THE EVALUATION OF KOPS NUT'S TOXICITY AND ANTIDIABETIC PROPERTIES: A PILOT STUDY

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

%	Percentage
°C	Degree Celsius
±	More or less
>	Greater than
<	Less than
g	Gram
g/cm²	Gram per Square Centimetre Pressure Unit
iu	International Unit
kcal	Kilocalories
mg	Milligrams
mg/kg	Milligram per Kilogram
mL	Millilitre
mmol/L	Millimoles per Litre
nm	Numbers represent wavelength
µg/mL	Microgram per Millilitre
µL	Microlitre
w/v	Weight in volume
ADA	American Diabetes Association
ARASC	Animal Research and Service Centre
BMI	Body Mass Index
BSLA	Brine Shrimp Lethality Assay
CDC	Centers for Disease Control and Prevention
CGM	Continuous glucose monitoring
CRP	C-Reactive Protein

DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DNSA	Dinitrosalicylic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-linked Immunosorbent Assay
FBG	Fasting Blood Glucose
FDA	Food and Drug Association
GAD	Glutamic acid decarboxylase
GLUT2	Glucose Transporter 2
GDM	Gestational Diabetes Mellitus
HbA1c	Glycated Haemoglobin A1c
HFD	High-Fat Diet
HIV-1	Human Immunodeficiency Virus 1
HIV-2	Human Immunodeficiency Virus 2
HLA	Human Leukocyte Antigen
IA-2	Islet antigen 2
IDF	International Diabetes Federation
IFG	Fasting Glucose Level
IFN-γ	Interferon-gamma
IGT	Impaired Glucose Tolerance
IL-1	Interleukin 1
IL-1β	Interleukin 1 Beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IP	Intraperitoneal

IR	Insulin Resistance
LT	Leukotriene
MIC	Minimum Inhibitory Concentration
MIF	Macrophage Migration Inhibitory Factor
MNU	Cytotoxic Nitrosourea
NHMS	National Health and Morbidity Survey
PBMCs	Peripheral Blood Mononuclear Cells
PPSK	Pusat Pengajian Sains Kesihatan
RBG	Random Blood Glucose
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAA	Serum Amyloid A
SD	Sprague Dawley
STZ	Streptozotocin
T1DM	Type-1 Diabetes Mellitus
T2DM	Type-2 Diabetes Mellitus
TFC	Total Flavonoid Content
TNF	Tumor Necrosis Factor
TPC	Total Phenolic Compound
UiTM	University Teknologi MARA
UPMS	Unit Pengurusan Makmal Sains
USM	University Sains Malaysia
WHO	World Health Organization
ZDF	Zucker Diabetic Fatty

PENILAIAN SIFAT TOKSISIITI DAN ANTIDIABETIK KOPS NUT: KAJIAN RINTIS

ABSTRAK

Diabetes mellitus (DM) adalah penyakit metabolik yang mengakibatkan peningkatan paras glukosa dalam darah yang tidak terkawal. *Ostodes pauciflora* Merr. (Kacang Kops) ialah spesis daripada keluarga *Euphorbiaceae*. yang dikenali oleh orang tempatan sebagai 'buah buantik', 'buah broti' (Komuniti Bidayuh), and 'buah merentik' (Komuniti Iban). Beberapa tumbuhan daripada genus yang sama telah menunjukkan kesan terapeutik terhadap penyakit seperti leukemia dan kanser. *O. pauciflora* dilaporkan mengandungi sebatian bioaktif seperti flavonoid dan fenolik yang diketahui menyumbang kepada potensi antidiabetik tumbuhan. Oleh itu, kajian rintis telah direka untuk menilai sifat hipoglisemik ekstrak minyak *O. pauciflora* pada tikus Sprague Dawley (SD) terinduksi diabetes oleh diet lemak-tinggi (HFD) dan streptozotocin (STZ). Ujian pengekangan α-amilase telah dijalankan untuk menilai potensi in vitro ekstrak Kacang Kop sebagai agen antidiabetik, sementara ujian kebolehtelapan udang garam (BSLA) dilakukan untuk mengukur toksisiti ekstrak tersebut. Dalam tempoh 4 minggu pertama, tikus eksperimen diberi HFD untuk mendorong obesiti, dan disuntik dengan satu dos STZ tunggal (35 mg/kg, intraperitoneal) pada minggu ke-5 untuk menginduksi diabetes (kadar glukosa dalam darah >11 mmol/L). Selepas 7 hari selepas induksi diabetes, tikus diabetes diberikan satu dos rawatan ekstrak minyak Kacang Kop (heksana): 100 mg/kg, 200 mg/kg, dan 400 mg/kg, masing-masing. Kesannya dalam menurunkan kadar glukosa dalam darah diperhatikan selama 24 jam berbanding dengan ubat komersial, metformin (300 mg/kg). Di antara konsentrasi ekstrak yang diuji, ekstrak 200 mg/kg adalah yang paling berkesan dalam menurunkan kadar glukosa dalam darah dalam tempoh pengamatan 24 jam, sebanding dengan metformin. Ujian pengekangan α-amilase menunjukkan hasil yang baik di mana ekstrak heksana dan dietil eter, mempunyai aktiviti pengekangan yang lebih tinggi berbanding acarbose (kawalan positif). Keputusan BSLA untuk ekstrak heksana juga menunjukkan bahawa ekstrak tersebut tidak toksik kerana tidak ada kematian yang dicatat. Oleh itu, dari semua bukti yang dikumpulkan dalam kajian ini, ekstrak O. pauciflora berpotensi untuk menurunkan kadar glukosa dalam darah pada tikus T2DM.

ABSTRACT

Diabetes mellitus (DM) is a metabolic disease resulting in uncontrollable elevation of the level of glucose in the blood. *Ostodes pauciflora Merr*. (Kop nuts) is a species from the family of *Euphorbiaceae*. which is known by local as 'buah buantik', 'buah broti' (Bidayuh community), and 'buah merentik' (Iban community). A few plants of the same genus have shown therapeutical effect against diseases such as leukaemia and cancer. *O. pauciflora* has been reported to contain bioactive compounds such as flavonoid and phenolic, which are known to contribute to the antidiabetic potential of a plant. Thus, a pilot study was designed to evaluate the hypoglycaemic properties of *O. pauciflora* oil extract on self-made high-fat diet (HFD) and streptozotocin (STZ) induced diabetic Sprague Dawley (SD) rats. α-amylase inhibition assays were conducted to assess the *in vitro* potential of Kops Nut's extract as an antidiabetic agent, while brine shrimp lethality assays (BSLA) were performed to gauge the toxicity of the extract. During the first 4 weeks, the experimental rats were given HFD to induce obesity, and injected with a single dose of STZ (35 mg/kg, intraperitoneal) on week 5 to induce diabetes (blood glucose >11 mmol/L). After 7 days of diabetes induction, the diabetic rats were given one dose of treatment of Kops Nut's oil extract (hexane): 100 mg/kg, 200 mg/kg, and 400 mg/kg, respectively. The blood glucose-lowering effects were observed for 24 hours compared to the commercial medication, metformin (300 mg/kg). Among the extract concentrations tested, extract of 200 mg/kg was the most effective in lowering the blood glucose level within the 24-hour observing period comparable to metformin. α-amylase inhibition assay shows favourable results where hexane and diethyl ether extracts, have higher inhibitory activity compared to acarbose (positive control). BSLA result for hexane extract also come out as nontoxic as there were no mortality recorded. Therefore, from all the evidence collected in this study, *O. pauciflora* extracts might potentially lower the blood glucose level in T2DM rats.

CHAPTER 1

INTRODUCTION

1.1 Background study

Diabetes mellitus (DM) is a metabolic disease resulting in uncontrollable elevation of the level of glucose in the blood. There are a few categories of DM which include type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes mellitus (GDM), and diabetes caused by secondary factors such as due to the use of steroids. Among these categories of DM, the most common type of DM is T1DM and T2DM, which are due to defective insulin secretion or action. T1DM affected children, while T2DM usually occurs in adults experiencing hyperglycaemia for a long time due to poor lifestyle (Sapra and Bhandari, 2022). Persistently elevated blood glucose levels result in prolonged harm, impairment, and breakdown of numerous bodily systems, notably affecting blood vessels, eyes, heart, kidneys, and nerves (Asmat et al., 2016; Tan et al., 2019).

In Malaysia, 1 in 5 adults have diabetes. This is equivalent to about 3.9 million people aged over 18 years old (NHMS, 2019). Approximately 90% of diabetes cases have been reported to be T2DM. T2DM is the prevalent manifestation of diabetes, attributed to risk factors such as obesity, advancing age, and familial history of the condition (ADA, 2020). As mentioned before, this disease is characterised by hyperglycaemia. This condition has been implicated to have a relation with oxidative stress as it causes an increase in the formation of reactive oxygen species (ROS) and diminished levels of antioxidants. Amidst oxidative stress, a profoundly harmful byproduct is discharged, culminating in oxidative damage to the organs such as the pancreas, liver, and kidneys, resulting in the pathogenesis of DM and its complications (Oguntibeju, 2019).

In recent years, the management of DM has been prioritising the utilisation of medicinal plants due to their reduced adverse effects in contrast to synthetic pharmaceutical drugs, which come with higher expenses. Therefore, more studies have been done emphasising harnessing the potential of medicinal plants.

The plant being investigated in this study is Kops Nut, scientifically known as *Ostodes pauciflora Merr*. This species is from the family of *Euphorbiaceae*. Locally known as 'buah buantik', 'buah broti' (Bidayuh community), and 'buah merentik' (Iban community). It is synonymous with two other species known as *Dimorphocalyx denticulatus* and *Tritaxis pauciflora* (Welzen and Winkel, 2015). This plant can be found throughout India up until Southern China and the Southeast Asian mainland, Sumatra and Java, yet to be found in the Malay Peninsula (Welzen and Winkel, 2015).

A few plants of the same genus have shown medicinal effect as they contain properties that are active against leukaemia and cancer cells (Handa et al., 1983). There is also a report that stated that a genus of this plant, *O. katharinae*, can inhibit the replication of wild-type human immunodeficiency virus-1 (HIV-1) and human immunodeficiency virus-2 (HIV-2) (Chen et al., 2017). Even though the plant of the same genus has shown therapeutical effects on specific medical condition, and it has also been reported that *O. pauciflora Merr* indeed possesses antioxidant as well as antimicrobial properties (Sakai et al., 2022), there is still yet further study done on *O. pauciflora Merr* on its antidiabetic properties.

Therefore, this pilot study aimed to evaluate the potential of the oil extract of *O. pauciflora Merr* as an antidiabetic agent in the T2DM rat model.

1.2 Problem statement

In Malaysia, *O. pauciflora Merr* can be found abundantly in the forest of Sarawak, and it also has been consumed for generations by the local community. This plant has been reported to possess antioxidant as well as antimicrobial properties (Sakai et al., 2022). But there has been, yet a study done in order to understand the mechanism of action of the crude *O. pauciflora Merr* associated with the reduction of hyperglycaemic properties in *in vivo* systems. Therefore, this study is proposed.

1.3 Rational of study

Even though there was a health benefits effect reported on a few genus of the species (such as *O. paniculata* and *O. katharinae*), there were still studies on *O. pauciflora Merr* although this nut can be easily found in the forest of Sarawak. The medicinal potential of Kops Nut is mostly unclear due to a dearth of research. It has been reported that Kops Nut possess antioxidant and antimicrobial properties (Sakai et al., 2022). As we know, pathogenesis of DM involves oxidative stress which also involved antioxidant compounds which can offset oxidative stress (Oguntibeju, 2019). As a result, this study will elucidate the antidiabetic profile of the Kops Nut.

1.4 Objective

1.4.1 General objective

To evaluate the effects of Kops Nut's extract in the T2DM rat model as a potential antidiabetic agent.

1.4.2 Specific objective

1. To determine the *in vitro* antidiabetic properties of Kops Nut's extract by Alpha-amylase inhibition assay.
2. To evaluate the toxicity of Kops Nut's extract using Brine Shrimp Lethality Assays.
3. To evaluate the effects of Kops Nut's extract on lowering blood glucose levels in diabetic-induced rats (acute study).

1.5 Hypothesis

1.5.1 Null hypothesis

1. The Kops Nut's extract did not have *in vitro* antidiabetic activity.
2. The Kops Nut's extract causes toxicity.
3. The Kops Nut's extract has no significant effect in lowering blood glucose levels in diabetic-induced rats.

1.5.2 Alternative hypothesis

1. The Kops Nut's extract has *in vitro* antidiabetic activity

2. The Kops Nut's extract did not cause any toxicity

3. Kops Nut's extract significantly lowers the blood glucose level in diabetic-induced rats

1.6 Significance of study

Diabetes mellitus constitutes a significant global health issue that has sparked worldwide apprehension. The current emphasis in diabetes care revolves around harnessing the potential of medicinal plants due to their convenient accessibility, cost-effectiveness, and reduced adverse effects in contrast to synthetic pharmaceutical medications (Yedjou et al., 2023). Therefore, the outcomes of this comprehensive examination are thus aimed at elucidating the potential antidiabetic impact of the medicinal plant *Ostodes pauciflora* Merr, as a prospective approach for managing type 2 diabetes mellitus (T2DM).

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes mellitus

Diabetes mellitus (DM) is a group of metabolic diseases result in uncontrollable elevation of the level of glucose in the blood (Sapra and Bhandari, 2022). The elevation of blood glucose levels can stem from either a deficiency in insulin secretion, resistance to insulin's effects, or an insufficient response to the insulin produced by the pancreas. Diabetes mellitus (DM) characterised by persistent hyperglycaemia is linked to damage, dysfunction, and eventual failure of organs and tissues such as the retina, kidney, nerves, heart, and blood vessels (Alam et al., 2014; Asmat et al., 2016; Tan et al., 2019).

2.2 Epidemiology

Diabetes presents an escalating global challenge, bearing implications for individuals, families, and nations. The IDF Diabetes Atlas (2021) underscores this concern, revealing that 10.5% of adults aged 20-79 have diabetes, with nearly half unaware of their condition. By 2045, projections by IDF indicate a significant surge, with 1 in 8 adults, around 783 million individuals, projected to grapple with diabetes, marking a 46% increase. Type 2 diabetes, accounting for over 90% of cases, is profoundly influenced by socio-economic, demographic, environmental, and genetic factors. The driving forces behind the surge in type 2 diabetes encompass urbanisation, an ageing populace, diminishing physical activity levels, and a mounting prevalence of overweight and obesity. The mitigation of diabetes' impact can be realised through preemptive actions against type 2 diabetes and the implementation of early diagnosis and optimal care across all diabetes types. By embracing these interventions, individuals managing the condition can potentially stave off or delay complications associated with diabetes.

Malaysia stands at the forefront of diabetes prevalence within the Western Pacific region, boasting one of the highest rates globally, leading to an annual expenditure of approximately 600 million US dollars. The incidence of diabetes has surged significantly, from 11.2% in 2011 to 18.3% in 2019, marking a substantial 68.3% increase. Based on a national survey, 2019 witnessed 3.6 million adults (18 years and above) grappling with diabetes in Malaysia, of which 49% (3.7 million) remained undiagnosed. Anticipated projections suggest that by 2025, diabetes will impact around 7 million Malaysian adults aged 18 and above, culminating in a formidable public health challenge, with a diabetes prevalence of 31.3% (Akhtar et al., 2022).

2.3 Type of diabetes mellitus

There are three common types of diabetes mellitus: type 1 DM, type 2 DM, and gestational diabetes mellitus.

2.3.1 Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disorder characterised by hyperglycaemia, which is an increase in the blood glucose level due to insulin deficiency as a result of the destruction of the insulin-producing beta cells which, also known as islet β-cells of the pancreas. T1DM is one of the most prevalent chronic endocrine and metabolic diseases, affecting millions of individuals worldwide. It is primarily diagnosed in children and adolescents but can also develop in adults. T1DM results from the immune system mistakenly attacking and destroying pancreatic beta cells, leading to insulin deficiency and uncontrolled blood glucose levels (Katsarou et al., 2017). In a small group of patients, there were no autoimmune reactions detected. This type of case is classified as idiopathic diabetic or also known as type 1B DM.

T1DM is considered an autoimmune disease with a complex interplay of genetic susceptibility and environmental triggers. Research has identified several genetic risk factors associated with T1DM, including specific human leukocyte antigen (HLA) genes. Environmental factors, such as viral infections and early childhood diet, are also believed to contribute to the disease's development (Paschou et al., 2018). The classic symptoms of T1DM include polyuria, polydipsia, polyphagia, weight loss, and fatigue. Rapid onset and severity of symptoms often necessitate urgent medical attention. However, some cases may present with a more gradual progression, making early diagnosis challenging. Diagnosing T1DM involves assessing clinical symptoms, blood glucose levels, and glycated haemoglobin (HbA1c) levels. Additional tests, such as the measurement of C-peptide and autoantibodies (Glutamic acid decarboxylase (GAD), Islet antigen 2 (IA-2), and others), help distinguish T1DM from other forms of diabetes and aid in identifying risk for future autoimmune diseases (Gregory et al., 2013).

T1DM management centres on achieving optimal glycemic control to prevent acute complications, such as diabetic ketoacidosis, and reduce the risk of long-term complications. Intensive insulin therapy through multiple daily injections or insulin pumps remains the mainstay of treatment. Continuous glucose monitoring (CGM) and closed-loop systems (artificial pancreas) have shown promising results in improving glucose control and quality of life for T1DM patients (Kahanovitz et al., 2017; Katsarou et al., 2017).

Long-term complications of T1DM include retinopathy, nephropathy, neuropathy, and cardiovascular disease. Tight glycemic control, blood pressure management, and lipid control are essential in reducing the risk of these complications (WHO, 2020).

2.3.2 Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is the commonest form of metabolic disorder that allocated 90% of all cases of DM compared to T1DM and GDM. The cause of T2DM is due to the combination of two factors which is insufficient insulin secretion due to defective pancreatic b cells and due to the insulin-sensitive tissue developing resistance to insulin resulting in the inability of the tissue to react to insulin (DeFronzo et al., 2015, Galicia-Garcia et al., 2020). Due to these two factors, there is dysregulation of carbohydrate, lipid and protein metabolism in individuals with T2DM (DeFronzo et al., 2015). The process of insulin secretion and action must be exact in order to fulfil metabolic demand. As a result, the molecular processes involved in insulin production and release, as well as insulin responsiveness in tissues, must be closely controlled. Defects in any of the systems affected might thus result in a metabolic imbalance, which contributes to the pathophysiology of T2DM (Galicia-Garcia et al., 2020).

Over the years, there has been rapid advancement in comprehending the development and progression of Type 2 Diabetes Mellitus (T2DM). Its primary origin lies in the gradual decline of insulin secretion by pancreatic β cells, typically occurring against a backdrop of pre-existing insulin resistance in skeletal muscle, liver, and adipose tissue. Prior to the onset of overt hyperglycaemia, there exists a phase known as prediabetes, a high-risk state that predisposes individuals to the eventual development of T2DM. Prediabetes is identifiable by a few conditions, such as elevated fasting glucose levels (IFG), impaired glucose tolerance (IGT), or increased levels of glycated haemoglobin A1c (HbA1c). The annual rate of conversion from prediabetes to T2DM varies between 3% and 11% per year (DeFronzo et al., 2015).

Besides that, there are also findings from research studies indicating a correlation between low-grade inflammation and the susceptibility to type 2 diabetes. Moreover, subtle inflammation contributes to insulin resistance and is intertwined with the characteristics of metabolic syndrome, a collection of traits that encompass hyperglycaemia. Within the realm of diabetic complications, oxidative stress has emerged as a recognised pathway. The oxidative stress induced by high blood sugar levels is thought to elevate the quantities of pro-inflammatory proteins, with macrophages that have infiltrated the affected areas releasing inflammatory cytokines. Consequently, this process gives rise to inflammation both locally and throughout the body. Notably, an increased release of tumour necrosis factor-alpha (TNF-alpha) has been observed in connection with insulin resistance related to obesity. As a matter of fact, obesity stands as a risk factor for the onset of type 2 diabetes (Oguntibeju., 2019).

2.3.3 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is a significant health concern affecting pregnant women worldwide. This review delves into various aspects of GDM, including its definition, risk factors, diagnosis, management, and long-term consequences on both mothers and offspring. The prevalence of GDM is notably high in specific populations, such as Asians, particularly Indians, where factors like obesity and advanced gestational age contribute to its incidence. Historically, GDM possessed significant risks, leading to high perinatal mortality and maternal death. However, advancements in management techniques, particularly insulin therapy, have improved outcomes, with perinatal mortality rates approaching those of the normal population (S, 2019).

Screening for GDM has evolved over time, with the adoption of the 50 g 1-hour oral glucose tolerance test as a reliable method used by many obstetricians in the United States. The

aetiology of GDM involves factors such as pancreatic beta-cell dysfunction, insulin resistance due to placental hormonal release, and clinical risk factors like obesity and a family history of diabetes. Management of GDM involves nonpharmacologic measures such as diet modifications, exercise, and glucose monitoring. Insulin therapy remains the standard treatment when glucose levels are unmanageable through lifestyle changes alone. Oral hypoglycaemic agents like metformin and glyburide are also increasingly used, despite lacking Food and Drug Association (FDA) approval. The prognosis for women with GDM involves ongoing monitoring, with recommendations for testing postpartum to rule out the development of type 2 diabetes. Women with GDM have an increased risk of developing diabetes mellitus in the years following pregnancy. Complications, both maternal and fetal, underscore the importance of effective management and patient education (Bryan, 2023).

Furthermore, GDM's impact extends beyond pregnancy, with both mothers and offspring at risk for long-term health issues. Offspring exposed to hyperglycaemia in the womb face a lifelong risk of becoming overweight or obese and developing type 2 diabetes. Pregnant women with GDM must maintain careful blood glucose control, often through a combination of healthy eating, exercise, and, if necessary, insulin therapy (Kc et al., 2015; Shen et al., 2019; CDC, 2020).

2.4 Hyperglycaemia

Elevated blood sugar levels following a meal trigger the generation and release of insulin by the islet β cells into the bloodstream. The interaction between insulin and insulin receptors located on cell membranes prompts the movement of glucose transporters to the cell membrane. This process enhances the absorption of glucose by the cells, leading to a reduction in blood glucose levels. If the pancreas cannot generate enough insulin, if insulin function is

impaired, or in cases where both factors are at play, it leads to elevated blood sugar (hyperglycaemia). Over time, this condition is linked to harm and dysfunction of various organs and tissues (Berbudi et al., 2020).

The human body employs remarkable mechanisms to safeguard itself against invasion by myriad bacteria, viruses, fungi, toxins, and parasites. Normally, these defence mechanisms create a formidable barrier that pathogens find challenging to breach. However, certain conditions and deficiencies can compromise the effectiveness of the immune system. Regrettably, diabetes is one of the conditions that disrupt the immune response within the host's body, and this disruption is attributed to insufficient insulin and hyperglycaemia (Tessaro et al., 2017).

An *in vitro* investigation exhibited that individuals with T1DM and T2DM exhibited diminished secretion of interleukin 1 beta (IL-1β) from peripheral blood mononuclear cells (PBMCs) and isolated monocytes in comparison to controls. Another study found that monocytes extracted from PBMCs of T1DM subjects released lower levels of interleukin 1(IL-1) and interleukin 6 (IL-6) compared to healthy donors. Considering the significance of IL-6 in safeguarding against pathogens and promoting adaptive immune responses, these investigations suggested that heightened glucose levels could restrain immune responses against invading pathogens (Tanaka et al., 2014; Berbudi et al., 2020).

Moreover, intracellular bacterial load was higher in PBMCs of diabetic individuals than in healthy controls, indicating impaired defence against invading bacteria due to hyperglycaemia. Hence, hyperglycaemia in diabetic individuals is believed to dampen the activity of macrophages and other leukocytes in eradicating pathogens (Tessaro et al., 2017).

2.5 Obesity-related insulin resistance

The prevalence of obesity has risen worldwide, evolving into an epidemic over recent decades along with its linked metabolic disorders, encompassing T2DM and the preliminary condition of insulin resistance (Johnson and Olefsky, 2013). The primary initiator behind the progression of T2DM is believed to be insulin resistance stemming from obesity (Luck et al., 2015). Obesity, categorised by a body mass index (BMI) of 30 or higher, significantly escalates the chances of metabolic syndrome and T2DM. Despite functioning as a primary reservoir for nutrients, adipose tissue takes on the role of an endocrine organ, releasing numerous factors to regulate immune cell activities (Daryabor et al., 2019).

In the course of obesity progression, there is an enlargement in both the size and quantity of adipocytes, leading to adipose tissue hypoxia and subsequent persistent inflammation. Moreover, obese individuals experience elevated levels of free fatty acids in their bloodstream, which encourages their accumulation in the adipose tissue, skeletal muscle, and liver. Obesity fosters the infiltration of neutrophils and macrophages into adipose tissue, triggering their pro-inflammatory behaviour, and ultimately causing tissue inflammation. Neutrophils are the initial immune cells to enter the adipose tissue of obese rodents and humans, followed by the subsequent recruitment of macrophages, whose numbers are linked to insulin resistance (Luck et al., 2015; Daryabor et al., 2019).

Obesity has been extensively demonstrated to heighten the presence of inflammatory agents, such as C-reactive protein (CRP), serum amyloid A (SAA), galectin-3, tumour necrosis factor (TNF)-α, IL-6, interferon-gamma (IFN-γ), macrophage migration inhibitory factor (MIF), IL-8, leptin, and leukotriene (LT)-B4 within both adipose tissue and the serum of obese animals. Additionally, inflammation triggered by obesity leads to an influx of B and T cells into AT over time. The immune activation related to obesity generates systemic insulin

resistance (IR), predisposing affected individuals to the development of T2DM (Daryabor et al., 2019).

2.6 *Ostodes pauciflora* Merr (Kops Nut)

2.6.1 General description

Kops Nut, scientifically known as *Ostodes pauciflora*, belongs to the family of Euphorbiaceae. This plant is called 'buah buantik' and 'buah broti' by the locals of the Bidayuh community. It is also known as 'buah merentik', mostly to the Iban community. It is synonymous with two other species known as *Dimorphocalyx denticulatus* and *Tritaxis pauciflora*. These species can be found in India, Southern China and the Southeast Asian mainland, such as Sumatra and Java (Welzen and Winkel, 2015). This nut can be consumed, and it has a creamy and fatty taste, which tastes like a combination of cashew and almond. Besides that, these nuts also have the same texture and also grown on a tree. Although there were abundant of these nuts, it lacked exposure to the communal and is frequently overseen as it is only known to a certain group of people (Xiao, 2015). Figure 2.1, 2.2, and 2.3 below shows the Kops Nut's seeds, kernels, and flesh.

Figure 2.1: Kops Nut's (*O. pauciflora*) seeds

Figure 2.2. Kops Nut's (*O. pauciflora*) kernels

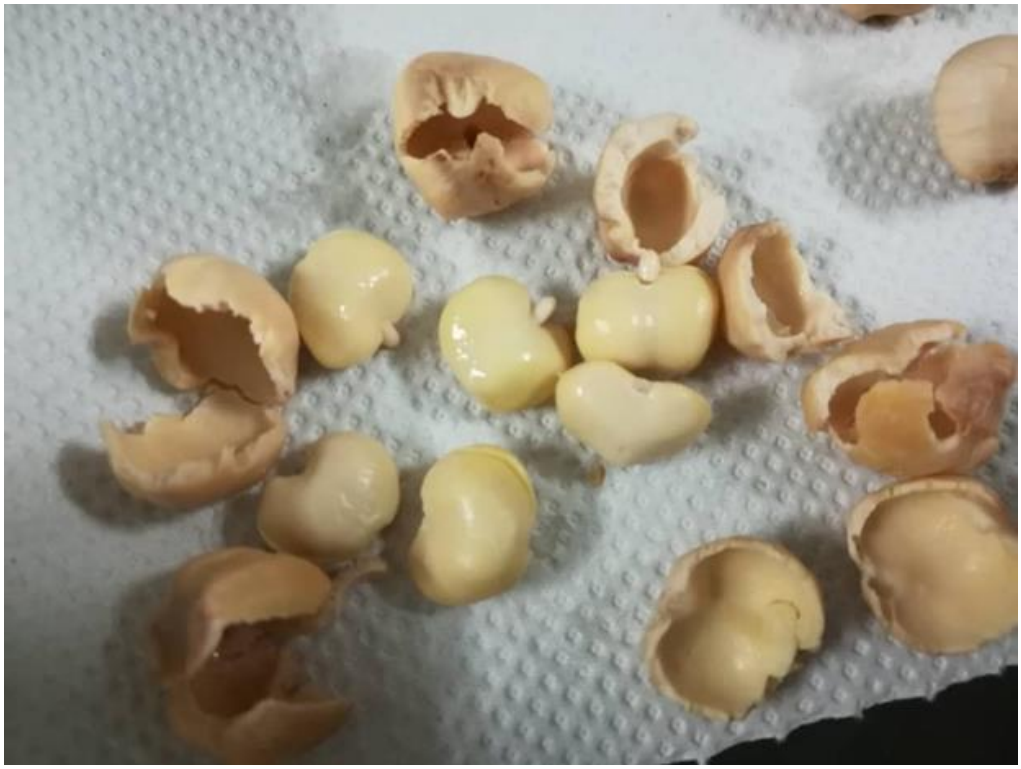

Figure 2.3. Kops Nut's (*O. pauciflora*) flesh

2.6.2 Phytochemistry

Alkaloids, tannins, flavonoids, and phenolic compounds stand out as the most noteworthy phytochemical constituents, each exerting specific physiological effects on the human body. Numerous plants have been identified for their antioxidant capabilities, and polyphenols emerge as a particularly abundant reservoir of these beneficial agents. The most recent investigation conducted on this plant reveals that *O. pauciflora* Merr possesses a higher total phenolic compound (TPC) content in comparison to another plant within the same genus, *O paniculata*. These variations in TPC levels could arise from genetic divergence between plants, disparities in growth environments, climatic variations, geographical origins, and distinct plant populations. The noteworthy elevation in TPC content observed in the methanol bark extract underscores the potential of this plant as a natural source for promising antioxidant

agents. This observation aligns with the findings of Soobrattee et al. (2005), who established the essential role of phenolic compounds possessing redox properties in conferring antioxidant activity within plant constituents (Sakai et al., 2022).

The total flavonoid content (TFC) within hexane, chloroform, and methanol extracts of both leaves and barks from *O. pauciflora* exhibited higher levels when compared to those found in *O. paniculata*, as reported by Rakesh et al. (2013). Flavonoids, an extensive subgroup of secondary metabolites in the phenolic compound category, are widely distributed throughout plants and prokaryotes. In the context of this research, the divergent climatic conditions strongly influenced the concentrations of phytochemicals and the antioxidant capacity. Specifically, *O. paniculata* was identified in India, whereas *O. pauciflora* was discovered in Sarawak, where precipitation rates are three times greater than in India. The presence of flavonoids in this studied plant suggests a potential for preventive effects in the context of cancer and heart disease development.

Furthermore, it is important to highlight that the *O. pauciflora* extract exhibited notable antioxidant and antimicrobial attributes. The scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by various solvent extracts obtained from *O. pauciflora's* leaves and barks displayed a range of efficacy, spanning from 51.91% to 65.18%. The outcomes of the Minimum Inhibitory Concentration (MIC) assessment demonstrated that all extracts exhibited effective inhibitory actions against the tested bacteria, showcasing values ranging from 56.25 to 1800 µg/mL (Sakai et al., 2022).

2.7 Other *Ostodes* genus

2.7.1 *O. paniculata*

Additional members of the *Ostodes* genus, such as *O. paniculata* Blume, have undergone investigation for their biological attributes. Notably, the chloroform extract from this species exhibited activity against P-388 lymphocytic leukaemia, both within *in vitro* and *in vivo* settings, as documented by Handa et al. in 1983. Furthermore, this research highlighted the presence of anticancer compounds, specifically a phorbol diester, originating from the stem and fruit of *O. paniculata*. This was further supported by a study done by Rakesh et al. (2013), that stated *O. paniculata* contains phytochemicals such as phenol and flavonoid which may contribute to the antioxidant activity of this plant.

2.7.2 *O. katharinae*

In a study by Chen et al. (2017), an inhibitor named 12-O-tricosanoylphorbol-20-acetate (hop-8) identified a novel ester of phorbol. This compound was isolated from the chloroform extract of *Ostodes katharinae*, a member of the Euphorbiaceae family. This compound demonstrated a remarkable ability to impede the replication of both wild-type HIV-1 and HIV-2 strains, as well as drug-resistant variants, exhibiting broad effectiveness in both C8166 cells and PBMCs. A notable mechanism underlying the action of hop-8 involves stimulating A3G expression within HIV-1 producing cells, thereby leading to an elevation in A3G levels present in the progeny virions. Consequently, this activity contributes to reducing the infectivity of the resulting progeny virus. This unique and innovative mechanism employed by hop-8 to hinder HIV replication holds significant promise as a potential avenue for the development of novel therapeutics aimed at combating HIV infections.

2.8 Oxidative stress (ROS)

Oxidative stress is a state in which the equilibrium between antioxidants and pro-oxidants tilts in favour of the latter due to diverse factors like ageing, drug effects, toxicity, inflammation, and even addiction. Essentially, it signifies the excess production or inadequate elimination of highly reactive molecules, including reactive nitrogen species (RNS) and reactive oxygen species (ROS). Oxygen possesses a remarkable reactivity, capable of integrating into molecules that hold the potential for harm and detriment, often referred to as Free Radicals. This oxidative stress disrupts the function and structural integrity of well-functioning cells within the body, mounting an assault upon them. To date, over 50 diseases have been linked to the influence of these free radicals in the onset of their pathogenesis. The harm extends to deoxyribonucleic acid (DNA), proteins, and other substantial molecules, as oxidation emerges as a contributory element in the development of a wide spectrum of diseases (Asmat et al., 2016).

Oxidative stress has been linked to the dysfunction of key glucose regulatory processes (insulin secretion and insulin action) in diabetes. Both of these processes are overseen by a molecular pathway recognised as the insulin signalling cascade (Ighodaro, 2018). The progression of disease within the body during diabetes mellitus (DM) is significantly influenced by the generation of reactive oxygen species (ROS), also known as free radicals, as a consequence of oxidative stress. Individuals with Type 2 DM exhibit indicators of oxidative stress in their serum, including ischemia-modified albumin. Elevated serum glucose levels, whether acutely or chronically, can lead to the release of ROS during the course of DM (Maiese, 2015).

The body's defence mechanisms, when weakened, lose their ability to effectively counteract the heightened production of reactive oxygen species (ROS). While a certain level of oxidative stress and ROS remains essential for normal metabolic processes, as they partake

in diverse regulatory functions within cells, their excessive presence proves detrimental. Neutrophils and macrophages generate ROS during the respiratory burst phase to eliminate antigens. Furthermore, ROS act as activating signals for numerous genes responsible for encoding transcription factors, differentiation, and developmental processes. They also facilitate cell-cell adhesion, signal transduction, engagement in vasoregulation, promotion of fibroblast proliferation, and heightened expression of antioxidant enzymes. Nevertheless, excessive and uncontrolled production of ROS poses harmful consequences (Tiwari et al., 2013).

Numerous experimental findings have established a connection between diabetes and oxidative stress, evidenced through the measurement of diverse biomarkers encompassing indicators of DNA damage and the byproducts of lipid peroxidation. It is widely recognised that the initiation and advancement of late-stage diabetic complications are significantly influenced by free radicals, primarily due to their capacity to harm lipids, proteins, and DNA. A range of pathological conditions, including Rheumatoid arthritis, Diabetes mellitus, and cancer, can be triggered by oxidative stress. The complications arising from diabetes that result from free radicals and oxidative stress encompass coronary artery disease, neuropathy, nephropathy, retinopathy, and stroke. *In vivo* investigations bolster the assertion that hyperglycaemia plays a pivotal role in instigating oxidative stress, thereby contributing to endothelial dysfunction within the blood vessels of diabetic patients (Asmat et al., 2016).

2.9 Animal model of T2DM

As the global incidence of diabetes mellitus continues to rise, diabetic rat models are considered pivotal in unraveling the mechanisms underlying human diabetes and its associated issues. Furthermore, these rat models hold indispensable significance in the exploration and creation of new pharmaceutical treatments targeting diabetes and its complications (Al-Awar et al., 2016). While facing criticism, the unequivocal reality remains that animal studies undeniably assist researchers in the discovery of medications and therapies aimed at enhancing human well-being. Furthermore, animal testing establishes drug safety measures and presents an alternative avenue of testing that corresponds to human conditions. Nonetheless, researchers bear the responsibility of employing animals in research solely when essential, adhering to the principles of replacement, reduction, and refinement (3R principles) in preclinical investigations (Munshi et al., 2014).

Tiny rodents, specifically rats and mice, stand out as the most commonly employed animal model for preclinical research on metabolic disorder. This is due to their mammalian nature, in which mice and rats share physiological similarities with humans, compared to non-mammalian creatures. Over recent decades, the prominence of mouse models has skyrocketed, with around 60% of preclinical animal investigations currently centred around Mus musculus. This heightened usage may be attributed to the molecular genetic tools accessible for introducing deliberate or unintentional mutations, ranging from single nucleotide modifications to rearrangements of chromosomes, facilitating gene functional assessments in mice. Furthermore, readily available, and continually enhanced standardised techniques and instruments for characterising mouse traits also contribute to this trend. Lastly, being small in stature, they typically produce 6–12 offspring (varying by mouse strain), attaining sexual maturity within 4–8 weeks of birth, having moderately short reproductive cycles, and a short

gestation period of just 3 weeks render mice a cost-effective selection for researchers (Kleinert et al., 2018).

The animal model of diabetes mellitus is categorised into two categories, the genetic model and the chemically induced model. There are two types of genetically mutated rats' models for T2DM, Zurker diabetic fatty (ZDF) rats and Goto-Kakizaki rats. As for chemically induced, the rats can become diabetic with high dose or multiple low doses of either Streptozotocin (STZ) or Alloxan (Al-Awar et al., 2016). Even though the genetically mutated rats' model is the easier option for diabetic study, as the rats don't have to be induced with obesity first by consumption of high-fat diets, researchers prefer to chemically induce the rats as it is much more cost-effective.

STZ and Alloxan are the most formidable diabetogenic compounds employed in diabetes-related investigations (Pourghasem et al., 2015; Rodrigues, 2016). STZ demonstrates its diabetogenic effect by selectively obliterating β cells, causing insulin deficiency, and hyperglycaemia, as well as inducing polydipsia and polyuria, mirroring the manifestations of human diabetes. Within the plasma membrane, glucose transporter 2 (GLUT2), a glucose transporter with low affinity, acts as the gateway for STZ to enter pancreatic β cells. Conversely, β cells that do not express GLUT2 resist STZ's influences. In contrast, STZ targets liver and kidney cells that do possess GLUT2 expression, causing harm. Structurally resembling n-acetyl glucosamine, STZ incorporates a glucose unit joined to a cytotoxic nitrosourea (MNU). As β cells have heightened glucose sensitivity, STZ gains access via the GLUT2 transporter, leading to eventual STZ toxicity (Goyal et al., 2016).

CHAPTER 3

METHODOLOGY

3.1 Plant material

The Kops Nut oil extract used in this study was provided by our collaborators from University Teknologi MARA, Sarawak (UiTM Samarahan). The plant sample from the hill area of Gunung Payang, Padawan at Serian Division (coordinate GPS 1.48799,110.33164). The plant was harvested around September and November, which was period when the plant was in their season. The plant was then identified and authenticated by the botanist in Universiti Teknologi MARA (UiTM) Samarahan. The voucher was deposited in the herbarium at UiTM Samarahan.

3.2 Plant extract preparation

The Kops Nut oil extraction process was done by our collaborators in UiTM Sarawak. The sample was carefully selected where only matured or ripped seeds were selected. It was then cleaned and boiled for 15-20 minutes. The sample then left to cool at room temperature before being stored at -20 ℃. After the sample cools down, it was further processed by extracting the white inner flesh from the kernel. The extracted flesh was washed and dried in the oven at a temperature of 45 °C until it was constantly dried and then stored at 4 °C before use.

To prepare the oil extract, the dried sample was grinded. In this study, two methods of extraction were used, mechanical extraction and solvent extraction. In mechanical extraction, the sample oil was extracted simply by applying pressure on the sample using screw grind hand pressure. As for solvent extraction, the samples were ground to a powdered form using a mortar

and pestle. Next, 5 g of each sample was weighed and placed into cellulose thimbles within the Soxhlet apparatus for extraction. The extraction was carried out using three different solvents - hexane, diethyl ether, and petroleum ether, with 150 ml of each solvent. The Soxhlet extraction lasted for 8 hours. Subsequently, the solvents were removed using a rotary vacuum evaporator at specific temperatures based on the solvent type: 50 °C for hexane, 55 °C for diethyl ether, and 60 °C for petroleum ether.

The resulting extracted oil was then stored in opaque containers and labelled corresponding to the extraction method or solvent used. Subsequently, the containers were covered with aluminium foil, and finally, the extracts were preserved at -20 °C until they were utilised for the study.

3.3 Materials

3.3.1 Drugs and Chemicals

The drugs and chemicals used in the study are summarised in Table 3.1.

Table 3.1. Drugs and chemicals used in this study.

Drugs/Chemical	**Brands**
Brine shrimp lethality assay:	
- Sea salt	- Sigma
- Brine shrimp (artemia)	- Supplied by UPMS
- Distilled water	- Supplied by UPMS
Alpha amylase assay:	
- 96-well plate	- Greiner Bio-one
- Acarbose	- Sigma
- Starch	- Sigma
- α-amylase enzyme	- Sigma
- Dinitrosalicylic acid (DNSA) reagent	- Sigma
- Distilled water	- Supplied by UPMS
High-fat diet (HFD) preparation:	
- Standard rat pellet	- Gold Coin Feedmills (M) Sdn. Bhd, Malaysia
- Ghee Oil	- CRISPO, Malaysia