IN VITRO EVALUATION OF POLYPHENOLIC-RICH FRACTION OF CORNSILK EXTRACT AS ANTI-HYPERGLYCAEMIC AGENT

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IN VITRO EVALUATION OF POLYPHENOLIC-RICH FRACTION OF CORNSILK EXTRACT AS ANTI-HYPERGLYCAEMIC AGENT

by

KOO JOANNE

Dissertation submitted in partial fulfilment of the requirements for the degree of Bachelor of Health Sciences (Honours) (Biomedicine)

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CERTIFICATE

This is to certify that the dissertation entitled '*In vitro* evaluation of polyphenolic-rich fraction of cornsilk extract as anti-hyperglycaemic agent' is the bona fide record of research work done by KOO JOANNE during the period from August 2024 to January 2025 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfillment for the degree of Bachelor of Health Science (Honours) (Biomedicine).

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Date: 27th January 2025

DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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(KOO JOANNE)

Date: 27th January 2025

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
*	Strength of significance
<	Smaller than
=	Equal
±	Plus-minus
°C	Degree celcius
ADMA	Asymmetric dimethylarginine
AFR	Africa
AGEs	Advanced glycation end products
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein-kinase
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSC	Biological safety cabinet
C	Constant
cAMP	Cyclic adenosine monophosphate
CEL	Carboxyethyl-lysine
CML	Carboxymethyl-lysine
CO_2	Carbon dioxide
ct	Cytoplasmic tail
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNSA	3, 5 – Dinitrosalicylic acid
DOLD	3-Deoxyglucosone lysine dimer
DPP-4	Dipeptidyl peptidase IV
EC_{50}	Half effective maximal concentration
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin-3-gallate
esRAGE	Endogenous secretory receptor of advanced glycation end product
ESRD	End-stage renal disease
ETC	Electron transport chain
EUR	Europe
FBS	Fetal bovine serum
FDA	Food and Drug Administration
g	Gram
GDM	Gestational diabetes mellitus
GFR	Glomerular filtration rate
GLO	Glyoxalase

GLP-1	Glucagon-like peptide-1
GLUT4	Glucose transporter-4
GOLD	Glyoxal-lysine dimer
HbA1c	Haemoglobin A1c
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
IC_{50}	Half-maximal inhibitory concentration
IDF	International Diabetes Federation
IL-1α	Interleukin- 1 α
kDa	Kilodaltons
L	Liter
L929 cells	Murine fibroblastic cells
LDL	Low-density lipoprotein
MAP	Mitogen-activated protein
mbar	Millibar
MENA	Middle East and North Africa
mg	Milligram
mG3PDH	Mitochondrial glycerol phosphate dehydrogenase
MGO	Methylglyoxal
mL	Milliliter
mM	Millimolar
MODY	Maturity-onset diabetes
MOLD	Methylglyoxal-lysine dimer
MRPs	Maillard reaction products
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	North America and Caribbean
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Sodium phosphate monobasic dihydrate
NF-κB	Nuclear factor -κB
nm	Nanometer
-OH	Hydroxyl group
PBS	Phosphate buffered saline
PenStrep	Penicillin streptomycin
pH	Potential of hydrogen
PRF	Polyphenolic-rich fraction
p-value	Significant value
R^2	Coefficient of determination
RAGE	Receptor of advanced glycation end product

RCS	Reactive carbonyl species
ROS	Reactive oxygen species
rpm	Revolution per minute
S.E.M	Standard error of means
SACA	South and Central America
SEA	South-East Asia
SGLT2	Sodium-glucose transport protein 2
sRAGE	Secretory receptor of advanced glycation end product
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TMD	Transmembrane domain
TNF-α	Tumour necrosis factor α
TZDs	Thiazolidinediones
V	Variable
WHO	World Health Organisation
WP	Western Pacific
x g	G-force
α	Alpha
β	Beta
μL	Microliter

PENILAIAN *IN VITRO* FRAKSI KAYA POLIFENOL DARI EKSTRAK SERAT JAGUNG SEBAGAI AGEN ANT-HIPERGLISEMIK

ABSTRAK

Diabetes mellitus (DM) adalah penyakit metabolik kronik yang dicirikan oleh peningkatan tahap glukosa darah akibat rintangan insulin atau pengeluaran insulin yang tidak mencukupi. Peningkatan kepekatan glukosa darah juga dikenali sebagai hiperglisemia yang menyumbang kepada pelbagai komplikasi diabetes seperti kardiomiopati diabetes dan nefropati diabetes. Metformin dan acarbose adalah ubat praktikal perubatan yang terkenal digunakan untuk merawat dan meringankan komplikasi. Walau bagaimanapun, ubatan antidiabetes tersebut memberikan kesan sampingan kepada pesakit diabetes semasa rawatan. Oleh itu, kajian ini bertujuan untuk menyiasat fraksi kaya polifenolik (PRF) dari ekstrak serat jagung sebagai calon berpotensi dalam kajian diabetes mellitus jenis 2 (T2DM). Kesan antiglikasi dan mekanisme PRF dari serat jagung dinilai menggunakan kajian dalam *vitro*. Ujian viabiliti sel dan EC_{50} PRF dari serat jagung ditentukan menggunakan sel fibroblas mencit (L929) melalui ujian MTT. PRF dari serat jagung menunjukkan perlindungan dan peningkatan viabiliti serta proliferasi sel L929 dengan signifikan secara statistik di bawah glukosa yang tinggi dengan nilai $p < 0.0001$ dan nilai EC_{50} ialah 3.84×10^{-12} mg/mL. Berdasarkan ujian MTT, PRF dari serat jagung lebih berkesan berbanding metformin dalam melindungi sel. Selain itu, PRF dari serat jagung menunjukkan perencatan enzim α-amilase yang signifikan secara statistic (IC_{50} = 8.38 mg/mL) menggunakan ujian perencatan α-amilase dalam *vitro*. Walau bagaimanapun, acarbose menunjukkan kesan perencatan yang lebih kuat (IC_{50} = 6.95 mg/mL) berbanding PRF dari serat jagung dalam ujian perencatan α-amilase. Akhir sekali, PRF dari serat jagung

menunjukkan pengurangan tahap produk akhir glikasi lanjutan (AGEs) secara signifikan menggunakan ujian antiglikasi BSA-glukosa dan -metilglioxal. PRF dari serat jagung menunjukkan aktiviti antiglikasi yang lebih kuat berbanding metformin dalam ujian antiglikasi ini. Secara ringkas, PRF dari serat jagung terbukti dengan sifat antiglikasi yang berkesan dan menunjukkan potensi sebagai calon menjanjikan untuk mengurangkan komplikasi diabetes melalui langkah antiglikasi. Walau bagaimanapun, penyelidikan yang banyak perlu dijalankan untuk mendapatkan pemahaman yang lebih lanjut mengenai kesan antiglikasi PRF dari serat jagung.

IN VITRO EVALUATION OF POLYPHENOLIC-RICH FRACTION OF CORNSILK EXTRACT AS ANTI-HYPERGLYCAEMIC AGENT

ABSTRACT

Diabetes mellitus (DM) is a chronic metabolic disease that characterised by elevated blood glucose level due to insulin resistance or inadequate insulin production. The increased blood glucose concentration also known as hyperglycaemia which contributes to various diabetic complications such as diabetic cardiomyopathy and diabetic nephropathy. Metformin and acarbose are the well-known medical practical drugs used to treat and alleviate the diabetes-related complications. However, those antidiabetic drugs bring side effects to the diabetic patients during treatment. Hence, this study is aimed to investigate the polyphenolic-rich fraction (PRF) of cornsilk extract as a potential candidate in the study of type 2 diabetes mellitus (T2DM). The antiglycation effect and mechanism of PRF of cornsilk was evaluated using *in vitro* study. The cell viability and EC_{50} of PRF of cornsilk were determined using murine fibroblast (L929) cell line via MTT assay. The PRF of cornsilk showed statistically significantly protected and enhanced the L929 cell viability and proliferation under high glucose with p value of < 0.0001 and EC_{50} value of 3.84×10^{-12} mg/mL. Upon the MTT assay, the PRF of cornsilk was more effective than the metformin in protecting cells. Furthermore, the PRF of cornsilk depicted statistically significantly (IC_{50} = 8.38 mg/mL) inhibited the α-amylase enzyme using *in vitro* α-amylase inhibition assay. However, acarbose demonstrated more robust with a greater inhibition effect (IC_{50} = 6.95 mg/mL) than the PRF of cornsilk in the α-amylase inhibition assay. Lastly, the PRF of cornsilk showed statistically significantly reduced the advanced glycation end products (AGEs) level using BSA-glucose and -

methylglyoxal antiglycation assays. The PRF of cornsilk showed stronger antiglycation activity than the metformin in these antiglycation assays. In short, the PRF of cornsilk is proved with its effective antiglycation property and showed to be a promising candidate to reduce diabetes-related complications by antiglycation pathway. However, more investigations have to carry out to gain further understanding on the antiglycation effect of the PRF of cornsilk.

CHAPTER ONE: INTRODUCTION

1.1 Research Background

Diabetes mellitus (DM) is a chronic metabolic disease, characterised by increased blood glucose level. DM can be categorised into several groups including Type 1, Type 2, gestational diabetes and neonatal diabetes. Type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM) are the main subtypes of DM. T1DM involves ruination of pancreatic β-cells, commonly due to autoimmune process, which in turns resulting to diabetes due to deficiency or extremely low insulin level. T1DM is typically found in children and adolescent. Besides, T2DM is aroused due to inequivalence between insulin level and insulin sensitivity causing a functional impaired of insulin. Moreover, insulin resistance also contributed to the development of T2DM, commonly seen in obesity and elderly groups. T2DM is more likely to affect middle-aged and older adults who have chronic hyperglycaemia due to unhealthy lifestyle and dietary choices (Sapra & Bhandari, 2023).

According to global findings of International Diabetes Federation (IDF) Diabetes Atlas 10th edition in 2021, 537 million adults (20 to 79 years), approximately 1 in 10 adults are living with diabetes. This number is estimated to increase to 643 million by 2030 and 783 million by 2045. Over 3 in 4 adults with diabetes are living in low- and middle-income countries. In addition, diabetes leads to 6.7 million deaths in 2021, can be demonstrated by 1 death case in every 5 seconds (IDF Diabetes Atlas, 2021). Based on a national survey report of Malaysia in 2019, it is found that 3.6 million adults (18 and above years) or 1 in 5 Malaysian adults are living with diabetes (Akhtar *et al.*, 2022; World Health Organisation [WHO], 2022). Diabetes is expected to affect 7 million Malaysian adults by 2025, posing a major public health risk with a diabetes prevalence of 31.3% (Akhtar *et al.*, 2022).

Non-enzymatic protein glycation, also known as Maillard reaction, is thought to be the major factor of different diabetic complications. When reducing sugars bind to amino groups in proteins, lipoproteins and nucleic acids, advanced glycation end-products (AGEs) are generated (Gutierrez, 2012). AGEs are the final metabolites of Maillard reaction. Higher blood sugar level will lead to more glycation reactions to occur, in turns making more AGEs which have a major role in diabetic complications such as macroangiopathy, neuropathy, nephropathy and diabetic foot (Singh *et al.*, 2014).

Cornsilk (*Stigma maydis*) is long, silky, and soft light green to reddish-brown hairy part of corn which enclosed the edible corn seed and is significant in management of various illnesses and diseases. Cornsilk is generally considered as agro-waste from corn plantation, hence it is predominantly discarded due to insufficient effective utilisation. Cornsilk consists lots of biologically active phytochemical components such as flavonoids and various pharmacological properties include antioxidant and antidiabetic (Chaudhary *et al.*, 2022). The anti-hyperglycaemic property of cornsilk is established from the presence of phytochemicals such as flavonoids, alkaloids, tannins and saponins. The phenolic compounds especially flavonoids in cornsilk extract possess high hypoglycaemic effects, whereby they are responsible for promoting the glucose uptake aided with mediators, increase insulin secretion, decrease intestinal glucose absorption, regenerate damaged pancreatic insulin-producing β-cells, and act on adipose cells for further inducing insulin-signalling pathways (Singh *et al.*, 2022).

1.2 Problem Statement

As in to date, the rates of morbidity and mortality of DM are kept increasing globally including Malaysia. According to Akhtar *et al.* (2022), Malaysia has one of the highest rate of diabetes in the world and in the Western Pacific region. In Malaysia, DM had been categorised into top 10 causes of death. The diabetes prevalence had elevated from 11.2% in 2011 to 18.3% in 2019, with a 68.3% increase. In addition, the treatment of diabetes is costing around 600 million US dollars per year (Akhtar *et al.*, 2022). The increasing trend of diabetes cases in population had determined the establishment of many diabetic therapies and medicines, whereby metformin is the popular first-choice drug to treat diabetes. The demand of herbal medicines and plant extracts for treating DM is higher due to lesser adverse reactions and better phytochemical benefits as compared to modern medicines (Singh *et al.*, 2022). From the ancient times, cornsilk is extensively used to treat diabetic complications and urinary tract infections. Hence, cornsilk formulations had obtained a great reputation among folk populations. The polyphenolic compounds of cornsilk demonstrate several medical characteristics such as antidiabetic, antioxidant and anti-inflammatory towards diabetic-related complications. Although the cornsilk formulations have been long use in the traditional medicine, the mechanism of cornsilk extract against diabetic complications has not yet been fully evaluated. Therefore, the effects of polyphenolic-rich fraction (PRF) of cornsilk extract as anti-hyperglycaemic agent is investigated in this study.

1.3 Objectives

1.3.1 Main Objective

To study the anti-hyperglycaemic mechanism of PRF of cornsilk extract using *in vitro* assay.

1.3.2 Specific Objectives

i. To determine the cell viability and half maximal effective concentration (EC_{50}) value of PRF of cornsilk extract on L929 cells by using MTT assay.

ii. To assess the effect of PRF of cornsilk extract on the inhibition of α-amylase by using *in vitro* α-amylase inhibition assay.

iii. To assess the effect of PRF of cornsilk extract in reducing the advanced glycation end-products (AGEs) level by using BSA-glucose assay and BSA-methylglyoxal assay.

1.4 Hypothesis

i. PRF of cornsilk extract will demonstrate significant protective effect on normal L929 cells compared to control drug, metformin.

iv. PRF of cornsilk extract will significantly inhibit α-amylase compared to control in the *in vitro* α-amylase inhibition assay.

ii. PRF of cornsilk extract will significantly reduce AGEs level compared to control in antiglycation assays.

1.5 Rational of the Study

This research study is important as T2DM reports kept increasing in Malaysia, and required to evaluate the effects and of PRF of cornsilk extract as potential anti-hyperglycaemic agent. Through this study, the viability and EC_{50} value of PRF of cornsilk extract on L929 cells can be obtained by using MTT assay. Furthermore, the effect of PRF of cornsilk extract on inhibition of α-amylase can be investigated by using *in vitro* α-amylase inhibition assay. Moreover, the effect of PRF of cornsilk extract in reducing AGEs can be assessed by using antiglycation assays. On the other hand, metformin as a standard diabetic medicine, has several undesired side effects including heartburn, headache, stomach pain and metallic taste in mouth (Yetman, 2024). Acarbose is an inhibitor of α-glucosidase and α-amylase in carbohydrate digestion, has gestational side effects such as diarrhoea, flatulence and bloating (Dong *et al.*, 2022). While cornsilk has anti-glycoxidation and anti-hyperglycaemic properties to reduce AGEs, blood glucose level and its complications in diabetic patients. Additionally, efficient utilisation of cornsilk is capable to maximise the employment of cornsilk not merely discarded as agro-waste from corn cultivation. Many advantages including easily accessible, available in large amount, lesser adverse reactions and better phytochemical benefits have made cornsilk to be a great potential candidate for diabetic study.

CHAPTER TWO: LITERATURE REVIEW

2.1 Diabetes Mellitus

The terms diabetes mellitus (DM) were taken from the Greek and Latin words respectively, where the word *diabetes* is defined as siphon, which means to pass through; whereas the word *mellitus* is defined as sweet (Sapra & Bhandari, 2023). DM is a non-communicable chronic metabolic disease characterised by elevated blood glucose level (hyperglycaemia) due to insufficient production of body insulin or inability of body to use insulin or both. Over time with hyperglycaemic condition, this will lead to severe destruction of heart, blood vessels, kidneys, eyes and nerves. Symptoms of T1DM and T2DM include frequent urination, constant hunger and thirst, weight loss, vision disturbances and fatigue. For T1DM, these symptoms might appear suddenly, whereas the symptoms are less marked for T2DM (WHO, 2019). Therefore, individuals with T2DM may be diagnosed several years after the onset of its complications. For this reason, early diagnose with early treatment is significant.

2.1.1 Prevalence of Diabetes Mellitus

DM as the one of the most serious global public health issues, offering a huge burden towards both public health and socioeconomic development (Akhtar *et al.*, 2022). WHO (2019) had reported that around 422 million people in the world living with diabetes, most of them living in low- and middle-income countries; and about 1.5 million deaths due to diabetes in each year. Both recorded morbidity and mortality of diabetes are increasing gradually over the years. The most common DM is T2DM, typically present in adults whom body is unable to make enough insulin or the body cells become resistant to insulin. According to IDF (2024), 1 in 8 adults (approximately 783 million) would be living with diabetes by 2045. Over 90% of people are diagnosed with T2DM due to genetic, socioeconomic, demographic and environmental factors. However, the key factors giving rise to T2DM including urbanisation, folk population, diminished levels of physical activity and increased obese population. Based on global findings of IDF Diabetes Atlas (2021), about 537 million adults in between 20 to 79 years old are living with diabetes, whereby most of them are unaware that they are living with diabetes. Furthermore, the diabetic population is predicted to rise to 643 million by 2030 and 783 million by 2045. As shown in Figure 2.1, the prevalence of diabetes will be increased from 2021 to 2045 with 24% in North America and Caribbean (NAC), 50% in South and Central America (SACA), 134% in Africa (AFR), 87% in Middle East and North Africa (MENA), 27% in Western Pacific (WP), 68% in South-East Asia (SEA), and 13% in Europe (EUR).

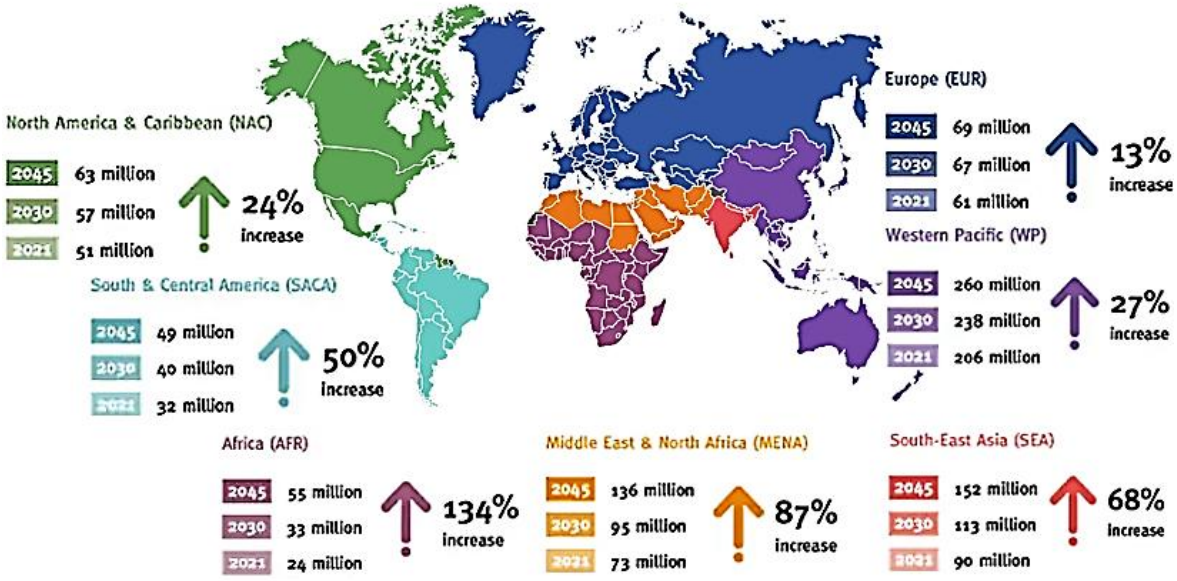

Figure 2.1: Global findings of prevalence of diabetes (International Diabetes Federation Diabetes Atlas, 2024)

2.1.2 Types of Diabetes Mellitus

DM is a complex, long-term metabolic disease with a great negative impact on the lives and health of individuals, families, and societies around the world. Based on Sapra and Bhandari (2023), DM is characterised with abnormal high blood glucose level, and it can be categorised into several groups including T1DM, T2DM, gestational diabetes, maturity-onset diabetes (MODY), neonatal diabetes and secondary causes due to endocrine disorders and improper of steroid use.

T1DM is characterised by destruction of islet of Langerhans of β-cells in pancreas, the place where producing body insulin to convert blood glucose to glycogen. The ruination of pancreatic β-cells commonly due to genetic and environmental factors (Sapra & Bhandari, 2023). When the body immune system mistakenly recognises the local β-cells as foreign cells, autoantibodies will be generated to attack and destroy the β-cells in the pancreas (Pietrangelo, 2024). This destruction of pancreatic β-cells is known as autoimmune disease, and it is permanent cell destruction. Hence, no or extremely low insulin production since there is no or almost absence of β-cells present in the pancreas. T1DM onsets from birth, increases gradually and peak at 4 to 6 years old, and continues increasing from 10 to 14 years old. T1DM appears approximately 45% in children before 10 years old, and the prevalence of T1DM under 20 years old is 0.23%. Since T1DM is an autoimmune disease, thus daily administration of insulin before a meal through insulin injection or insulin pump is mandatory (Sapra & Bhandari, 2023).

T2DM is characterised by imbalance of insulin level and insulin sensitivity in the body, resulting in abnormal high of blood glucose level (Sapra & Bhandari, 2023). When the insulin concentration failed to keep pace with blood glucose concentration or the cells are less likely to be sensitised by the insulin, indicating low glucose uptake by the cells, causing redundant blood glucose remains in the bloodstream. To add on, insulin resistance is one of the factors that contributing to diabetes. It can be described as the cells in muscles, adipose tissues and liver are unable to respond well to insulin, leads to hyperglycaemia. Insulin resistance is a short-term condition; however, it will develop into diabetes if left untreated for a longer period of time (Booth, 2024). T2DM typically occurs in middle-aged and obese population who have prolonged hyperglycaemia due to poor eating practices and manner of living, the prevalence is 90% higher in all over the world. Furthermore, T2DM has a stronger association to hereditary inheritance as compared to T1DM, indicating a more complicated interaction between genetic factors and lifestyle. Most of the T2DM patients have at least one parent with same type of diabetes (Sapra & Bhandari, 2023). This statement had been greatly supported by Thorn *et al.* (2008), which demonstrated that the descendent of a T2DM parent will have a 40% lifetime risk of T2DM, while offspring of both parents with T2DM even has a higher lifetime risk of T2DM.

Gestational diabetes mellitus (GDM) is a common hyperglycaemia that is established during pregnancy. GDM can be categorised into classes, there are primarily diet-controlled GDM known as class A1GDM, and GDM that requires anti-hyperglycaemic medication known as class A2GDM (Rodriguez *et al.*, 2024). GDM is caused by insulin-blocking hormones such as proinsulin, in which excessive proinsulin might trigger β-cell stress. Furthermore, high titre of hormones such as progesterone, cortisol, prolactin, human placental lactogen and oestrogen will contribute to functional deficit of β-cells and impaired peripheral insulin sensitivity (Sapra & Bhandari, 2023). Moreover, Thorn *et al.* (2008) also reported that abnormal hormone concentration can affect maternal pancreatic β-cell dysfunction and delayed β-cell response, in turns reduced insulin production and ultimately leads to maternal hyperglycaemia. Besides, a maternal obesity in early pregnancy period will promote insulin resistance. This is because the higher level of free fatty acid is able to inhibit maternal uptake of glucose and induce hepatic gluconeogenesis (Thorn *et al.*, 2008). Through diet-controlled plan or assisted by anti-hyperglycaemic medications, the diagnosed GDM can be alleviated or eradicated. GDM will turn into T2DM if prolonged postpartum hyperglycaemia is left untreated.

2.1.3 Treatment of Type 2 Diabetes Mellitus

A combination of lifestyle changes and pharmacological treatment will be the cornerstone of diabetes therapy (Marín-Peñalver *et al.*, 2016). Lifestyle changes such as dietary modification and increased physical activity can be very effective in the regulation of blood glucose. Diet containing refined carbohydrates, high fructose corn syrup and saturated fat should be reduced or avoided, while a meal consists of high fiber and monosaturated fats is highly encouraged. Furthermore, regardless the intensity of exercise, increased physical activity is the dominant task in glycaemic control, especially for those T2DM patients who are obese. To illustrate, an aerobic exercise about 90 to 150 minutes per week is recommended (Goyal *et al.*, 2023).

If the maintenance of glycaemic control cannot be achieved, therapeutic drugs should be considered and prescribed to chronic hyperglycaemic individuals. At present, oral and injectable (non-insulin) pharmacological choices are available for treatment of T2DM. There are ten classes of orally available drugs to treat T2DM, including metformin, inhibitors of α-amylase and α-glucosidase, sulfonylureas, meglitinides, thiazolidinediones (TZDs), dipeptidyl peptidase IV (DPP-4) inhibitors, bile acid sequestrants, dopamine agonists, sodium-glucose transport protein 2 (SGLT2) inhibitors and oral glucagon-like peptide-1 (GLP-1) receptor agonists (Goyal *et al.*, 2023). Besides, among numerous diabetic medications, metformin is the first-line therapy to treat T2DM.

Metformin, a dimethyl biguanide, is a standard anti-hyperglycaemic agent to manage elevated blood glucose level. Due to its proven clinical efficacy on glycaemic management as monotherapy and in combination with many other medicines, the utilisation of metformin as therapeutic practical drug has been far beyond the sulfonylureas (Feingold, 2024). Liver is the most significant organ that in charge of blood glucose regulation. After metformin is transported from intestine to liver, there are four different mechanisms induced by metformin to inhibit the hepatic gluconeogenesis and increased hepatic insulin sensitivity (Marín-Peñalver *et al.*, 2016). Metformin is able to retard the formation of mitochondrial ATP through inhibition of NADH coenzyme Q oxidoreductase (Complex I) in mitochondrial electron transport chain (ETC) or inhibition of mitochondrial glycerophosphate dehydrogenase which is needed to transport reducing equivalents from cytoplasm into mitochondria for redox reaction. This results in reduction of ATP production which relatively decreases the hepatic gluconeogenesis. In addition, it also leads to a rise in AMP to ATP ratio, in turn activating mucosal AMP-activated protein-kinase (AMPK) (Feingold, 2024).

Furthermore, intake of metformin can increase the hepatic AMP level which reduce gluconeogenesis and glycogenesis. This is because AMP is a strong allosteric inhibitor of fructose 1,6-bisphosphatase which is a vital enzyme involved in gluconeogenesis. Plus, elevated level of AMP has inhibitory effect on adenylyl cyclase, resulting inhibition of glucagon-induced cyclic AMP (cAMP) production, and hence decreases glycogenesis. To add on, a high AMP level also activates the AMPK. Besides, metformin-activated AMPK has the potency to trigger and activate the catabolic pathways that lead to diminished gluconeogenesis, decreased production of fatty acid and increased oxidation of fatty acid. It is firmly believed that changes in metabolism of fatty acid can improve the hepatic insulin sensitivity and reduce the serum triglyceride level (Feingold, 2024). Moreover, blocking of mitochondrial glycerol phosphate dehydrogenase (mG3PDH) by metformin will influence the transport of NADH from cytoplasm into mitochondria, this results in a suppression of gluconeogenesis from lactate (Marín-Peñalver *et al.*, 2016).

On the contrary, many research investigations have shown that metformin does bring side effects to the diabetic patients. Sakouhi *et al.* (2022) had stated that metformin caused frequent diarrhoea, followed by nausea and vomiting. The symptoms of abdominal pain, bloating and epigastric cramps are associated with metformin consumption. While metallic taste is rarely happened in the metformin-treated subjects, vitamin B12 deficiency is reported in the case of long-term metformin therapy.

2.2 Non-Enzymatic Protein Glycation and AGEs formation

Non-enzymatic glycation, also known as Maillard reaction, is a spontaneous post-translational modification of proteins or amino acids by reducing sugars (Yeh *et al.*, 2016). It is a random, cascading, non-enzymatic and site-specific complex reaction, able to cause spontaneous destruction to both cellular and extracellular proteins in physiological systems. This non-enzymatic glycation is different from glycosylation (enzymatic glycation), it can bind specifically to the site of sugar at lower temperatures. Non-enzymatic protein glycation is able to generate AGEs at the final phase of reaction, in which the AGE is a key contributor to development of chronic diabetic complications. In diabetic patients, the protein glycation will be exacerbated due to consequences of higher levels of glucose and other saccharides derivatives in bloodstream and vasculatures (Ahmed & Thornalley, 2006; Ma *et al.*, 2021). As illustrated in Figure 2.2, Maillard reaction is a multi-steps and time-consuming glycation process, it often requires several days or up to several weeks to fully complete. The reaction is divided into three phases, including initiation phase, propagation phase and advanced phase.

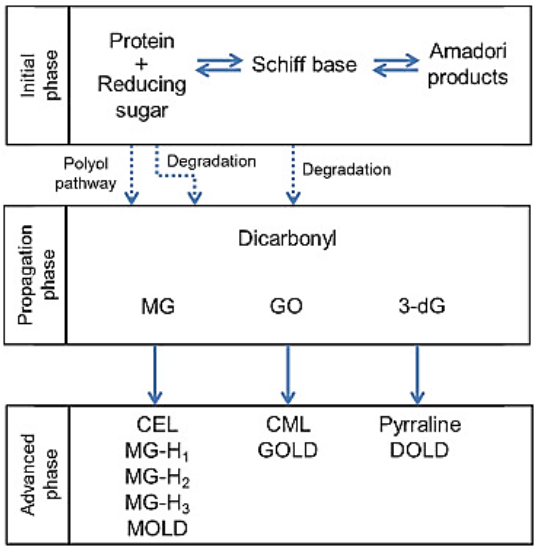

Figure 2.2: Maillard reaction (Yeh *et al.*, 2016)

Move on, besides Maillard reaction, autoxidation of glucose and peroxidation of lipids are able to produce AGEs. These oxidation pathways can turn the substrates into dicarbonyls derivatives by an elevated level of oxidative stress, where the dicarbonyls derivatives which known as α-oxaldehydes (glyoxal, methylglyoxal, and 3-deoxyglucosone) can react with monoacids and thus generating AGEs. Moreover, polyol pathway is also one of the mechanisms involved in production of AGEs. In polyol pathway, aldose reductase reduced glucose to sorbitol then the sorbitol is concerted to fructose by sorbitol dehydrogenase. After that, the fructose metabolites such as fructose 3-phosphate are converted to α-oxaldehydes, then interacting with monoacids to form AGEs. Therefore, as shown in Figure 2.3, there are at least four pathways to generate AGEs: Maillard reaction, oxidation of glucose, lipid peroxidation and polyol pathway (Luevano-Contreras & Chapman-Novakofski, 2010). On the other hand, Yeh *et al.* (2016) stated that methylglyoxal is the most potent glycating agent for the formation of AGEs during Maillard reaction. Methylglyoxal is one of the most highly reactive carbonyl species (RCS) in plasma. Methylglyoxal is related to many chronic metabolic diseases, whereby the titre of methylglyoxal in plasma is observed to be significantly higher in diabetic patients compared to the non-diabetic patients.

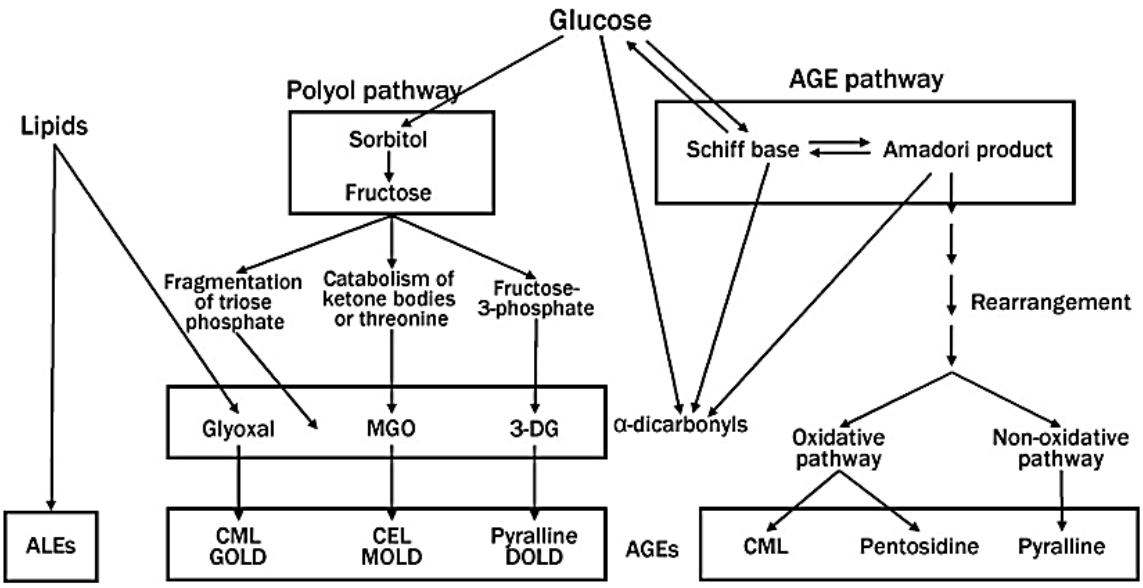

Figure 2.3: Possible pathways for production of AGEs (Luevano-Contreras & Chapman-Novakofski, 2010)

2.2.1 Initiation Phase

In initiation phase, the carbonyl group of reducing sugars (glucose, fructose, and ribose) will attach to amino groups (mainly lysine and arginine) of proteins to form a Schiff base. It is can also be formed through polyol pathway. A Schiff base is an unstable compound characterised by a formation of double bond between a carbon and nitrogen where the nitrogen is not connected to hydrogen. Next, the unstable Schiff base undergoes Amadori rearrangement to produce more stable ketoamines known as Amadori products, in which they can generate free radicals and irreversibly crosslink with proteins and peptides (Khan *et al.*, 2023). To add on, haemoglobin A1c (HbA1c) is the most well-known Amadori product in diabetic study. Generally, the initiation phase depends on glucose concentration and it happens within hours (formation of Schiff base) or days (formation of Amadori product). This phase is reversible if the glucose concentration reduces (Luevano-Contreras & Chapman-Novakofski, 2010).

2.2.2 Propagation Phase

In propagation phase, Amadori products undergo further chemical modifications (such as dehydration and degradation) in the presence of transitional metal ions to produce active carbonyl intermediate groups known as dicarbonyls which are the precursors for synthesis of AGEs at an advanced stage of Maillard reaction. Furthermore, glucose and Schiff base in the previous initiation phase can be converted and stored into dicarbonyls, which are known as carbonyl stress, have capacity to interact with amino and sulfhydryl groups of proteins resulting in browning and crosslinking (Khan *et al.*, 2023). Moreover, hydroxymethylfurfural (HMF) fission products including pyruvaldehyate and diacetyl are also produced in this phase. This phase can be exhibited as slight yellow or colourless (Tamanna & Mahmood, 2015).

2.2.3 Advanced Phase

The advanced phase is the last phase of Maillard reaction, it is irreversible and takes place in several weeks to months. The dicarbonyls undergo a series of chemical modifications such as oxidation, non-oxidation, hydration, dehydration, glycation, glycosylation, fructosylation and acid hydrolysis to generate stable and irreversible AGEs. For illustration, the AGEs are 3-deoxyglucosone lysine dimer (DOLD), glyoxal lysine dimer (GOLD) and methylglyoxal-derived imidazolium crosslinking (Khan *et al.*, 2023). The AGEs are characterised by thermally stable compounds, some of them have fluorescent properties (Yeh *et al.*, 2016). In this phase, aldol condensation occurs and eventually forming brown coloured heterocyclic nitrogenous compounds, melanoidins (Tamanna & Mahmood, 2015).

2.3 Interaction Between AGEs and Its Receptor (RAGE)

Above 20 different AGEs have been investigated in human blood, tissues and even in food. AGEs can be roughly categorised into 2 types, fluorescent and non-fluorescent AGEs. The well-known fluorescent AGEs including pentosidine and methylglyoxal-lysine dimer (MOLD), whereas the important non-fluorescent AGEs including carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL) and pyrraline. The AGEs have shared a common characteristic which is the presence of lysine residue in their molecular structure. When the AGEs production is beyond the normal baseline, the effective mechanism of AGEs detoxification will be activated to overcome the inequivalence of body AGEs. However, an exacerbated accumulation of AGEs brings cumulative metabolic burden, inflammation and oxidative stress (Perrone *et al.*, 2020). In fact, these negative implications are associated with the interaction between AGEs and their receptors (RAGEs).

RAGE is first defined as a receptor of AGEs, it is later described as a multiligand receptor that recognises a pattern or general motif (Tan *et al.*, 2007). RAGE, is an approximately 45-kDa protein, belongs to a multiligand member of immunoglobulin superfamily. It is composed of three extracellular domains, which include a variable (V) immunoglobulin-like domain and followed by two constant domains (types C and C′); and a single transmembrane domain followed by a cytosolic tail as shown in Figure 2.4. The N-terminus of V domain is responsible for the ligand-binding site, while the cytosolic tail is responsible for RAGE-induced intracellular signalling. In addition, there are four known types of RAGE, including full-length RAGE, N-truncated RAGE, and C-truncated RAGE, in which the C-truncated RAGE has two isoforms, secretory RAGE (sRAGE) and endogenous secretory RAGE (esRAGE) (Prasad *et al.*, 2017).

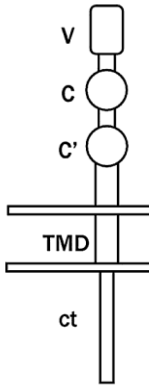

Figure 2.4: Structure of RAGE (Prasad *et al.*, 2017). V is variable immunoglobulin-like domain; C and C′ are constant domains respectively; TMD is transmembrane domain; and ct is cytoplasmic tail.

Furthermore, according to Perrone *et al.* (2020), RAGE is widely expressed in many cell types (such as endothelial cells, neurons, microglia, smooth muscle cells and macrophages) and various tissues (such as heart, lungs, skeletal muscle and vessel wall). RAGEs generally are expressed at basal levels under healthy condition, but the expression of RAGEs is upregulated under pathological conditions such as DM, cardiovascular disease, Alzheimer's disease, cancer and natural senescence. The statement is supported by Prasad *et al.* (2017) where RAGE can interact with numerous ligands, enrolling its role in the aetiology of chronic diseases related with cellular stress and inflammation. To illustrate, accounted in AGEs, other ligands such as amyloid-β peptide which found accumulated in Alzheimer's disease, and amyloid A which found accumulated in systemic amyloidosis.

Move on, RAGE is a common signal transduction receptor for CML, which is the significant non-fluorescent AGE *in vivo*, it is also interacting with other AGEs as well. The AGEs only bind to the V domain of RAGE, inducing a sustained period of cellular activation which can lead to inflammation. The activation of RAGE is strongly responsible for the pathogenicity related with AGEs. Besides, RAGE is highly expressed in certain cells during diabetes and inflammation. The interaction of AGEs and RAGE on macrophages can activate p21ras and mitogen-activated protein (MAP) kinase signalling pathway, resulting in production of oxidative stress and activation of nuclear factor -κB (NF-κB). The NF-κB is capable to regulate gene transcription for endothelin-1, tissue factor and formation of pro-inflammatory cytokines such as interleukin-1α (IL-1α) and tumour necrosis factor-α (TNF-α). During glycoxidative stress, the NF-κB mediates formation of TNF-α which causes additional reactive oxygen species (ROS) generation. Notably, ROS is significantly participated in the pathogenesis of T2DM, neurodegenerative and cardiovascular diseases. The direct exposure of endothelial cells to hyperglycaemic condition elevates the formation of ROS, which in turn activates ROS-producing NADPH oxidase (Singh *et al.*, 2014). As a result, the interplay between AGEs and RAGE promotes the RAGE expression in a positive feed-forward loop, which apparently contributing to various pathophysiologies (Rungratanawanich *et al.*, 2021).

2.4 Role of AGEs in Pathogenesis of Diabetic Complications

AGEs are the final metabolites of non-enzymatic glycation between reducing sugars and amino groups in proteins, lipoproteins and nucleic acids. *In vivo* accumulation of AGEs has played a significant role in pathogenesis of DM and its complications such as diabetic atherosclerosis, neuropathy, nephropathy, retinopathy and cataract. Additionally, AGEs are related to other health issues including Alzheimer's disease and normal senescence (Gutierrez, 2012). Furthermore, according to Singh *et al.* (2014), non-enzymatic glycation of plasma proteins such as albumin and fibrinogen might form various defective implications including alteration in drug binding in the plasma, platelet activation, production of ROS, impairment in immune system regulation. Besides, AGEs can function as cross-linkers between proteins, leads to formation of proteinase-resistant aggregates that increase the difficulty to remove them.

2.4.1 Diabetic Cardiomyopathy

Luevano-Contreras and Chapman-Novakofski (2010) desrcibed that *in vivo* accumulation of AGEs over time leads to alteration in the structure and function of cardiovascular system, demonstrates as arterial stiffening, myocardial relaxation abnormalities, formation of atherosclerotic plaque and endothelial dysfunction. The study stated that one of the mechanisms for the alterations is additional cross-linking on collagen by glycation of local free amino acids in which the normal structure of such collagen already consists of cross-linking. The collagen-AGEs cross-linking contributes to the stiffness of vasculature. The statement is supported by Singh *et al.* (2014) where the study described that AGEs increases the degree of cross-linking beyond the basal physiological levels. The AGEs can interact and cross-link with matrix proteins such as collagen, laminin, vitronectin and elastin. Such additional cross-linking greatly interrupts the flexibility property of the matrix protein, resulting in rigidness of matrix proteins, in turn decreases cardiac contractility and triggers diastolic dysfunction in heart. On the other hand, another mechanism contributes to cardiomyopathy is AGEs-mediated reduction of low-density lipoprotein (LDL) uptake by cellular receptors. The condition happens through the glycation of LDL on the apolipoprotein B and in the phospholipid components of LDL. After that, it is found that the glycated LDL is more likely to cross-linking with the collagen on the arterial wall compared to non-glycated LDL, additionally, it is not taken up into the cells and accumulates in the vessel lumen. The macrophages then taken up the glycated LDL leads to foam cell formation and the establishment of atheroma (Luevano-Contreras & Chapman-Novakofski, 2010).