2 transporter at the plasma membrane. According to the results, STZ induction alone increased intracellular reactive oxygen species in INS-1 β cells but was inhibited upon post-treatment with MELF. Therefore, the ability of MELF to inhibit intracellular reactive oxygen species in STZ-induced INS-1 was further investigated for its scavenging activity, induction of antioxidant enzymes and proteins.

Plants contain a wide variety of antioxidants such as ascorbic acid, glutathione, tocopherol, or carotenoid, which can overcome the oxidative stress. Having antioxidant activity is an added advantage to pharmaceutical treatment where it can function as multifunctional drugs (Dehghan et al., 2016). Therefore, adding medicinal plants to the diet, can inhibit the intracellular reactive oxygen species or scavenge the free radicals within the β cells, and further protects from diabetes mellitus complications. Based on the results, MELF was shown to possess the highest antioxidant activities compared to other extracts. For example, MELF was revealed to scavenge DPPH and superoxide free radicals and was able to reduce ferric ion based on FRAP assay. Ascorbic acid and gallic acid have been used as positive control as described by Kong et al. (2012). Several studies have reported that high antioxidant activity can lead to antidiabetic property (Dehghan et al., 2016). Furthermore, post-treatment with MELF has been shown to induce translocation of Nrf-2 protein expression in the nuclear site of STZ-induced INS-1 β cells. These findings were in line with the therapeutic effects of pterostilbene in STZ-induced INS-1E β cells by regulating the Nrf-2 translocation (Bhakkiyalakshmi et al., 2014). Translocation of Nrf-2 protein further induced production of several antioxidant protein. Post-treatment with MELF showed an increase in HO-1 antioxidant proteins with elevated catalase activity in STZ-induced INS-1 β cells. Therefore, these results show that MELF can attenuate oxidative stress by the scavenging activity of DPPH and
superoxide free radicals, reduced ferric ion, induced translocation of Nrf-2, induced HO-1 protein expression and increased catalase activity.

In the drug discovery protocol, evaluation of toxicity profile of plant extracts or bioactive compounds is a crucial step. The toxicity data helps to determine the dosage of the treated plant extracts so that it can be used in animals and humans thereafter (Padgaonkar et al., 2018). So far, there is no report available on the toxicity of MELF. Hence, the toxicity of MELF was investigated in SD rats by following the international OECD 423 guidelines. The SD rats were orally administered with 2g/kg of MELF and 1% CMC (control) and observed for any behavioral or physical changes for the duration of 14 days. At the end of period, there was no mortality or significant difference in the body weight between MELF treated and the control. The toxicity was further investigated through biochemical and histopathological analysis. The abnormalities of liver and kidney organs can be detected by several biochemical tests such as liver or kidney function test (Christapher et al., 2017). When liver cells are damaged, abnormal amounts of enzymes such as ALT, ALP or ALT were released to the bloodstream. The increased amount of these enzymes resemble that the liver function is impaired (Abou Seif, 2016). According to the present results, ALP and ALT enzymes show not significantly difference in treated MELF compared to controls. Although the amount of ALP was slightly increased in treated MELF, the amount of ALP is not exceeded to 135 U/L according to reference of liver toxicity (Yi et al., 2018). In kidney function test, the breakdown of certain proteins produced a waste substance such as urea nitrogen or creatinine and excreted by kidney. If the kidney become impaired, the waste products are not eliminated and rise the blood toxic (Weiner et al., 2015). Based on the present results, treated MELF shows no significant difference of creatinine and total proteins levels in SD rats. However, the amount of urea was slightly decreased compared to controls, in which explain that MELF had improved in urea excretion. In addition, the
histopathological analysis showed no changes in the liver and kidney tissues of MELF treatment rats compared to control. Collectively, the overall toxicity data claims that MELF shows no toxic effects in SD rats.

One of the important therapeutic approaches to prevent T2D is by inhibition of postprandial hyperglycemia through inhibition of carbohydrate hydrolysis enzymes such as α-glucosidase and α-amylase in the digestive organs. The inhibition of carbohydrate breakdown can inhibit glucose absorption in intestinal tract and therefore induced hypoglycemia in the blood (El Abed et al., 2017). Hence, all extracts were evaluated for their enzyme inhibitory effects of α-glucosidase and α-amylase using acarbose as a positive control. Acarbose has been used as positive control for α-amylase and α-glucosidase inhibition enzyme activity as described by Justino et al. (2018). Based on the data, MELF showed greater inhibition against α-glucosidase and α-amylase enzymes compared to other extracts. Moreover, the inhibition of α-glucosidase by MELF was higher than acarbose. Although, inhibition of these enzymes can bring certain side effects such as diarrhea or flatulence, but it shows no side effects against cardiovascular or liver safety risk when compared to others antidiabetic drugs (DeFronzo et al., 2015). Similar results were reported using aqueous ethanolic extracts of P. dactylifera fruits, which demonstrated a potent inhibition of α-glucosidase compared to acarbose and the fruit confirmed the anti-postprandial hyperglycemia activity. To further corroborate the hypoglycemic activity of MELF in in vivo model, non-diabetic SD rats were orally administered with MELF followed by glucose. The fasting blood glucose was determined by using a glucometer every 30 min. Accordingly, after induction of hyperglycemia, the fasting blood glucose was increased in the control rats but was decreased in treated MELF and acarbose after 30 minutes. These results indicated that MELF induced hypoglycemic and anti-postprandial hyperglycemic through the inhibition of glucose uptake in the intestine and further avoided deterioration of diabetes mellitus. Furthermore, these results
were also similar of Phrynium imbricatum leaves which showing the α-amylase inhibition activity and the hypoglycemic effects in non-diabetic hyperglycemic mice (Uddin et al., 2014).

In the *in vivo* experiments of this study, diabetes was induced by a single *ip* injection of nicotinamide followed by STZ to create a T2D model. STZ causes the complete destruction of pancreatic β-cells which leads to decreased insulin synthesis and secretion. Prior to STZ injection, nicotinamide was given to partially protect the pancreatic β cells from destruction. Once fasting blood glucose reached more than 10 mmol/L, SD rats were treated with 1% CMC, MELF (125 and 500 mg/kg) and glyburide for 4 weeks. Each week the body weight and fasting blood glucose of all groups were recorded. In the final weeks, the fasting blood glucose of diabetic control has increased up to 15.90 ± 1.74 mmol/L, while in MELF and glyburide treated rats, the fasting blood glucose has decreased to 7.96 ± 0.74 and 7.57 ± 0.87 mmol/L, respectively. The reduction in fasting blood glucose of MELF and glyburide treated rats was 31.67% and 23.38%, respectively, when compared to the 0 weeks of treatment. Therefore, these results indicated that treatment with MELF showed greater anti-hyperglycemic activity compared to the anti-diabetic drug, glyburide, in STZ-NA-induced SD rats. Furthermore, the results are in line with N-Trisaccharide and methanol extracts of Bersama engleriana which exhibited higher anti-hyperglycemic activity compared to anti-diabetic drugs in STZ-NA-wistar rats (Kavishankar & Lakshmidevi, 2014; Pierre et al., 2012). In addition, treatment with MELF helped in recovering the body weight of diabetic rats after 4 weeks. These results showed that MELF did not affect the body weight. The reduction of fasting blood glucose in MELF treated rats was mainly due to the recovery of insulin secretion from pancreatic β cells in NA-STZ-induction. Therefore, the insulin proteins were investigated through immunohistostaining assay. Based on the results, MELF treated rats showed an increase of insulin protein expression inside the pancreatic islets. These results also corroborated
in vitro data when MELF induced insulin secretion in STZ-induced INS-1 β cells. Hence, these results suggested that MELF can protect pancreatic β cells and induce insulin secretion against deleterious STZ as evidenced by the in vitro (STZ-induced INS-1 β cells) and in vivo (STZ-NA-induced SD rats) models. The results are also in line with the increase in RIN-5 β cells viability and insulin secretion as well as decreased fasting blood glucose and increase plasma insulin induced by saffron in STZ-induced SD rats (Dehghan et al., 2016).

The anti-apoptosis and autophagy activity of MELF has been proven through in vitro assays, therefore, the pancreatic islets of SD rats were investigated for apoptosis- and autophagy-related proteins such as cleaved caspase-3 and LC3A/B protein through immunohistostaining assay. According to the results, MELF treated rats were shown to decrease activation of caspase 3 and LC3A/B protein expression in STZ-NA-induced SD rats. These results are in parallel with the in vitro MELF treatment showing a decrease in the activation of caspase 3 and LC3A/B protein expression in STZ-induced INS-1 β cells. Therefore, treatment with MELF was found to have anti-apoptotic and autophagy activity based on the in vitro (STZ-induced INS-1 β cells) and in vivo (STZ-NA-induced SD rats) models. These results are in agreement with the anti-apoptotic effects of hexarelin resulting in decreased cleaved of caspase 3 and 9 protein expression in STZ-induced MIN6 β cells with concurrent decrease in Bax and caspase 9 protein expression of pancreatic islets in STZ-induced wistar rats (Zhaou et al., 2016). Furthermore, the results are also in line with the anti-apoptosis and autophagy impairment of Exendin-4 in decreased active caspase 3 and decrease ubiquitin, p62, LC3A/B proteins expression in tacrolimus-induced INS-1 β cells and diabetes SD rats (Lim et al., 2016). In addition, MELF also has been shown to decrease cholesterol levels in the blood. Although STZ did not induced hyperlipidemia, but the lipid levels of MELF treatment improved in STZ-NA induced SD rats. For instance, treated MELF, decreased total cholesterol and low-density
lipoprotein levels with increased high-density lipoprotein levels in STZ-NA-induced SD rats. Therefore, these results indicated the ability of MELF in decreased lipid levels. The results are also the same as Borneol which induced anti-hyperglycemia and anti-hyperlipidemia in STZ-induced wistar rats (Madhuri & Naik, 2017).

From all the extracts, MELF was found the highest in biological activity according to our in vitro and in vivo assays. The qualitative and quantitative phytochemical analysis reveals, MELF has the highest phenol and flavonoids content compared to other extracts. Therefore, the presence of phenol and flavonoids groups in L. flavescens, might contribute to the antidiabetic properties in in vitro and in vivo.
CHAPTER 6: CONCLUSION

As conclusion, these works remarkably present the protective effects of MELF towards STZ-induced INS-1 β cells damage. These effects were observed by increased anti-apoptotic and decreased impaired autophagy by activating AKT proteins expression. Furthermore, MELF able to reduce intracellular ROS and thus promotes catalase enzyme with modulating HO-1 and Nrf-2 proteins expression. MELF able to inhibit α-amylase and α-glucosidase enzyme and decrease fasting blood glucose in non-diabetic SD rats. The fasting blood glucose of treated MELF was found to decreased in STZ-NA induced diabetic rats. MELF also shown to induced pancreas-protective effects through anti-apoptotic and autophagy mechanism in diabetic-induced SD rats. Therefore, MELF has shown its anti-diabetic activity based on the in vitro and in vivo assay (Figure 6.1).
Figure 6.1: Overall schematic diagram of the antidiabetic activity of *L. flavescens*. (A) The *in vitro* study of MELF treatment in STZ-induced INS-1β cells. (B) The *in vivo* study of MELF in with/without STZ-NA-induced SD rats.
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APPENDIX

Gallic acid standard graph

\[ y = 0.0044x - 0.0848 \]
\[ R^2 = 0.9933 \]

Quercetin standard graph

\[ y = 0.0007x - 0.027 \]
\[ R^2 = 0.9398 \]
H₂O₂ standard curve for catalase assay

Insulin standard graph
BSA standard graph using Bradford proteins quantification

\[ y = 0.0004x + 0.0838 \]
\[ R^2 = 0.9906 \]

BSA standard graph using BCA proteins quantification

\[ y = 0.0007x + 0.0319 \]
\[ R^2 = 0.9947 \]