INVESTIGATING THE ANTIOXIDANT POTENTIAL, TOXICITY, AND HYPOCHOLESTEROLEMIC EFFECTS OF KOP NUT IN A HIGH-FAT DIET-INDUCED TYPE 2 DIABETES RAT MODEL.

NUR ZAKIAH AMANI BINTI ZAMZURI

2023

INVESTIGATING THE ANTIOXIDANT POTENTIAL, TOXICITY, AND HYPOCHOLESTEROLEMIC EFFECTS OF KOP NUT IN A HIGH-FAT DIET-INDUCED TYPE 2 DIABETES RAT MODEL.

by

NUR ZAKIAH AMANI BINTI ZAMZURI

Dissertation submitted in partial fulfillment of

the requirements of the degree of

Master of Science (Biomedicine) Mixed Mode

AUGUST 2023

ACKNOWLEDGEMENT

I begin my acknowledgement with 'In the name of Allah, the Most Gracious the Most Merciful'. First and foremost, Alhamdulillah all praise to Allah, the Almighty for providing me with this opportunity and granting me the capability to proceed successfully. With the help and encouragement of many people, this thesis has been completed on schedule. Therefore, I would like to offer my utmost sincere thanks to all of them.

First of all, I would like to express my sincere gratitude to my supervisor, Associate Prof Dr. Wan Amir Nizam bin Wan Ahmad, for his great suggestions, wonderful guidance, ongoing encouragement, and unwavering support in making this research possible. I also would like to thank my co-supervisor, Dr Liza Noordin, as well as Dr Noraini Abdul Ghafar and Dr Rafidah Husen. They also had taken a lot of effort to meticulously go through my thesis and came up with helpful suggestions.

Besides, I am extremely indebted to my partners for this research, Liyana Nursyahirah binti Johari and Shahrina binti Shah Jahan, for their cooperation and suggestion throughout this research study. I also would like to extend my gratitude to all my lecturers, lab mates, staff members of the Faculty of Health Science Laboratory, and the Animal Research and Service Center (ARASC) for their kindness, excellent cooperation, and inspirations in many ways throughout this research study. Finally, I would like to express my heartfelt gratitude to my loving parents and siblings for their love, dream, and sacrifice throughout my life. I cannot find the appropriate words that could properly convey my appreciation for their devotion, support and faith in my ability to attain my goals.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

%	Percentage
°C	Degree Celsius
µg/mL	Microgram per mililitre
µL	Microlitre
ACC1	Acetyl-CoA carboxylase 1
ACC2	Acetyl-CoA carboxylase 2
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ARASC	Animal Research and Service Centre
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
BMI	Body mass index
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPX	Distyrene, plasticizer, and xylene
FDA	Food and Drug Administration
FFA	Free fatty acid
GDF15	Growth differentiation factor 15
GFRAL	GDNF family receptor alpha-like
GLUT2	Glucose transporter 2

H&E	Hematoxylin and eosin
HCl	Hydrogen chloride
HFD	High-fat diet
HIV-1	Human immunodeficiency virus 1
HIV-2	Human immunodeficiency virus 2
HMGR	Hydroxy-methylglutaryl-CoA reductase
kg/m^2	Kilogram per square metre
mg/kg	Milligram per kilogram
mmol/L	Milimoles per litre
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NHMS	National Health and Morbidity Survey
OECD	Organisation for Economic Cooperation and Development
ROS	Reactive oxygen species
rpm	Revolution per minute
SD	Sprague Dawley
SEM	Standard error of the mean
STZ	Streptozotocin
T1DM	Type-1 diabetes mellitus
T2DM	Type-2 diabetes mellitus
UV	Ultraviolet

PENYIASATAN POTENSI ANTIOKSIDAN, TOKSISIITI, DAN KESAN HIPOKOLESTEROLEMIK KOP NUT DALAM MODEL TIKUS DIABETES JENIS 2 DIABETES DIET TINGGI LEMAK

ABSTRAK

Diabetes mellitus Jenis-2 (T2DM) kebiasaannya berkait dengan obesiti, peningkatan pengeluaran asid lemak bebas dan rintangan insulin. Dalam kajian ini, ketoksikan dan potensi terapeutik *Ostodes pauciflora* Merr atau ekstrak minyak kacang Kop sebagai agen penurun kolesterol dalam model tikus T2DM, telah diterokai. Aktiviti antioksidan bagi berbagai jenis ekstrak minyak kacang Kop telah dinilai menggunakan ujian DPPH. Ia menunjukkan peratusan perencatan radikal DPPH yang tinggi pada kepekatan terendah (10 µg/mL) ekstrak dietil eter pada 61.44%, diikuti oleh ekstrak petroleum eter pada 60.58%, dan terakhir, ekstrak heksana dengan 59.33%. Dalam ujian ketoksikan akut, setiap lima (n = 5) ekor tikus jantan Sprague Dawley (SD) telah diberi ekstrak minyak kacang Kop pada dos yang berbeza dan ia diberi hanya sekali sahaja sepanjang tempoh kajian mengikut protokol OECD 425. Berdasarkan pemerhatian ini, ekstrak kacang Kop dianggarkan mempunyai dos toksik melebihi 2000 mg/kg. Dalam kajian rintis ini, dua belas (n = 12) ekor tikus SD jantan telah digunakan dalam kajian subakut untuk menilai sifat penurun kolesterol oleh ekstrak minyak kacang Kop. Selepas memberi rawatan selama 4 minggu, keputusan menunjukkan semua kumpulan tikus tiada perubahan ketara dalam paras glukosa dan paras kolesterol darah. Selain itu, pemeriksaan histologi menunjukkan pemulihan sel hepatik dengan seni bentuknya yang lebih baik dalam kumpulan Metformin dan Ekstrak jika dibandingkan dengan kumpulan Tidak Dirawat

(tikus diabetes), seperti berkurangnya pembentukan macrovesikular dan mikrovesicular. Dapatan menunjukkan bahawa ekstrak minyak kacang Kop mempunyai potensi antioksidan dengan LD50 melebihi 2000 mg/kg pada masa yang sama menunjukkan penambahbaikan dalam histologi hepatik.

INVESTIGATING THE ANTIOXIDANT POTENTIAL, TOXICITY, AND HYPOCHOLESTEROLEMIC EFFECTS OF KOP NUT IN A HIGH-FAT DIET-INDUCED TYPE 2 DIABETES RAT MODEL

ABSTRACT

Type-2 diabetes mellitus (T2DM) is usually associated with obesity, with the increased production of free fatty acids and insulin resistance. In this study, the toxicity and therapeutic potential of *Ostodes pauciflora* Merr or Kop nuts oil extract as a cholesterol-lowering agent in a T2DM rat model, was explored. The antioxidant activity of different Kop nut oil extracts was evaluated using a DPPH assay. It revealed high inhibition percentage of DPPH radical at the lowest concentration (10 µg/mL) where diethyl ether extract inhibits at 61.44%, followed by petroleum ether extract at 60.58%, and lastly, hexane extract with 59.33%. In acute toxicity, each five (n = 5) male Sprague Dawley (SD) rats were given different dose of Kop nut oil extract and were administered once only throughout the study according to the OECD 425 protocol. Based on these observations, the toxic dose of Kop nut extract is estimated to exceed 2000 mg/kg. In this pilot study, twelve (n = 12) male SD rats were used in the subacute study to evaluate the cholesterol-lowering properties of Kop nut oil extract. After giving treatment for 4 weeks, all groups show no significant changes in blood glucose and cholesterol levels. Other than that, the histological examination shows restoration of hepatic cells with an improved architecture in the Metformin and Extract group compared to the Untreated group (diabetic rats), such as less formation of macrovesicular and microvesicular. The finding

indicates that Kop nut oil extract poses an antioxidant potential with LD50 above 2000 mg/kg while showing improvement in liver histology.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Obesity is characterized by excess body fat accumulation in adipose tissue which is also intertwined with cholesterol level that collectively impacts cardiovascular health and overall well-being. In 2022, it was stated by the World Health Organization (WHO) that the number of people worldwide who suffer from obesity is more than 1 billion and it is estimated that the number will escalate by 2025 (WHO, 2022). The excess adipose tissue, a hallmark of obesity, contributes to increasing circulating free fatty acids in the blood which can be associated with health problems such as hypercholesterolemia, dyslipidemia, and cardiovascular disease (Juanola et al., 2021; Liang et al., 2022). Furthermore, excess fat cells also contribute to chronic low-grade inflammation that further causes insulin resistance and promotes diabetes (Yaribeygi et al., 2020).

Diabetes mellitus is a complex and prevalent metabolic disorder that has garnered significant attention in medical research. Type-2 diabetes mellitus (T2DM) is usually known to be associated with obesity, contributing to 90% of diabetes mellitus patients (Reed et al., 2021). It is characterized by elevated blood glucose levels brought on by insufficient insulin production or ineffective insulin use, posing a significant global health challenge (Oguntibeju, 2019). Diabetes prevalence is predicted to increase by 12.2% by 2045 from the current global prevalence of 10.5% in 2021 (International Diabetes Federation, 2021). The correlation between T2DM and obesity not only can be accounted

for by the associations between obesity and insulin resistance, but also by the apoptosis of pancreatic beta-cells (Oguntibeju, 2019).

The intricate relationship between these two conditions amplifies the blood cholesterol level imbalance that contributes to metabolic problems such as hyperlipidemia, dyslipidemia, and hyperglycemia (Oguntibeju, 2019; Reed et al., 2021). This also contributes to T2DM-related oxidative stress which plays a pivotal role in the development of various health complications such as non-alcoholic fatty liver disease (NAFLD) (Mishra et al., 2012; Oguntibeju, 2019). These health conditions amplify the risk of NAFLD and the transformation of non-alcoholic steatohepatitis (NASH) into severe liver complications, as they share underlying mechanisms and metabolic disturbances (Oguntibeju, 2019).

Studies involving plants in the management of T2DM have drawn a lot of attention, as scientists investigate the possible medical advantages of phytochemicals in controlling and preventing disease. Numerous plants, including herbs, spices, fruits, and vegetables, have been investigated for their bioactive components that may influence glucose metabolism, lipid metabolism, insulin sensitivity, and inflammation (Unuofin & Lebelo, 2020). These studies have revealed the presence of phytochemicals like polyphenols, flavonoids, and alkaloids with potential antioxidants, antihyperlipidemic, and antidiabetic properties (Unuofin & Lebelo, 2020).

In this study, we explore the potential of Kop nuts, which originated from a plant with the scientific name *Ostodes pauciflora* Merr. and can be found in Sarawak. Despite being

consumed by the locals, this safety profile of the plant has not been evaluated and there is a lack of study regarding its therapeutical potential (Xiao, 2020; Sakai et al., 2022). The Kop nut oil extracts were obtained from our collaborators in Universiti Teknologi MARA (UiTM) Sarawak. These include the various oil extractions of Kop nuts, including petroleum ether extract, hexane extract and diethyl ether extract. These extracts were assessed for their antioxidant properties. Then, it was followed by an *in vivo* study where selected Kop nut oil extract was assessed on their toxicity and cholesterol-lowering properties in T2DM animal model.

1.2 Problem Statement

Among 3 major risk factors for non-communicable disease, Malaysian are highly risk to high blood cholesterol levels according to National Health and Morbidity Survey (NHMS) 2019 (National Institutes of Health Malaysia, 2020). Furthermore, about 8 million adults in Malaysia have raised their total cholesterol level and this is equal to 4 out of 10 people will have high cholesterol level (National Institutes of Health Malaysia, 2020). Increase in cholesterol levels is strongly related to diabetes mellitus due to insulin resistance caused by accumulation of cholesterol and fats in adipose tissue which increases blood glucose levels (Oguntibeju, 2019). A report on the Malaysian health care expenses of 2017 reveals that diabetes was a chronic disease with the largest health care spending with an estimated yearly cost of RM 4.4 billion (Zainuddin & Su-Lyn, 2022). This cost will increase by year because of an increase in obesity and diabetic prevalence among Malaysians from 2011 to 2019 as reported by NHMS 2019 (National Institutes of Health Malaysia, 2020).

The drug used in diabetic treatment such as metformin, was reported to have cholesterol-lowering properties (Rena et al., 2017). However, some diabetic drugs pose limitations of usage as they are not suitable for certain people, such as patients with chronic kidney disease and people prone to lactic acidosis (Corcoran & Jacobs, 2023). This also includes some drugs required for long-term usage and some are only effective for a short time (Reed et al., 2021). Diabetic drugs also pose a various of adverse effects which can range from nausea, vomiting, diarrhea to hypoglycemia and risk for cancer (Reed et al., 2021). In short, the cost of treatment will increase as the prevalence of obesity and diabetes increases. Despite treating diabetes and reducing cholesterol levels, some diabetic drugs pose various limitations and adverse effects. Considering the issues mentioned, an investigation on medicinal plants has been conducted to look for optional diabetic treatment that might reduce cholesterol levels, treat diabetes affordably, effectively and with less adverse effects (Unuofin & Lebelo, 2020).

1.3 Rational of Study

As far as we know, although the Kop plant is reported to have potential antioxidants and antimicrobial properties, there is scarce information regarding its toxicity, and ability to lower cholesterol in T2DM (Sakai et al., 2022). In order to produce new plant-based anti-diabetic medication, it must be safe, and the substance is not likely to be hazardous when used as intended, taking into consideration any accumulative effects on consumers and their probable consumption (U.S. Food and Drug Administration, 2000). Furthermore, there is a strong correlation between fats and diabetes because insulin resistance is linked to increased circulating fatty acid levels and fatty acid production in the liver which could lead to cholesterol-related health complications (Oguntibeju, 2019; Reed et al., 2021). T2DM also leads to an increase in oxidative stress that induces most diabetic-related complications, which makes it important to assess its antioxidant potential (Oguntibeju, 2019). Therefore, current research was aimed at investigating the antioxidant, toxicological, and cholesterol-lowering potential of Kop nut oil extract in in T2DM rat model.

1.4 Objective

General Objective

To study the toxicity, antioxidant, and therapeutic effect of Kop nut oil extract as a cholesterol-lowering agent in a Type-2 diabetes mellitus (T2DM) rat model.

Specific objective:

1. To determine the acute toxicity of Kop nut oil extract *in vivo* (OECD 425).
2. To determine the antioxidant property of Kop nut oil extract *in vitro* using 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay
3. To evaluate the effects of Kop nut oil extract on cholesterol profile in T2DM rats.
4. To determine the Kop nut oil extract effects on the histology of the liver in T2DM rats.

1.5 Hypothesis

The study hypothesis is that the Kop nut's oil extract has antioxidant and has cholesterol-lowering properties in the T2DM rat model with less toxicity effects.

Specific hypothesis:

1. Kop nut oil extract is less toxic.
2. Kop nut oil extract does have antioxidant property.
3. Kop nut oil extract does improve cholesterol profile in T2DM rats.
4. Kop nut oil extract does improve the histology of the liver in T2DM rats.

1.6 Significant of the Study

The significance of studying the effects of Kop nut extract treatments on T2DM lies in the potential to uncover novel and sustainable therapeutic approaches that can alleviate the burden of this chronic condition. Uncovering the Kop nut therapeutical potential for lowering cholesterol opens new ways to reduce circulating fatty acid that is associated with obese-induced insulin resistance. Plus, findings on the Kop plant antioxidant properties are useful to alleviating the complications related to oxidative stress caused by obesity and diabetes. The toxicological outcomes will determine whether the Kop nuts can be consumed safely in higher doses. These hold promises for creating a cost-effective complementary intervention that could improve obesity and diabetes by lowering cholesterol levels, reducing oxidative stress and less adverse effects.

CHAPTER 2

LITERATURE REVIEW

2.1 Cholesterol, Obesity and Type-2 Diabetes Mellitus

2.1.1 Cholesterol

Cholesterol is an organic compound that consists of a hydrophilic part, a hydrophobic part, and four rings structure (Figure 2.1) (Schade et al., 2020). It has an important effect on human health such as the production of steroid hormones, preventing miscarriage, essential vitamin distribution, and membrane cell permeability (Schade et al., 2020). Other than food, the source of cholesterol or lipids in our body comes from the liver through the production of fatty acids by *de novo* lipogenesis of acetyl-CoA and malonyl-CoA that originate from carbohydrates (Schade et al., 2020; Geng et al., 2021).

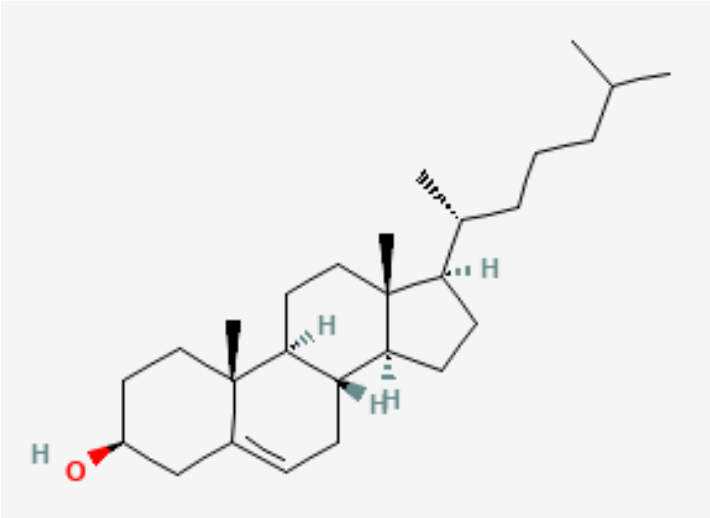

Figure 2.1 Chemical structure of cholesterol (National Center for Biotechnology Information, 2023)

Cholesterol in the body can also be associated with various health problems and metabolic issues. Reports from National Health and Morbidity Survey (NHMS) 2019 stated that 38.1% of Malaysians have hypercholesterolemia, and 24.6% do not know that they have hypercholesterolemia where the total cholesterol is at or above 5.2 mmol/L (National Institutes of Health Malaysia, 2020). Plus, the elevated level of low-density lipoprotein (LDL) and decrease of high-density lipoprotein (HDL) cholesterol appear to be related to a higher risk of cardiovascular disease (Juanola et al., 2021). In addition, abnormal levels of cholesterol become indicators for metabolic diseases such as dyslipidemia which is associated with the alteration in the levels of very low-density lipoprotein (VLDL), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein (HDL) (Juanola et al., 2021).

2.1.2 Obesity

Excessive accumulation of fats in the body is highly associated with obesity as excess energy from food consumption and lack of activities lead to the storing of energy in fat cells (Reed et al., 2021; Liang et al., 2022). Obesity is a non-communicable disease that can be treated or avoided but is prone to recurrence and can be determined with body mass index (BMI) value of more than 30 (kg/m^2) or using a waist-to-hip ratio (World Health Organization, 2021). When the adipose tissue already reaches its limit, lipids are stored in non-adipose tissue, also known as ectopic fats, like muscle, liver, and pancreas (Godoy-Matos et al., 2020; Tomic et al., 2022).

According to the WHO (2021), the prevalence of obesity has tripled from 1975 to 2016, and about 13% of adults across the globe are obese while another 39% are overweight. In

addition, several non-communicable diseases, such as cardiovascular disease, diabetes, musculoskeletal disorders, and some cancers, are associated with an increase in BMI (WHO, 2021). In Malaysia, the prevalence of obesity according to NHMS 2019 has increased from 17.7% (2015) to 19.7% (2019) while abdominal obesity has increased from 48.6% (2015) to 52.6% (2019) (National Institutes of Health Malaysia, 2020). The increase in obesity prevalence will also cause an increase in obesity incidents.

Accumulation of fats and lipids in obesity promotes insulin resistance, a condition where the insulin-induced cell does not react well to insulin, resulting in reduced glucose uptake from blood (Oguntibeju, 2019). As lipids accumulate in adipose tissue and ectopic fats, it produces a low-intensity inflammation that triggers macrophage infiltration in the tissue more than usual, and this cycle continues as the macrophages secrete more inflammatory factors that attract more macrophages (Oguntibeju, 2019; Liang et al., 2022). Other than inflammatory factors, macrophages also produce reactive oxygen species (ROS) that alter adipocyte metabolism and increase the production of free fatty acids (FFA), thus promoting insulin resistance and lipotoxicity (Liang et al., 2022). The effect of insulin resistance is also enhanced by macrophages through the secretion of exosomes and activation of inflammasomes due to ROS-induced oxidative stress (Liang et al., 2022). With the increase in insulin resistance, less glucose uptake in cells contributes to T2DM (Oguntibeju, 2019).

Furthermore, the formation of ectopic fats in specific organs leads to organ-specific insulin resistance (Tomic et al., 2022). In the pancreas, the accumulation of fat and the effect of insulin resistance disrupts mitochondrial respiratory chains and further increases the

production of NADPH oxidase that causes oxidative stress, thus resulting in beta-cell dysfunction, which contributes to reduced insulin production (Yaribeygi et al., 2020; Tomic et al., 2022). The onset of dysfunction of pancreatic beta-cells and increase in insulin resistance in obese leads to a spike in blood glucose levels which is associated with T2DM (Oguntibeju, 2019; Tomic et al., 2022). Besides, about 80% of T2DM patients are either overweight or obese, which indicates a strong association between T2DM and obesity (Reed et al., 2021; Liang et al., 2022).

2.1.3 Type-2 Diabetes Mellitus

Diabetes mellitus (DM) is a disease with multiple factors that are related to a disorder in carbohydrate, fat, and protein metabolism, which results in the increase of blood glucose (hyperglycemia) and lipid (hyperlipidemia) (Oguntibeju, 2019). Type-1 diabetes mellitus (T1DM) which is characterized by the inability of the pancreas to produce insulin as they lack about 90% of pancreatic beta-cell due to autoimmune reaction (Reed et al., 2021). While T1DM consists of 10% of DM patients, another 90% consist of Type-2 diabetes mellitus (T2DM) that lacks insulin secretion due to pancreatic beta-cell dysfunction together with insulin resistance (Oguntibeju, 2019; Yaribeygi et al., 2020).

Diabetes mellitus is one of the non-communicable diseases listed as the third highest risk factor for premature death and it is estimated that more than 590 million people will be diagnosed with T2DM by 2035 (Oguntibeju, 2019; Reed et al., 2021). According to International Diabetes Federation (IDF) (2021), the global prevalence of diabetes is 10.5% in 2021 and is estimated to increase to 12.2% by 2045 with 94% of the increase contributed by low and middle-income countries. In Malaysia, NHMS 2019 shows the prevalence of

high blood glucose associated with diabetes (18.3%) is higher in 2019 compared to high blood glucose in 2011 (11.2%) (National Institutes of Health Malaysia, 2020).

Diagnosis of diabetes comes with blood glucose analysis, which poses specific symptoms. A person is considered pre-diabetes when the fasting blood glucose is between 6.1-6.9 mmol/L or 2 hours post-prandial blood glucose between 7.8-11 mmol/L, even though they pose no symptoms (Reed et al., 2021). However, once the fasting blood glucose exceeds 7 mmol/L or 2 hours post-prandial blood sugar exceeds 11 mmol/L, the person is diagnosed with DM (Reed et al., 2021). In general, diabetes poses various symptoms, including dehydration, fatigue, infection susceptibility, excessive urination, weight loss, disturbances in metabolism, and vision impairment. (Prabhakar et al., 2014). Also, diabetes leads to high blood glucose and increased lipid or fats in the blood which are linked to hyperglycemia and hyperlipidemia, respectively (Oguntibeju, 2019; Reed et al., 2021).

In obese T2DM patients, insulin levels are higher compared to T1DM, but the presence of insulin resistance leads to reduced glucose uptake causing blood glucose levels to increase which is associated with hyperglycemia (Yaribeygi et al., 2020; Noordin et al., 2022). Obesity with a sedentary lifestyle requires less ATP in muscle, which means less glucose uptake for ATP generation, which results in enhanced insulin resistance, impairs fat oxidization, and increased fat storage (Reed et al., 2021). Insulin resistance also facilitates free fatty acids (FFA) production and results in an increase of FFA in the blood causing dyslipidemia, especially when there is impairment in fat uptake in the liver (Oguntibeju, 2019; Al-Harbi et al., 2021). Finally, the uptake of fats in the liver causes hepatic insulin

resistance due to fat accumulation, and this results in increased glucose production from gluconeogenesis (Godoy-Matos et al., 2020). Despite higher insulin levels, the blood glucose level is high due to reduced glucose uptake caused by insulin resistance and increased production of glucose in the liver by gluconeogenesis of fats.

2.2 Oxidative Stress in Type-2 Diabetes Mellitus

Normal cellular metabolism will produce highly unstable molecules or free radicals, which is important as they involve various molecular pathways such as cellular signaling, synaptic plasticity, apoptotic processes and defense mechanism against infection (Yaribeygi et al., 2020). However, a high amount of these radicals exceeding the defense mechanism of a cell will cause damage to the cell, which is called oxidative stress (Oguntibeju, 2019). Oxidative stress is highly related to T2DM as it is involved in its pathogenesis. In obesity, lipolysis of adipose tissue produces reactive oxygen species (ROS) which causes oxidative stress that results in inflammation, pancreatic beta-cell dysfunction, and insulin resistance, which leads to T2DM (Oguntibeju, 2019; Liang et al., 2022).

The presence of diabetes and hyperglycemia further increases the production of free radicals (Tomic et al., 2022). In hyperglycemia, glucose is reduced to sorbitol by aldose reductase and NADH is generated in the process which results in ROS production (Oguntibeju, 2019). High blood glucose levels allow the formation of advanced glycation end-products (AGEs) that are involved in inducing oxidative stress (Oguntibeju, 2019). Such oxidative stress leads to cell damage that includes damage to the deoxyribonucleic

acid (DNA) and impairs mitochondrial function, which contributes to ROS production (Yaribeygi et al., 2020).

Further increases in oxidative stress are known to involve diabetic-induced complications. Oxidative stress contributes to the progression of non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH) as it induces cell death or cell injury in the liver (Oguntibeju, 2019; Juanola et al., 2021). The formation of inflammation due to oxidative stress leads to damage to retina blood vessels and light-sensitive tissue in the eye causing visual impairment, also known as diabetes retinopathy (Oguntibeju, 2019). Other than that, damage caused by oxidative stress also leads to several other health problems including nephropathy, neuropathy, cancer, age-related disease, and cardiovascular disease (Mishra et al., 2012; Oguntibeju, 2019).

2.3 Non-Alcoholic Fatty Liver Disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is a disease that is caused by the accumulation of fats in the liver without the influence of alcoholism, where more than 5% of hepatocytes have accumulated with fat (hepatic steatosis) (Juanola et al., 2021). A variety of fatty liver diseases, including simple hepatic steatosis, non-alcoholic fatty liver, non-alcoholic steatohepatitis (NASH), and NASH cirrhosis, have been referred to collectively as NAFLD (Geng et al., 2021). NAFLD is usually asymptomatic, while NASH have symptoms such as steatosis, hepatocellular ballooning, lobular inflammation, and often fibrosis that causes liver complications like cirrhosis, hepatocellular carcinoma, hepatic decompensation, and liver-related death (Juanola et al., 2021; Powell et al., 2021).

The global prevalence of NAFLD increased from 25.3% to 38.2%, with the highest prevalence reported in Latin America, and the Middle East and North Africa (MENA) (Younossi et al., 2023). The prevalence of obesity and T2DM is rising along with NAFLD. About 51% of NAFLD and 82% of NASH patients are obese, while diabetes is present in 23% of NAFLD patients and 47% of NASH patients (Younossi et al., 2016). In addition, the overall incidence of NAFLD among T2DM patients is 55.5% worldwide, with Africa reporting the smallest percentage of 30.4%, followed by the United States with 51.8% (Younossi et al., 2019).

Obese-induced insulin resistance in T2DM aids in FFA production causing an increase of circulating FFA that is being delivered to the liver (Godoy-Matos et al., 2020; Liang et al., 2022). The formation of ectopic fats also causes insulin resistance in the liver that leads to reduced hepatic glycogen synthesis, increased glucose production (gluconeogenesis), and increased de novo lipogenesis, where fatty acid is produced from acetyl CoA (Godoy-Matos et al., 2020). The increase of fats in the liver leads to lipotoxicity which contributes to endoplasmic reticulum stress, mitochondrial dysfunction, hepatocyte damage, cell apoptosis and fibrosis in the liver (Godoy-Matos et al., 2020; Reed et al., 2021). Due to the regenerative nature of the liver, any injured cell will be replaced, thus contributing to NASH progression becoming fibrosis, cirrhosis and hepatocellular carcinoma (Juanola et al., 2021).

NAFLD can be diagnosed using blood biomarkers, imaging and liver biopsy assessment in individuals with at least 5% of hepatocytes infiltrated with steatosis (Powell et al., 2021). Blood biomarkers using a scoring system to analyze the liver enzyme such as serum

alanine aminotransferase for estimation of liver fibrosis and steatosis degree (Godoy-Matos et al., 2020; Powell et al., 2021). Furthermore, there are various diagnostic methods using imaging techniques such as ultrasonography, computed tomography, transient elastography, and magnetic resonance imaging (MRI) (Godoy-Matos et al., 2020). Ultrasonography is one of the imaging methods primarily used in diagnosis where hepatic steatosis can be observed with a bright liver echo texture and blurring of the hepatic vasculature (Powell et al., 2021). Another diagnosis method is a liver biopsy, an invasive method, where only patients with a specific condition are allowed. The liver biopsy can detect NAFLD progression to NASH by observation of histological features of steatosis, inflammation, hepatocyte ballooning and fibrosis (Godoy-Matos et al., 2020).

2.4 Streptozotocin (STZ)

Chemicals such as streptozotocin (STZ) have been used to induce diabetes in animal study. STZ is a nitrosourea that originated from *Streptomyces achromogenes* and has been used in diabetic induction, both T1DM and T2DM, in rat and mice studies (Zakaria et al., 2021; Furman, 2021). It is a deoxyribonucleic acid (DNA) alkylating agent with molecular formula $C_8H_{15}N_3O_7$ (Figure 2.2)

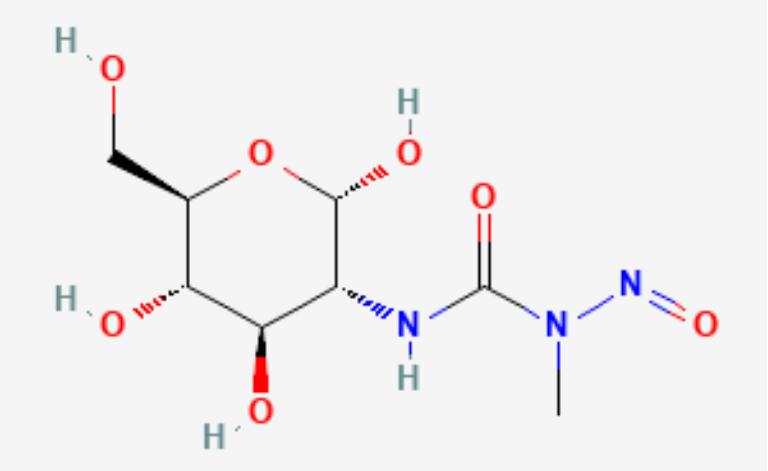

Figure 2.2 Chemical structure of streptozotocin (National Center for Biotechnology Information, 2023)

The administration of STZ in diabetic induction can be done intravenously or intraperitoneally. STZ can be administered with multiple low or single high doses to induce T1DM (Furman, 2021). On the other hand, administration of STZ with nicotinamide leads to T2DM condition without insulin resistance, while the addition of high-fat diet followed by a dose of STZ leads to T2DM condition with insulin resistance (Furman, 2021). STZ must be prepared at the time of the injection and injected immediately after being diluted with citrate buffer to avoid degradation due to its unstable characteristic (Furman, 2021).

This chemical induces diabetics in animals by targeting the pancreatic beta-cell. STZ enters beta-cells by binding to glucose receptor, GLUT2 (Zakaria et al., 2021). As the STZ in the beta-cell, it causes the fragmentation of DNA by alkylation (Zakaria et al., 2021). This causes damage to pancreatic beta-cells, which results in a condition similar to diabetes, where insulin secretion is decreased (Zakaria et al., 2021). Due to this

mechanism of action, STZ is harmful and carcinogenic to humans, which requires careful handling (Furman, 2021; National Center for Biotechnology Information, 2023).

Besides STZ, another chemical known to target pancreatic beta-cells in diabetic animal studies is alloxan (Zakaria et al., 2021; Dhamsaniya & Raval, 2023). Compared to STZ, alloxan causes a high mortality rate, which will likely to reverse diabetes into a non-diabetic state (Dhamsaniya & Raval, 2023). Plus, it has less selectivity toward pancreatic beta-cells compared to STZ (Furman, 2021; Dhamsaniya & Raval, 2023). Finally, STZ has many more advantages because it can induce both T1DM and T2DM. (Furman, 2021). Thus, due to many benefits, STZ is now frequently used in diabetic animal studies instead of alloxan (Furman, 2021).

2.5 Metformin

Metformin is a drug that was approved by the FDA in 1994 and used as first-line therapy to treat people with T2DM according to the American Diabetes Association (ADA) (Corcoran & Jacobs, 2023). The drug was derived from an organic substance used as an herbal remedy with a molecular formula of $C_4H_{11}N_5$ (Figure 2.3) (Rena et al., 2017). It is now widely used as a biguanide drug with various molecular mechanisms and sites of action for diabetes treatment (Rena et al., 2017). In diabetic treatment, metformin is usually prescribed as a monotherapy, either on its own or in conjunction with other glucose-lowering treatments such as insulin. Though recent studies reveal various potentials in metformin, it appears to be able to treat diabetes while also reducing fat and body weight (Kaneto et al., 2021).

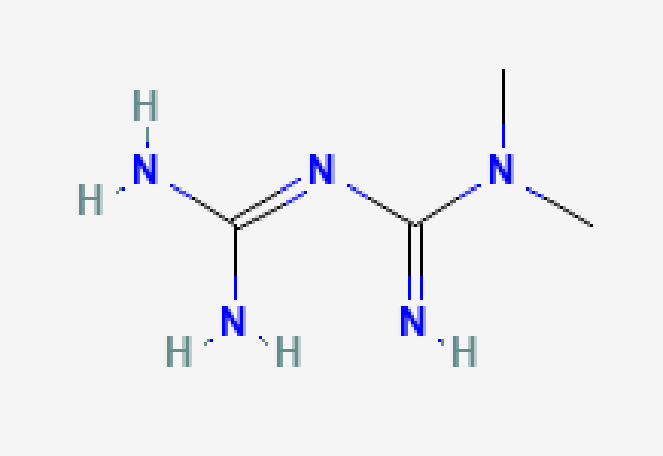

Figure 2.3 Chemical structure of metformin (National Center for Biotechnology Information, 2023)

As a biguanide medication, metformin inhibits the liver's ability to produce glucose in gluconeogenesis. Gluconeogenesis requires ATP to synthesize glucose from non-carbohydrates such as lactate and amino acids (Bhagavan & Ha, 2015). However, metformin affects gluconeogenesis by inhibiting Complex I in the mitochondria respiratory chain, thus reducing ATP production (Rena et al., 2017). This increases ADP: ATP and AMP: ATP ratios, which further inhibits gluconeogenesis through the activation of AMP-activated protein kinase (AMPK) (Rena et al., 2017). On the contrary, it is also suggested that metformin facilitates lysosome in a mechanism to activate AMPK, thus affecting gluconeogenesis (Rena et al., 2017).

The metformin-induced activation of AMPK in the liver will lead to the phosphorylation of both isoforms of acetyl-CoA carboxylase (ACC1 and ACC2) and hydroxy-methylglutaryl-CoA reductase (HMGR) (Schimmack et al., 2006). This leads to the oxidation of fatty acids and inhibits fat synthesis (Rena et al., 2017). Thus, metformin in

diabetes treatment can reduce body weight due to lipid metabolism in mitochondria (Diabetes Prevention Program Research Group, 2012). Hence, the amount of lipids will decrease resulting in the reduction of obesity-induced insulin resistance (Fullerton et al., 2013).

Plus, consuming metformin also affects body weight through the presence of growth differentiation factor 15 (GDF15), a peptide hormone that regulates food intake. GDF15 is produced when cells experience stress and inhibit appetite by attaching to a group of receptors in the hindbrain. A study in mice showed that metformin can induce GDF15 in various types of cells, reducing food intake and preventing weight gain (Coll et al., 2020). However, the effects can become less effective due to the lack of GDF15 and GDNF family receptor alpha-like (GFRAL) (Yang et al., 2017). The lack of GFRAL, a type of GDF15 receptor in the hindbrain, decreases the effects of GDF15, including the amount of food intake, body weight, fat mass and glucose homeostasis (Yang et al., 2017).

On the other hand, metformin has a common adverse effect involving gastrointestinal symptoms such as nausea and vomiting (Corcoran & Jacobs, 2023). Some people are at risk of lactic acidosis and are discouraged from consuming metformin. This includes those with liver disease, heart failure, and chronic kidney disease. Other groups of people who are at risk include the elderly, surgery patients, hypoxia and alcohol addicts. Plus, long-term and high doses of metformin may cause vitamin B12 deficiency in one's body, though scientists have not fully understood the mechanism (Infante et al., 2021).

Besides metformin, there are a few other treatments used for T2DM, such as insulin secretagogues and insulin therapy (Reed et al., 2021). Insulin-secretagogues drugs such as sulfonylureas and meglitinides promote insulin production in the blood. However, it also causes weight gain, is prone to hypoglycemia and is only effective for a short time (Reed et al., 2021). Insulin therapy or insulin injection is a daily treatment that also causes adverse effects such as increased weight, hypoglycemia and risk of colorectal cancer (Reed et al., 2021). However, these drugs are options and can be given when treatment using metformin and losing weight is not effective in treating T2DM (Reed et al., 2021).

2.6 Type-2 Diabetes Mellitus and Medicinal Plant

Humans and plants have a relationship that lasts from an early age as the plants and their products are fully integrated into human lives. Plants have a variety of bioactive compounds which make them used as medicine and these plants are called herbs or medicinal plants. Among the examples of medicinal plants known for their use in diabetes treatment are cinnamon (*Cinnamomum verum* and *Cinnamomum zeylanicum*), *Gymnema sylvestre*, and *Panax ginseng* (Unuoffin & Lebelo, 2020). There are also various studies conducted to seek any plants that have the potential for diabetes treatment (Unuoffin & Lebelo, 2020).

2.7 Kop Nut (*Ostodes pauciflora* Merr.)

Kop nut or ‘kacang Kop’ also called ‘buah merentik’ or ‘buah buantik’ by native speakers and originated from a plant that can be found in Sarawak Malaysia (Sakai et al., 2022). The plant’s scientific name is *Ostodes pauciflora* which is also synonym for *Dimorphocalyx pauciflorus,* and it belongs to the Euphorbiaceae family (Van Welzen &

Winkel, 2015). The fruit grows in pairs and has a light-yellow appearance that turns greyish as it ripens, with a light green interior and black round seeds that break open to reveal a white kernel (Figure 2.4) (Xiao, 2020). On top of that, the nut is also consumed by natives and has a creamy flavor with a hint of cashew and almond texture (Xiao, 2020; Sakai et al., 2022).

A study on Kop was conducted on the bark and leaves of the plant, which revealed antioxidant properties of the Kop plant with a 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition percentage is between 51.9% to 65.2% (Sakai et al., 2022). In addition, the total phenolic and flavonoid content was higher than *O. paniculata* (Sakai et al., 2022). Last but not least, Kop nut also poses antimicrobial activity which shows that chloroform and methanol extract of Kop nut has potent inhibition against *Streptococcus pyogenes, Escherichia coli*, and *Pseudomonas aeruginosa* (Sakai et al., 2022).

Figure 2.4 Kop nut. (a) Picture of Kop nut with its coating (Source from Xiao, 2020). (b) Picture of Kop nut taken during the laboratory process revealing its white inner.

Plants of Euphorbiaceae are rich in secondary metabolites like phenolics, flavonoids, alkaloids and other substances. For instance, *Plukenetia conophora* research shows the leaf's potential for use in herbal medicine because it contains vital vitamins, alkaloids, and minerals like potassium, sodium, magnesium, and calcium (Onawumi et al., 2013). On

top of that, the *P. conophora* nut contains phytochemicals like sterols and flavonoids that can be linked to hypolipidemic properties (Ajayi et al., 2020). Similarly, research findings demonstrate that the flavonols and phenol present in Euphorbiaceae plant family such as *Euphorbia cotinifolia* and *Euphorbia hirta* plant, provide antioxidant, antidiabetic, and antihyperlipidemic properties (Agrawal et al., 2020; Silalahi, 2021). In relation to that, traditional medicine uses *E. hirta* to treat ulcers, overcoming dysentery, digestive tract infections, skin infection, diabetes, respiratory disorder and gastrointestinal disorder (Silalahi, 2021).

Moreover, genus *Ostodes* which belongs to the family Euphorbiaceae is known for the three species that have been recognized namely *Ostodes paniculata, Ostodes katharinae,* and *Ostodes kuangii* (Van Welzen & Winkel, 2015). Findings from the study on *O. paniculata* show that it has anticancer agents like phorbol diester besides having other compounds such as acetylaleuritolic acid, stigma-4-en-3,6-dione, stearic acid, vanilin and octacosanol (Handa et al., 1983; Shuhua et al., 2004). On top of that, it was discovered that *O. katharinae* contains phorbol ester, which is helpful in preventing HIV-1 and HIV-2 replication in C8166 cells (Chen et al., 2017).

However, the pharmacological potential of *O. pauciflora* is not thoroughly explored. A study by Sakai et al. (2022) on the bark and leaves of the Kop plant is the only one that has been done so far. Based on the phytochemical profile of members of the Euphorbiaceae family, it is hypothesized that *O. pauciflora* may contain potent phytochemicals with a variety of bioactivities that have advantages and present new opportunities for pharmacological investigations.