EVALUATION OF ANTIDIABETIC ACTIVITY OF *Christia vespertilionis* (BAKH.F.) LEAVES EXTRACT USING METABOLOMICS APPROACH

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by

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LIST OF SYMBOLS

α	Alpha
Å	Angstrom
°C	Degree Celcius
eV	Eletronvolt
g	Gram
kcal	Kilocalorie
kcal/mol	Kilocalorie per mole
kg	Kilogram
<	Less than
µL	Microlitre
µm	Micrometre
mL	Milimetre
mm	Milimetre
mg	Milligram
mg/kg	Milligram per kilogram
mL/kg	Millimol per kilogram
mmol/L	Millimol per litre
mM	Millimolar
min	Minutes
M^+	Molecular weight
>	More than
nm	Nanometre
%	Percentage
π	pi
±	Plus-minus sign

pH	Potential of Hydrogen
®	Registered trademark
rpm	Revolutions per Minute
™	Trademark

LIST OF ABBREVIATIONS

2D	Two-dimensional
3A4A	Crystal structure of isomaltase from *Saccharomyces cerevisiae*
3D	Three-dimensional
3TOP	Crystal Structure of the C-terminal Subunit of Human *Maltase-Glucoamylase* in Complex with Acarbose
ACD	Advanced Chemistry Development
ADG	α -D-glucose
ACUC	Animal Care and Use Committee
ADT	AutoDockTools
AGI	α-Glucosidase inhibition assay
ARG	Arginine
ASH	Protonated Aspartic acid
ASN	Asparagine
ASP	Aspartic acid
AUC	Area under curve
b.w	Body Weight
cAMP	Cyclic adenosine 3′,5′-monophosphate pathways
CMC	Carboxymethyl Cellulose
CON	Control group
CV-EAH	Ethyl acetate: hexane extract of *C. vespertilionis* leaves
CV-EAH I	50 mg/kg b.w of CV-EAH
CV-EAH II	250 mg/kg b.w of CV-EAH
CV-EAH III	550 mg/kg b.w of CV-EAH
DMSO	Dimethyl Sulfoxide
EA	Ethyl Acetate extract
EAH	Hexane: Ethyl Acetate extract
EAM	Methanol: Ethyl Acetate extract
ESI	Electrospray Interface
FBG	Fasting blood glucose
FXR	Farnesoid X receptor
FC	Fold Change

GC-MS	Gas Chromatography-Mass Spectrometry
GLN	Glutamine
GLU	Glutamic acid
H	Hexane extract
HFD	High-Fat Diet
HIS	Histidine
i.p	Intraperitoneal
IC_{50}	Half-Maximal Inhibitory Concentration
ILE	Isoleucine
LC- MS/Q-ToF	Liquid Chromatography- Quadrupole Time-Of-Flight Spectrometry
LEU	Leucine
LYS	Lysine
M	Methanol extract
m/z	Mass-to-Charge Ratio
MET	Methionine
MET	300 mg/kg b.w of metformin group
MSTFA	*N*-Methyl-*N* – (trimethylsilyl) trifluoroacetamide
MVDA	Multivariate Data Analysis
NC	Negative control group
NIST14	National Institute of Standards and Technology
NHMS	National Health and Morbidity Surveys
OB-DB	Obese-Diabetic
OPLS	Orthogonal Partial Least Square
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
PDB	Protein data bank
PHE	Phenylalanine
PKA	Protein kinase A
PNPG	*p*-Nitrophenyl-β-D-glucopyranosidase
PRO	Proline
RMSECV	Root-mean-square error of Cross-Validation
RMSEE	Root-mean-square error of Estimation
ROC	Receiver Operating Characteristic
RT	Retention Time
SER	Serine

STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TGR5	G protein-coupled receptor 5
THR	Threonine
TIC	Total ion chromatography
TRP	Tryptophan
TYR	Tyrosine
UGT	UDP-glucuronyltransferase
VAL	Valine
VIP	Variable Importance Rojection
vs	Versus

LIST OF APPENDICES

PENILAIAN ANTIDIABETIK EKSTRAK DAUN *Christia vespertilionis* (BAKH.F.) MENGGUNAKAN PENDEKATAN METABOLOMIK

ABSTRAK

Christia vespertilionis (Bakh.f.) (*C. vespertilionis*), tumbuhan yang berasal dari Asia Tenggara, menunjukkan potensi antidiabetik yang memberangsangkan. Kajian ini menyelidik potensi antidiabetik daun *C. vespertilionis* melalui pendekatan bersepadu yang menggabungkan metabolomik *in vitro* dan *in vivo* dengan simulasi pemautan molekul. Ekstrak kasar daun *C. vespertilionis* disediakan menggunakan pelarut dengan polariti berbeza dan disaring untuk aktiviti antidiabetik. Inhibitor *α*-glukosidase dikenal pasti melalui metabolomik GC-MS dan LC-MS/Q-ToF. Pemautan molekul digunakan untuk mengkaji interaksi dengan protein sasaran (PDB: 3A4A dan 3TOP). Kajian *in vivo* menggunakan tikus obes-diabetik yang diinduksi dengan STZ menilai kesan antidiabetik ekstrak melalui metabolomik serum LC-MS/Q-ToF. Metabolomik berasaskan GC-MS mengenal pasti tiga penanda kimia utama: asid palmitik, asid α-linolenik, dan 7-tetradekanal, manakala LC-MS/Q-ToF mendedahkan aloe-emodin-8-monoglukosida, bruceine I, dan sanjidin B. Keputusan pemautan menunjukkan semua sebatian yang dikenal pasti berinteraksi dengan residu protein melalui interaksi hidrofobik dan ikatan hidrogen. Sebatian ini diramalkan bertindak sebagai perencat kompetitif, menunjukkan mereka mungkin berkongsi mekanisme perencatan yang serupa dengan enzim. Dalam kajian *in vivo*, dos 500 mg/kg berat badan (b.w) CV-EAH (CV-EAH III) menunjukkan penurunan signifikan paras glukosa serum (137.80 mg/dL ; $p < 0.001$) dan peningkatan berat badan (412.33 g; $p < 0.05$) dalam tikus obes-diabetik yang diinduksi STZ, setanding dengan metformin,

ubat rujukan standard untuk diabetes. Analisis metabolomik mengenal pasti sembilan biomarker yang berubah secara signifikan ($p < 0.05$, VIP ≥ 1.0). Pentadbiran CV-EAH III memodulasi laluan berkaitan metabolisme purin, pertukaran pentosa dan glukuronat, serta biosintesis asid hempedu primer. Analisis komponen utama mengesahkan pemulihan metabolik dalam tikus yang dirawat dengan CV-EAH III, yang dikelompokkan rapat dengan kumpulan kawalan sihat. Secara kesimpulannya, kajian ini telah membuktikan potensi antidiabetik daun *C. vespertilionis* melalui penyelidikan menyeluruh *in vitro*, *in vivo*, dan *in silico* dengan bantuan pendekatan metabolomik.

EVALUATION OF ANTIDIABETIC ACTIVITY OF *Christia vespertilionis* (BAKH.F.) LEAVES EXTRACT USING METABOLOMICS APPROACH

ABSTRACT

Christia vespertilionis (Bakh.f.) (*C. vespertilionis*), a plant indigenous to Southeast Asia, has demonstrated promising antidiabetic properties. This study investigates the antidiabetic potential of *C. vespertilionis* leaves through an integrated approach combining *in vitro* and *in vivo* metabolomics with molecular docking simulations. The crude extract of *C. vespertilionis* leaves was prepared using solvents of varying polarity and screened for antidiabetic activity. α-glucosidase inhibitors were identified through GC-MS and LC-MS/Q-ToF metabolomics. Molecular docking was used to study interactions with target proteins (PDB: 3A4A and 3TOP). An *in vivo* study using STZ-induced obese-diabetic rats assessed the extract's antidiabetic effects via LC-MS/Q-ToF serum metabolomics. GC-MS-based metabolomics identified three key chemical markers: palmitic acid, α-linolenic acid, and 7-tetradecanal, while LC-MS/Q-ToF revealed aloe-emodin-8-monoglucoside, bruceine I, and sanjidin B. The docking results indicated that all identified compounds interact with the protein residues through hydrophobic interactions and hydrogen bonds. These compounds are predicted to exhibit a competitive inhibition mode, suggesting they might share a similar inhibition mechanism with the enzyme. *In vivo*, a 500 mg/kg body weight (b.w) dose of CV-EAH (CV-EAH III) significantly reduced serum glucose levels (137.80 mg/dL ; $p < 0.001$) and improved body weight (412.33 g, $p < 0.05$) in STZ-induced obese-diabetic rats, comparable to metformin, the standard reference drug for diabetes. Metabolomics analysis identified nine significantly altered biomarkers ($p < 0.05$, VIP ≥ 1.0). CV-EAH III administration modulated pathways related to purine metabolism,

pentose and glucuronate interconversions, and primary bile acid biosynthesis. Principal component analysis confirmed metabolic restoration in CV-EAH III-treated rats, clustering closely with healthy controls. Conclusively, this study has demonstrated the antidiabetic potential of *C. vespertilionis* leaves through comprehensive *in vitro, in vivo*, and *in silico* investigations with the aid of the metabolomics approach.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

A natural product derived from natural sources, especially from medicinal plants, has been observed in a wide range of pharmacological studies and commercialised by established pharmaceutical industries. Medicinal plants have been discovered and utilised for various purposes to cure and combat diseases since ancient times. Consequently, pharmaceutical companies are increasingly producing products derived from nature for diverse medical applications.

Diabetes is a global health concern, becoming increasingly prevalent worldwide. According to the International Diabetes Federation (IDF), 537 million people aged 20 - 79 had diabetes in 2021. If current trends continue, this number is expected to increase to 643 million by 2030 and 783 million by 2045 (Sun *et al*., 2021). Diabetes is most prevalent in regions such as North America, the Middle East, Southeast Asia, India, and China, which are particularly impacted due to their large populations. (Teo *et al*., 2021). In Malaysia, the overall prevalence of diabetes was 15.8%, indicating that about one in seven people is affected. Among different age groups, the 20–29 age group had the lowest prevalence at 3.16%, whereas the highest rate, 33.46%, was observed in individuals aged 60 and above. This means that those aged 60 and older are more than ten times as likely to have type 2 diabetes compared to individuals in their twenties (Akhtar et al., 2022). The rising prevalence of type 2 diabetes mellitus (T2DM) among children and teenagers is especially troubling. This trend, driven by sedentary lifestyles, high-calorie diets, and increasing rates of childhood obesity, underscores the urgent need for early prevention and education. If not properly managed, diabetes can lead to severe complications, including heart disease, stroke, kidney failure, and

blindness (Sunny, 2021). Diabetes not only poses a significant health burden but also strains economies and healthcare systems. According to the American Diabetes Association, the average healthcare costs for people with diabetes are 2.3 times greater than for those without diabetes (McEwen and Herman, 2021).

Dietary carbohydrates increase postprandial plasma glucose levels, causing hyperglycemia in T2DM patients. A rapid increase in blood glucose can lead to hyperglycemia in individuals with T2DM, typically driven by enzymes such as pancreatic α-amylase and α-glucosidase, which are located on the brush border of intestinal cells (Ayoub *et al*., 2023). Monosaccharides, such as glucose and fructose, are absorbed from the intestinal lumen into the bloodstream. In contrast, complex carbohydrates like disaccharides and oligosaccharides must first be enzymatically broken down into monosaccharides before they can be absorbed through the duodenum and upper jejunum (Nightingale and Spiller, 2023).

The prevention of chronic vascular problems and the early management of diabetes mellitus both depend heavily on controlling hyperglycemia. The most effective ways to treat T2DM are by strongly inhibiting intestinal *α*-glucosidases and mildly inhibiting pancreatic *α*-amylase. Inhibitors of these enzymes delay carbohydrate digestion, prolong digestion time, and slow glucose absorption, which contributes to lowering the postprandial rise in blood sugar. Acarbose, miglitol, and voglibose are some of the *α*-glucosidase inhibitors used as oral antidiabetics, with metformin being the most frequently prescribed medication for T2DM patients in Malaysia (Dzamic and Matejic, 2022). However, these pharmacological therapies have several drawbacks, including limited efficacy and potential long-term adverse effects, which pose challenges for the healthcare system.

Numerous natural substances have been investigated for their potential to inhibit glucose production from carbohydrates in the gut or glucose absorption in the intestine. Recently, there has been increasing interest in managing diabetes through dietary restrictions and medicinal plants (Rahman *et al*., 2022). In many developing countries, such as Malaysia, traditional medicine using medicinal plants is a more economical form of healthcare. This has sparked interest in using medicinal plants as an alternative management strategy for T2DM. The development of complementary and alternative medicine is significantly supported by medicinal plants, often used as natural remedies and alternatives to conventional therapies for common diseases. Incorporating medicinal plant medicines into basic healthcare can support the Sustainable Development Goals and World Health Organization's initiatives, aid new drug development, improve healthcare access, preserve forest resources, and reduce socioeconomic disparities (Pathak et al., 2024). Plants are a popular choice for alternative and complementary medicine due to the synergistic effects of their complex mixtures of substances on the intricate human body system. Additionally, unlike some modern medications that can be metabolized into harmful toxins potentially causing chronic disorders with repeated use, certain chemicals in medicinal plants may help reduce toxicity in the human body.

Numerous traditional medicinal plants have been investigated for their potential as antidiabetic agents (Kasole *et al*., 2019; Akbar, 2020). Similarly, *Christia vespertilionis,* the green butterfly wing known locally as 'daun rerama', is a medicinal plant from the *Fabaceae* family. It is commonly found in Asian countries (Farizan *et al*., 2023). This plant has been widely used to treat ailments such as tuberculosis, bone fractures, snake bites, bronchitis, inflamed tonsils, and circulatory problems (Murugesu *et al*., 2020b). The crushed leaves of this plant are frequently applied topically to

alleviate scabies (Norazhar *et al*., 2022). Numerous studies have highlighted the potential of *C. vespertilionis* by showcasing its antiproliferative, antimalarial, and antidiabetic properties alongside its antioxidant effects (Ismail *et al*., 2020; Yamin *et al*., 2022). The plant's phytocompounds have been identified to contain alkaloids, triterpenes, fatty acids, phenols, long-chain alkanes, and alcohols that contribute to their antioxidant and anticancer activities (Smitha and Jain, 2019; Zambari *et al*., 2021). Consequently, further studies utilizing chromatography analytical techniques could help identify the bioactive compounds responsible for the antihyperglycemic effects. Additionally, the efficacy of *C. vespertilionis* leaves extract against diabetes should be evaluated *in vitro*, *in silico*, and *in vivo* studies.

Integrated with chromatography and multivariate data analysis (MVDA), a metabolomic approach has been utilized to identify bioactive metabolites. On top of that, metabolite profiling aids the understanding of metabolic routes and mechanisms in cells, tissues, organs, or organisms. This strategy has been utilized to identify bioactive substances in medicinal plants and to support the therapeutic value of many plant extracts over solitary isolated metabolites. Further, metabolomics enables the identification and quantification of numerous targets, offers data on all metabolites, and offers data on natural products by connecting probable bioactivity with metabolites in the target plants (Rinschen *et al*., 2019). Another advantage of using this approach is the possibility of identifying new biomarkers causing a particular disease and revealing the mode of action of medicinal plants in treating this disease (Zeki *et al*., 2020; Yang *et al*., 2022).

Isolation of the compound targets on detailed characterisation of individual compounds, while metabolomics analyses the complete profile of the extract/biological system. Hence, the current research employed a holistic integrated metabolomics

approach that aims to evaluate the antidiabetic effects of *C. vespertilionis* leaves extract and reveal its action mechanism. This approach was used to profile and identify *α*-glucosidase inhibitors in ethyl acetate: hexane extract of *C. vespertilionis* leaves (CV-EAH) and to develop a validated model based on gas chromatography-mass spectrometry (GC-MS) and liquid chromatography- quadrupole time-of-flight spectrometry (LC-MS/Q-ToF) metabolomics approach. The mechanism of action of these chemical markers with enzymes was explored, revealing their binding strength interaction sites and potential inhibition mechanisms within receptor-binding pockets using 3A4A and 3TOP enzymes. Additionally, this research aims to elucidate the metabolic pathway and identify the potential biomarkers of CV-EAH using a streptozotocin (STZ) -induced obese-diabetic (OB-DB) rat model for its antidiabetic activity through an LC- MS/Q- ToF-based metabolomics approach.

1.2 Problem Statements

Currently available synthetic drugs for diabetes, such as voglibose, metformin, and acarbose, often cause side effects with long-term use. Prolonged use of antidiabetic drugs like metformin, acarbose, and voglibose often causes gastrointestinal side effects due to their effects on the digestive system. Metformin alters gut microbiota and slows glucose absorption, leading to diarrhoea and bloating. Acarbose and voglibose delay carbohydrate digestion, causing undigested carbs to ferment in the colon, which produces gas and draws water into the bowel, resulting in flatulence, bloating, and diarrhea over time (Blahova *et al*., 2021). Consequently, researchers are exploring medicinal plants to develop drugs that have no side effects and are less toxic.

In addition, medicinal plants are made up of a complex mixture. The complex mixture involves synergistic and/or antagonistic interactions among the compounds to

affect the plant's bioactivities. A major problem faced by the phytochemists is overlooking the discovery of many possible active compounds during bioassay-guided fractionation. It could be due to the low abundance of the active compounds in natural sources, omitting the synergistic/ antagonistic interaction among the metabolites in the plants and loss of bioactivity during fractionation and purification of bioactive compounds (Vidar et al., 2023). Currently, the issue has been effectively tackled and overcome through the application of a metabolomics approach (Wolfender et al., 2014).

C. vespertilionis leaves extract has been reported to possess various pharmacological actions, including traditional use for treating diabetes (Dash, 2016). However, no proper metabolite profiling of this plant has been reported, particularly regarding the compounds responsible for suppressing metabolic alterations. The metabolomics approach can evaluate the efficacy of the sample by analyzing all possible metabolites in biofluids. Additionally, the structural interaction of the enzyme protein with its inhibiting metabolites has yet to be visualized through any research work.

1.3 Research Objectives

1.3.1 General Objectives

The general objectives of the study are to elucidate the mechanism of action of *C. vespertilionis* leaves extract as a potential antidiabetic plant and to identify its putative chemical markers and biomarkers via a metabolomics approach.

1.3.2 Specific objectives

The specific objectives of this study are:

1. to evaluate antidiabetic activity using a α-glucosidase inhibition and identification of its putative chemical markers using GC-MS and LC-MS/Q-ToF based metabolomics approaches.
2. to visualize the protein-ligand interactions of α-glucosidase inhibitors from *C. vespertilionis* leaves extract using PDB: 3A4A and PDB: 3TOP crystal structures.
3. to evaluate the antidiabetic activity of *C. vespertilionis* leaves extract using *in vivo* STZ-induced OB-DB rat model.
4. to elucidate the metabolic pathway of *C. vespertilionis* leaves extract for its antidiabetic activity through metabolomics approach.

1.4 Research Questions

1. Does *C. vespertilionis* leaves extract show α-glucosidase inhibitory activity, and can its bioactive compounds be identified using GC-MS and LC-MS/Q-ToF metabolomics?
2. Do the compounds in *C. vespertilionis* leaves extract interact visually with α-glucosidase based on PDB: 3A4A and 3TOP structures?

3. How effective is the antidiabetic activity of *C. vespertilionis* leaves extract when evaluated using STZ-induced OB-DB rat model?
4. What metabolic pathways are involved in the antidiabetic activity of *C. vespertilionis* leaves extract?

1.5 Research Hypothesis

1. *C. vespertilionis* leaves extract exhibits significant *α*-glucosidase inhibitory activity and contains distinct putative chemical markers that can be identified using GC-MS and LC-MS/Q-ToF metabolomics approaches.
2. The *α*-glucosidase inhibitors from *C. vespertilionis* leaves extract show specific and significant visual interactions with the PDB: 3A4A and PDB: 3TOP crystal structures.
3. The extract from *C. vespertilionis* leaves exhibits significant antidiabetic activity when evaluated using STZ-induced OB-DB rat model.
4. LC-MS/Q-ToF-based metabolomics analysis reveals significant alterations in metabolic pathways associated with antidiabetic activity following treatment with *C. vespertilionis* leaves extract.

1.6 Significance of the Study

The significance of this research highlighted that GC-MS and LC-MS/Q-ToF techniques can identify *α*-glucosidase inhibitors from this plant extract, including some compounds that have not been previously reported. Such discoveries could lead to the developing of novel, plant-derived medications that offer an alternative or complementary approach to existing antidiabetic drugs.

Through detailed molecular docking simulations and metabolic pathway analysis, the research provides a deeper understanding of the interaction of bioactive

compounds in *C. vespertilionis* leaves with key enzymes and their metabolic processes. This knowledge is crucial for designing effective antidiabetic therapies and predicting potential side effects or interactions with other medications. Exploring plant- based therapeutics aligns with the growing interest in sustainable and environmentally friendly healthcare solutions. *C. vespertilionis*, as a natural resource, could provide a renewable and low-cost source of antidiabetic compounds, contributing to the sustainability of healthcare systems, especially in resource-limited settings.

CHAPTER 2

LITERATURE REVIEW

2.1 Origin and Botanical Classification

The *Fabaceae* family plant *Christia vespertilionis* (L.f.) Bakh. f. has recently attracted the attention of researchers from tropical Asia due to its medicinal properties. *C. vespertilionis* has different synonyms which include *Hedysarum vespertilionis* (L.f.), *Lourea vespertilionis* (L.f.) *Desv*, and *Christia lunata Moench* (Quattrocchi, 2016). *C. vespertilionis* is locally known as "*Pokok Rerama*" or Butterfly Wing plant which is native to some of the tropical Asian countries and found abundantly in Malaysia, Vietnam, Thailand, Indonesia, Cambodia, Myanmar, North America, and China. Other vernacular names include "Dau Canh Doi" in Vietnam. (Table 2.1).

Table 2.1 Vernacular names of *C. vespertilionis*

Country	Language	Vernacular Names	References
Malaysia	Malay, English	"Pokok rerama" Butterfly wing	(Dash, 2016)
Vietnam	Vietnamese	Dau Canh Doi	(Nguyen-Pouplin *et al*., 2007)

2.2 Morphology and Structure

C. vespertilionis is a medicinal plant that grows between 0.6 and 1.2 meters tall and has upright branches that droop. Its foliage often takes the form of stalked leaves with three leaflets: a greenish one with stipes, bigger than the two lateral leaflets, and a boomerang-shaped terminal leaflet. At night, the leaves become bend downwards. When a light breeze disturbs them, the butterfly-like leaves flutter, resembling butterflies. The branching inflorescence bears an off-white flower about 6 mm in diameter. The fruits are in the form of 4 or 5 jointed legumes. *C. vespertilionis* is a divot shrub plant that can be propagated via stem cutting (Shah *et al*., 2020). (Figure 2.1).

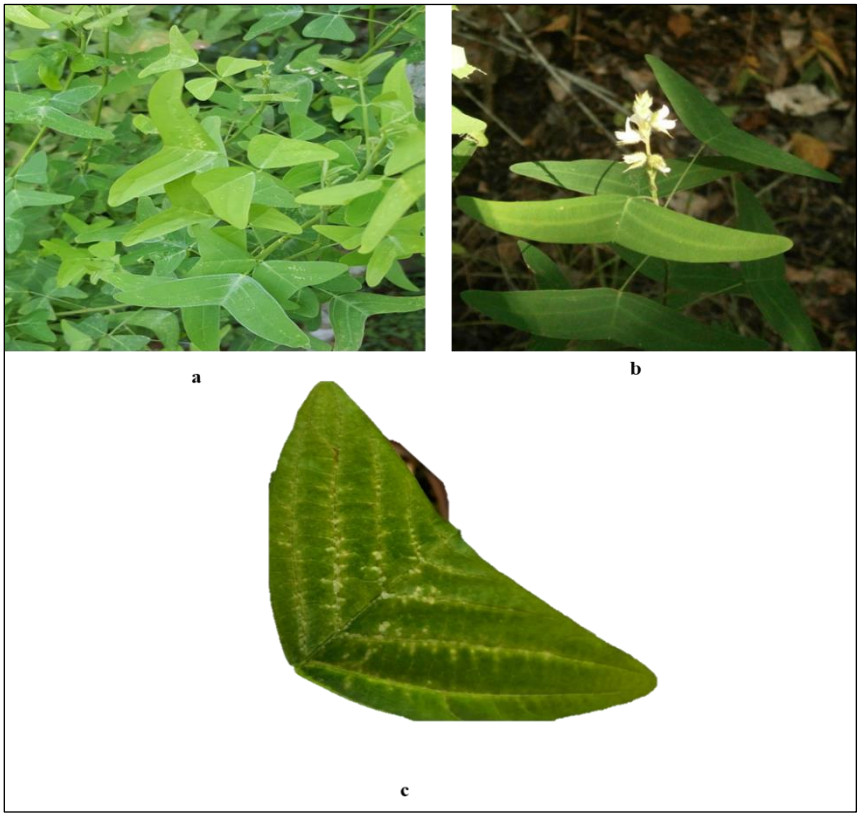

Figure 2.1 Structure of *C. vespertilionis*; (a) the whole plant (b) the flower of the plant, (c) simple leaves.

2.3 History and Ethnobotanical Uses

In the region of South Asia, the plant *C. vespertilionis* is a well-known medicinal plants. The plant has long been used to cure tuberculosis and snakebite wounds, and the leaves are frequently applied topically to repair bone fractures (Dash, 2016). *C. vespertilionis* leaves have also been used to treat tonsillitis, muscle weakness, colds, and poor blood circulation (Lee *et al*., 2020). According to one research, the *C. vespertilionis* plant may have anti-inflammatory characteristics (Zambari *et al*., 2023). The plant is often boiled, or some parts are diced and made into a paste by soaking them in water. The resulting paste is then applied locally to treat conditions like wounds and sprains, or it is taken orally to treat gastrointestinal problems (Chassagne *et al*., 2016). This has made a wide range of commercial goods, such as teas, drinks, and powders readily available (Figure 2.2).

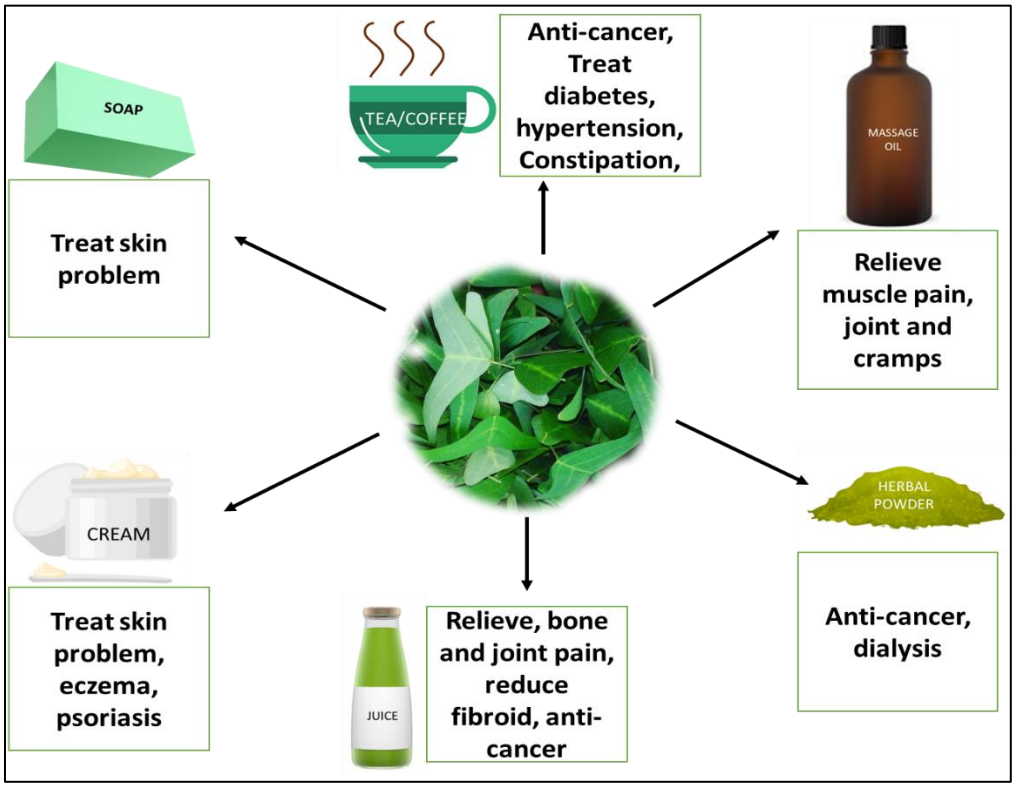

Figure 2.2 Commercial products made with *C. vespertilionis* are available on the market.

2.4 Phytochemical Contents of *C. vespertilionis*

In the leaves of *C. vespertilionis*, there are compounds from the phytochemical classes: flavonoids, alkaloids, terpenoids glycosides, tannins, diterpenes, coumarin, and quinine. It is interesting to note that Norazhar employed a molecular network approach to identify the 60 metabolites in the leaves. The study's findings showed that two novel derivatives (apigenin-6-*C*-*β*-glucoside 4′-*O*-*α*-apiofuranoside and apigenin-6-*C*-*β*-[(4″,6″-*O*-dimalonyl)-glucoside] 4′-*O*-*α*-apiofuranoside) were effectively extracted using a mass-targeted isolation approach (Norazhar *et al*., 2021).

A study showed how Fourier-transform infrared spectroscopy (FT-IR) analysis combined with multivariate analysis to quickly find the best metabolite data (Joshi et al., 2021). Upadhya and his team used repeated chromatographic separation of the *n*-hexane fraction to isolate two new chemicals (Upadhyay *et al*., 2013).

LC-MS chromatogram of *C. vespertilionis* samples detected nicotinamide, psolaren, ibuprofen, zerumbone and linoleate. Linoleate, a salt derivative of linoleic acid, is a polyunsaturated omega-6 fatty acid commonly used for its anti-inflammatory properties and its ability to reduce acne, particularly in skin treatments (Osman *et al*., 2017). Zerumbone may be useful as an anti-cancer compound. Research shows that zerumbone has anti-tumor effects in neuroendocrine tumor cells and significantly inhibits the growth of cervical cancer cells without harming normal cell substances (Hofer *et al*., 2013; Ghasemzadeh *et al*., 2017). Figure 2.3 illustrates the chemical structure while Table 2.2 lists the specifics of sub-phytochemical.

Table 2.2 The phytochemical classes and compounds present in *C. vespertilionis* leaves.

Phytochemical Class	Phytochemical Compound	References
Flavanoids	Quercetin-3-*O*-glucoside, Catechin-3-*O*-*β*-Dglucopyranoside, Orobol 2, 3-dihydro-2'-hydroxy-Genestin, mono- and dihydroxyflavones, Flavonol 3-*O*-glycosides, Flavone-*C*,*O*-diglycosides, apigenin-6-*C*-*β*-glucoside apigenin-6-*C*-*β*-[(4″,6″-*O*-dimalonyl)-glucoside] 4′-*O*-*α*-apiofuranoside Isoorientin, Schaftoside	(Upadhyay *et al*., 2013; Abd Latip and Abd Mutalib, 2019; Norazhar *et al*., 2021)
Terpenes	D:C-friedoolean-8-en-29α-ol, Ursolic acid methyl ester, Oleanolic acid methyl ester, Erythrodiol, Geraniol, 7-Isopropylidene-1-methyl-1,2,6,7,8,9-hexahydronaphthalene (Christene), Zerumbone, Phytol	(Upadhyay *et al*., 2013; Osman *et al*., 2017)
Fatty acid	Methyl linolenate, 10-undecenoic acid, Pentadecyl acrylate, Methyl palmitate	(Osman *et al*., 2017; Yasin *et al*., 2020)
Polysterol	Stigmasterol, β-sitosterol acetate, β-sitosterol	(Upadhyay *et al*., 2013)
Coumarin	Psoralen	(Osman *et al*., 2017)
Amide	Nicotinamide	(Osman *et al*., 2017)
Oil	2'-hydroxydecanylpentadec-5, 8, 10, 12-tetraenoate (Christanoate)	(Upadhyay *et al*., 2013)
Sulphur containing compounds	Tetrahydro-2-methyl-thiophene, 2-(2-benzothiazolylthio)-1-(3,5 dimethylpyrazolyl)-ethanone	(Yasin *et al*., 2020)

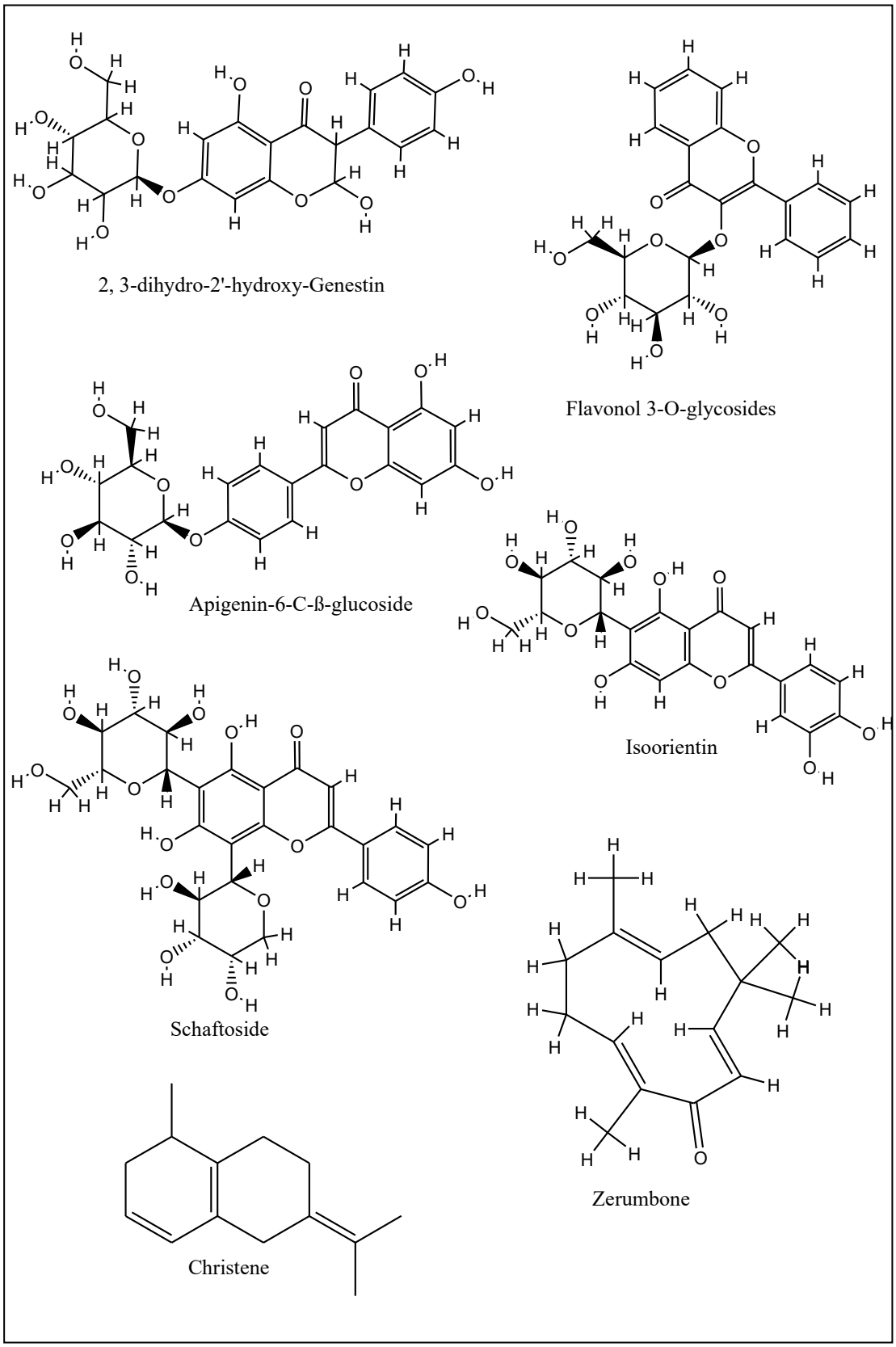

Figure 2.3 The 2D-chemical structure from various phytochemical classes and compounds present in *C. vespertilionis* leaves (diagram was reconstructed using Chem-Draw Ultra-Structure Software) (continue).

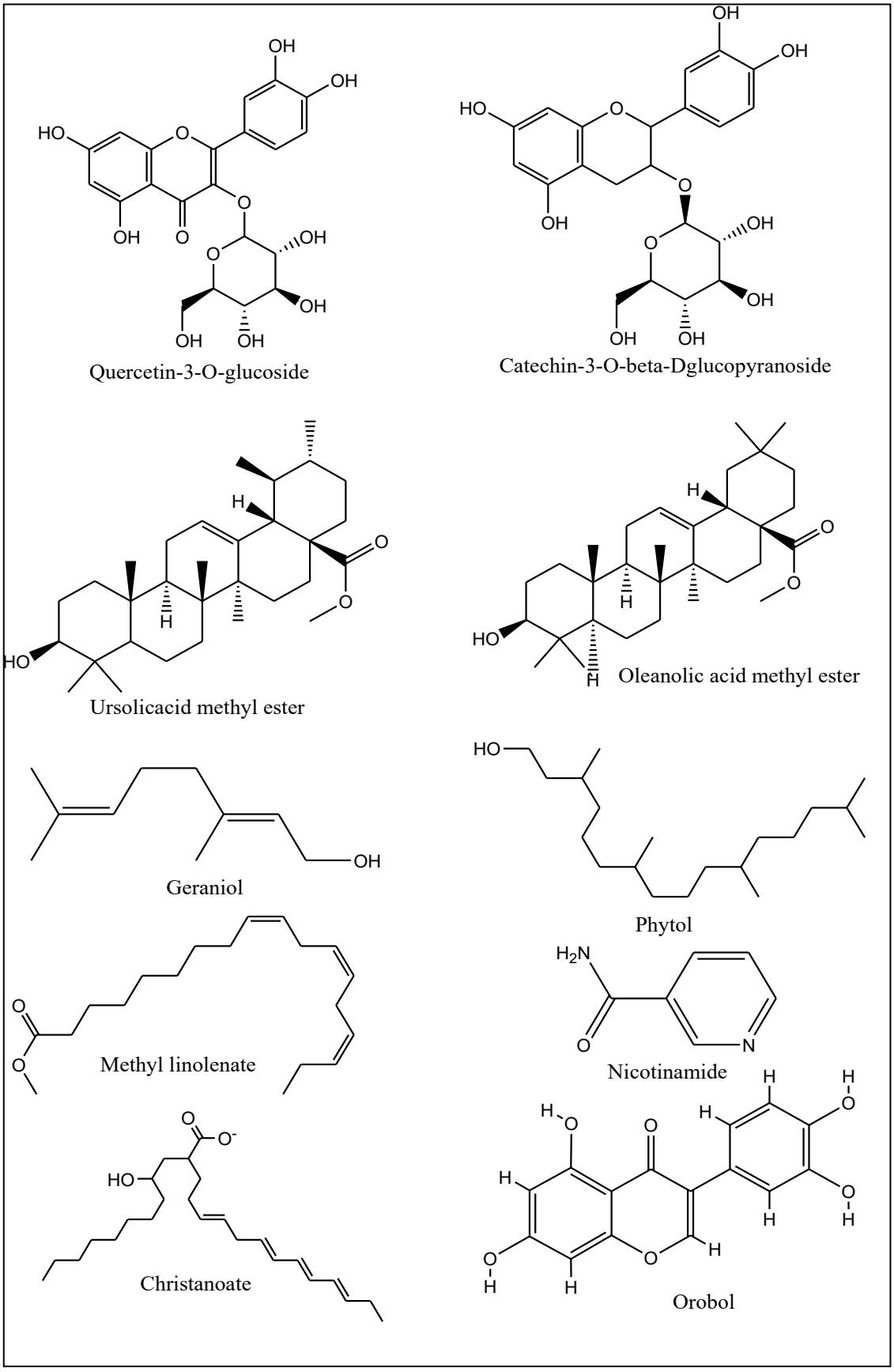

Figure 2.3 2D-chemical structure from various phytochemical classes and compounds presents in *C. vespertilionis* leaves (diagram was reconstructed using Chem-Draw Ultra-Structure Software).

2.5 Pharmacological and Medicinal Properties

The phytochemical components of medicinal plants have attracted the attention of researchers from all over the world because they are rich in active secondary metabolites that have pharmacological effects on people. This is because the pharmaceutical and nutraceutical sectors greatly benefit from the extracted bioactive chemicals. Additionally, several pharmacological activities have been identified, including antidiabetic, antioxidant, anticancer, antimalaria, and antihyperuricemia effects (Süntar, 2020).

2.5.1 Antimalaria Properties

Novel chemical compounds isolated from *C. vespertilionis* demonstrated significant antiplasmodial activity in an *in vitro* study (Upadhyay *et al*., 2013). Among the identified compounds were christene and christanoate. Christene, in particular, exhibited notable anti-malarial activity against *Plasmodium falciparum*, with an IC_{50} value of 9.0 µg/mL. These findings were further supported by Yasin *et al*. (2020), who reported that methanolic leaves extracts of *C. vespertilionis* also showed anti-malarial activity against the *P. falciparum* strain. Additionally, cyclohexane extracts of *C. vespertilionis* demonstrated antiplasmodial effects, with IC_{50} values ranging from 10 to 20 µg/mL. However, it is important to note that these compounds exhibited high cytotoxicity toward mammalian cells, with a selectivity index (SI) of less than 2 (Nguyen-Pouplin *et al*., 2007).

2.5.2 Anticancer Properties

According to a study published by Hofer *et al*., (2013), human fibroblasts (HF-SAR) analysed utilising extracts with a concentration of 10 µg/mL of ethyl acetate were used to evaluate the antitumor activities of *C. vespertilionis* leaves using antiproliferative in MTC-SK cells, pro-apoptotic effects in KRJ-I cells, and

antiproliferative effects in KRJ-I cells. They claimed that both MTC-SK and KRJ-I cells significantly responded to the ethyl acetate extract from *C. vespertilionis* leaves in an antiproliferative and pro-apoptotic manner. The extracts employed for tumor cells, however, did not reduce proliferation when tested on human fibroblasts (HF-SAR), demonstrating that the phytoconstituents of this fraction do exclusively influence tumor cells. According to a gene expression study, ethyl extract of *C. vespertilionis* treatment increased the expression of MTDH and decreased the expression of PDCD5 and TNFRSF10b in MTC-SK cells, and it did the opposite in KRJ-I cells according to a GAPDH gene expression analysis.

Ismail *et al*., (2021) have also discovered that *C. vespertilionis* treated with MCF7 cells possesses antitumor properties. Based on the results of this study, which employed non-polar to polar solvents to evaluate the antitumor activities, it was discovered that the dichloromethane extract had the strongest IC_{50} value when used on MCF7 cells and the highest selectivity index value, with an extract with an index of 8.2. Investigations revealed that the P53, BAX, cytochrome C, caspase-8, and caspase-3 genes were elevated, and the genes for BCL-2 and PCNA were downregulated in the MCF7 cells after treatment.

The cytotoxicity of ethanol extract on a panel of cell lines, including CRL2522, HaCaT, HepG2, MCF-7, and WRL68, demonstrated low cytotoxicity against all cell lines as the IC_{50} values above 1 mg/mL, according to Latip findings. As a result, their attempt to combine ethanol extract with cyclophosphamide at IC_{10} concentration led to antagonism, as shown by the combination index value of 1.359 (Abd Latip and Abd Mutalib, 2019). More research is necessary to confirm and understand the synergistic mode of action. Considering the outcomes so far, further scientific research is required to interpret these plant activities.

2.5.3 Antioxidant Properties

According to a study by Abd Latip and Abd Mutalib, (2019), total flavonoid content and total phenolic content were used to examine the antioxidant effects of *C. vespertilionis* aqueous and ethanol extract. Although the percentage yield of the aqueous extract is lower than the ethanol extract, they reported that the flavanoid and phenolic content of *C. vespertilionis* ethanolic extract are higher than the aqueous extract. It concluded that the high extraction yield might not necessarily translate into high levels of the phytochemicals phenolic and flavonoids. Similarly, the antioxidant activity of leaves extract revealed a substantial number of phenolic compounds ranging from 1.52 to 5.82 mg, as well as a considerable number of flavonoid compounds ranging from 3.45 to 27.57 mg. Based on the results, water extract for leaves had a greater level of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (72%).

However, the study claimed that antioxidant activities were examined using leaves that had been extracted with methanol. DPPH test with butylated hydroxytoluene standard and the Folin-Ciocalteu method were both used in the study to assess the total phenolic content. According to the biochemical test, the total phenolic content of the leaves extract is 128.852± 3.90 mg, and the DPPH test indicates that the IC_{50} for scavenging free radicals is 39.987 mg/mL (Yamin *et al*., 2022). This demonstrates the antioxidant activity of *C. vespertilionis* leaves extracts.

2.5.4 Antidiabetic Properties

Yamin *et al.* (2022) have reported utilizing *α*-amylase inhibition to have antidiabetic benefits. According to the research, they investigated the *α*-amylase inhibition using the metformin standard at three distinct concentrations (500, 250, and 125 mg/mL), using methanol extracts. According to Yamin's findings, the antidiabetic

potentiality was shown to be present in 23.33, 20.14, and 15.34, respectively, with an IC_{50} of 35.2 mg/mL.

Similarly, Murugesu *et al*., (2020a) examined the antidiabetic capabilities utilizing the important enzyme *α*-glucosidase, which is involved in the digestion and absorption of carbohydrates. The enzyme *α*-glucosidase has been demonstrated to be most potently inhibited by the hexane: ethyl acetate extract, whereas methanol extracts have the lowest levels of enzyme inhibition when using quercetin as a standard. They concluded that the polarity and the chemical present in the extract affected the inhibition of *α*-glucosidase. It's interesting to note that the team also used qualitative analysis to explore phytochemical screening of *C. vespertilionis* leaf extracts, and the detected compounds were simulated using an *in silico* approach with PDB:3TOP and PDB:3A4A protein structure. Further research is necessary to fully understand the biological effects of *C. vespertilionis* leaves on antidiabetic activities, both *in vitro* and *in vivo*.

2.6 Diabetes Mellitus

2.6.1 Definition and Epidemiology

Diabetes mellitus (DM) is one of the most chronic metabolic disorders, characterized by hyperglycemia, a condition where blood sugar levels are consistently high. This disrupts the normal metabolism of carbohydrates, fats, and proteins in the body. DM is considered a serious health issue due to its significant morbidity and mortality rates, primarily caused by complications in both microvascular (such as retinopathy, nephropathy, and neuropathy) and macrovascular (such as coronary heart disease, peripheral vascular disease, and stroke) systems (Alam *et al*., 2021) (Figure 2.4).

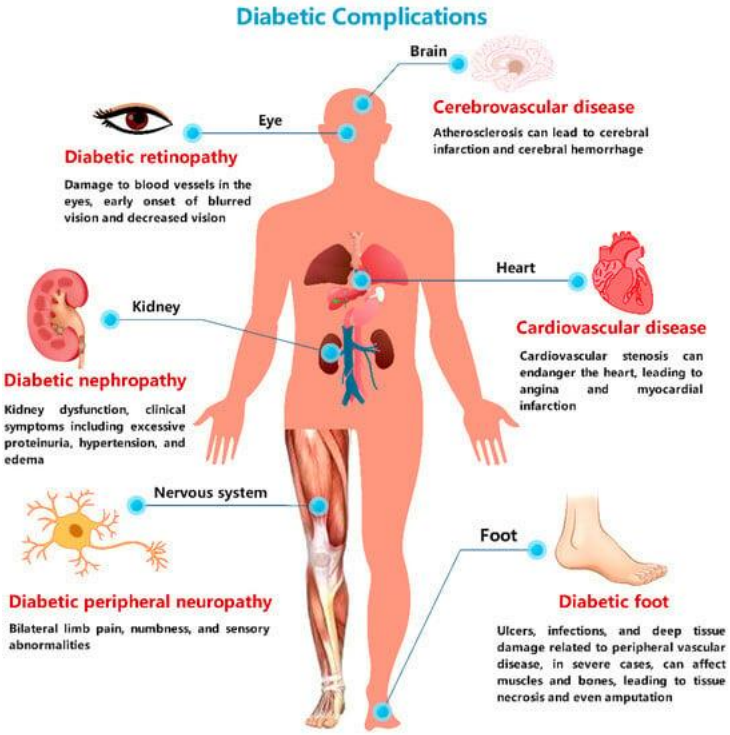

Figure 2.4 Complication of diabetes.
(Zhang *et al*., 2024)

DM is defined as a heterogeneous metabolic disorder with hyperglycemia as its main clinical feature (Misra *et al*., 2021). It results from defects in insulin secretion and/or insulin action, or a combination of both (Katsiki *et al*., 2020). Insulin is a hormone responsible for maintaining normal blood glucose levels (80 to 126 mg/dL). Unlike monosaccharides, which are absorbed directly into the bloodstream from the intestinal lumen, complex starches, oligosaccharides, and disaccharides must be broken down into monosaccharides before absorption (Figure 2.5). This digestion of complex sugars is facilitated by enteric enzymes, primarily pancreatic α-amylase and α- glucosidases, which are in the brush border of intestinal cells (Harmon and Swanson, 2020).

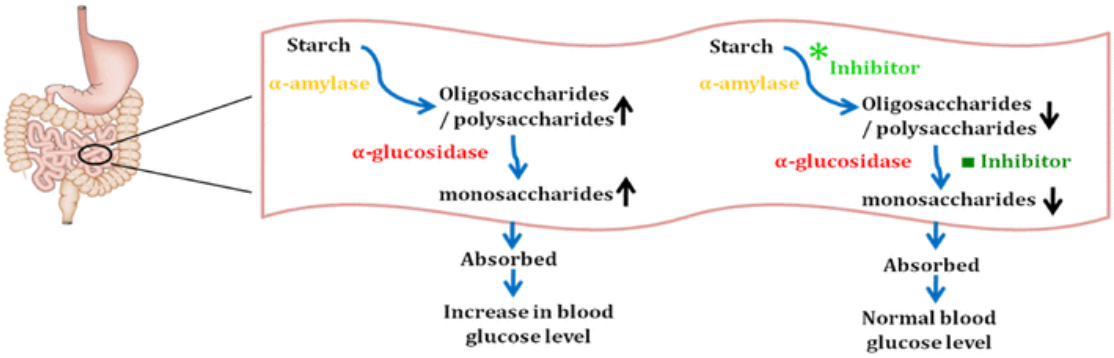

Figure 2.5 The function of *α*-amylase and *α*-glucosidase in starch digestion and metabolism.
(Sohrabi *et al*., 2022)

2.7 Prevalence of Diabetes Mellitus

The global prevalence of diabetes mellitus has been increasing over the past few decades. According to the International Diabetes Federation (IDF), the number of adults living with diabetes rose from 108 million in 1980 to 573 million in 2021, and it is projected to reach 783 million by 2045 (Pikala & Burzyńska, 2024). This rise can be attributed to several factors, including population growth, ageing, urbanisation, and the increasing prevalence of obesity and physical inactivity. The majority of adults with diabetes (68%) are concentrated in the ten countries with the highest diabetes prevalence. Figure 2.6 illustrates diabetes around the world as reported by IDF 2021.

In addition, 541 million people are estimated to have impaired glucose tolerance in 2021. It is also estimated that over 6.7 million people aged 20–79 will die from diabetes-related causes in 2021. The number of children and adolescents (i.e. up to 19 years old) living with diabetes increases annually. In 2021, over 1.2 million children and adolescents had T1DM. Direct health expenditures due to diabetes are already close to one trillion USD and will exceed that by 2030. The IDF Diabetes Atlas 10th edition also shows that hyperglycaemia in pregnancy (HIP) affects approximately one in six

pregnancies. Another cause for alarm is the consistently high percentage (45%) of people with undiagnosed diabetes, which is overwhelmingly T2DM (Sun *et al*., 2021).

The prevalence of diabetes mellitus in Malaysia has been increasing over the past few decades. According to the National Health and Morbidity Survey (NHMS) 2023, the prevalence of diabetes among Malaysian adults was 18.3%, up from 13.4% in 2019. This represents nearly one in six adults living with diabetes, making it a major public health issue. The report notes that the prevalence of diabetes increases with age, yet many cases remain undiagnosed.

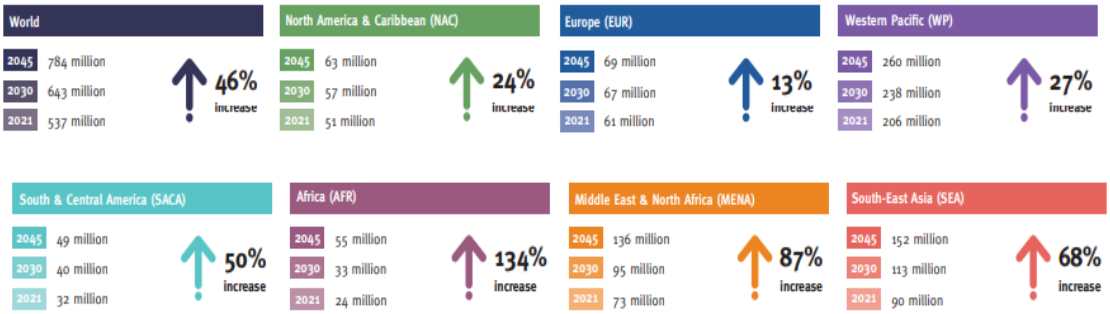

Figure 2.6 Prevalence of diabetes around the world in 2021 (IDF 2021).

2.7.1 Types of Diabetes Mellitus

2.7.1(a) Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) often diagnosed in children and young adults, was previously known as juvenile onset diabetes (JOD) or insulin-dependent DM. This type of diabetes requires patients to rely heavily on insulin replacement therapy (Figure 2.7). T1DM is classified into two categories: immune-mediated DM and idiopathic DM (Catarino *et al*., 2020).

Immune-mediated DM is caused by an autoimmune response that destroys the pancreatic β-cells, leading to insulin deficiency. Markers of this autoimmune activity include islet cell autoantibodies, autoantibodies to GAD (GAD65), autoantibodies to insulin, and autoantibodies to tyrosine phosphatases IA-2 and IA-2b (Mourad *et al*., 2021). This form of DM is often associated with other autoimmune diseases such as autoimmune hepatitis, Graves' disease, pernicious anemia, Addison's disease, and Hashimoto's thyroiditis (Hare and Topliss, 2022). Idiopathic T1DM, on the other hand, has no known cause and is characterized by permanent insulinopenia, meaning the patients have a severe insulin deficiency (Kakleas *et al*., 2015).

2.7.1(b) Type 2 Diabetes Mellitus

Type 2 diabetes, also known as non-insulin dependent diabetes mellitus or maturity-onset diabetes, is the most prevalent form of DM. It primarily affects older individuals but is increasingly seen in obese adolescents (Kumar *et al*., 2020). Unlike T1DM, T2DM does not involve the autoimmune destruction of β-cells.

Most T2DM patients are obese, with obesity being a significant factor in the development of this metabolic disease. However, individuals with a normal body mass