IDENTIFICATION OF ALPHA-GLUCOSIDASE INHIBITORS AND ANTIOXIDANTS IN *Zingiber officinale* RHIZOME USING LC-MS BASED METABOLOMICS AND MOLECULAR DOCKING

BY

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ABSTRACT

Zingiber officinale (ginger) rhizome has widely used as a spice and folk medicine for anti-diabetic. Though some researchers have investigated the pharmacological aspect of the plant's anti-diabetic properties, there is a need for metabolite profiling to reveal the bioactive chemicals responsible for the plant's anti-diabetic effects via inhibition of α-glucosidase. The aims of the present study are to evaluate the α-glucosidase inhibitory activity of the ginger ethanolic extract, to identify the putative α-glucosidase inhibitors from the rhizome using LC-MS based metabolomics, and to analyse the molecular interaction of these inhibitors to the enzyme receptor via in silico molecular docking analysis. The ginger rhizome was extracted using solvents with different concentrations of methanol in water (100, 75, 50, 25, and 0%, v/v). The extracts were tested via in vitro α-glucosidase inhibitory assay and analysed using LCMS-QTOF. The data obtained were subjected to multivariate data analysis to pinpoint the putative α-glucosidase inhibitors. The molecular interaction between the putative α-glucosidase inhibitors and the enzyme was investigated using in silico molecular docking. The ginger methanol extract exhibited the highest α-glucosidase inhibitory (AGI) activity (IC_{50}= 185.2 µg/mL) compared to the other extracts. This extract also showed antioxidant activities with DPPH-IC_{50} and FRAP value of 125.0 µg/mL and 16.95 mmol TE/mgDW, respectively. The LCMS-based metabolomics revealed several α-glucosidase inhibitors in the ginger rhizome, namely 7-methoxycoumarin, supinine and 12-hydroxycorynoline. Although the existence of these compounds has been reported in other plants, the presence of these compounds in ginger is being reported for the first time in this study. The activity of these compounds was supported by computational study using in silico molecular docking. These compounds displayed binding energy values less than the control ligand (< -6.0 kcal/mol) indicating their strong affinity to the enzyme. Methoxycoumarine and supine formed interactions with ASP 352 and ARG 422 through π -anion interaction hydrogen bond, respectively. Similarly, supinine interacted with ARG 442 through a hydrogen bond. These interactions took place at the active site of an enzyme while, 12-hydrocoryline interacted with an allosteric binding site of the enzyme. This study highlights the potential of the ginger rhizome as anti-diabetic agent via α-glucosidase inhibitory activity and anti-oxidant mechanisms, which is beneficial in the development of future anti-diabetic nutraceuticals.

ملخص البحث

الخالصة

يستخدم زنجبير أوفيسينال)المسن(rheizome على نطاق واسع لتوابل وأدوية شعبية لمكافحة السكر. وعلى الرغم من أن بعض الباحثين قد بحثوا عن الجانب الصيدالذي من خصائص المصنع المضادة للتشخيص، إال أن هناك حاجة إلى تحديد المكافئات للكشف عن المواد الكيميائية الحيوية النشطة المسؤولة عن آلثار المضادة للسكر في المصنع من خالل منع ألفا - غلوكوسيداز. وتتمثل أهداف هذه الدراسة في تقييم النشاط التثبيطي ألفا - غلوكوسيدازي في المستخلص اإليثانولي الزنجبيلي، وتحديد مثبطات الغلوكوسيداز المكثوفة من الركيزوم باستخدام المستقلبات القائمة على أساس LC-MS ، وتحليل التفاعل الجزيئي لهذه المثبطات في مستقبل االنزيم عن طريق تحليل الرسو الجزيئي السيليكو. وتم استخراج الريزوم الزنجبيل باستخدام مذيبات بتركيزات مختلفة من المي ثانول في الماء (100 و75 و50 و25 و0 في المائة، v/v). وقد اختبرت المستخلصات عن طريق تحليلها وتحليلها باستخدام LCMS-QTOF. وقد خضعت البيانات التي تم الحصول عليها لتحليل بيانات متعدد المتغيرات من أجل تحديد المثبطات العثومية لـ Alfu-glucosidase. وقد تم التحقيق في التفاعل الجزيئي بين مثبطات الغلوكوسيداز المكثوفة والنزيمات التي استخدمت في الرسو الجزيئي للسيليكو. وقد أظهر مستخلص المي ثانول الزنجبيل أعلى نشاط مثبط للغلوكوسيداز (AGI) (IC50=185.2 ميكروغرام/ملغم) بالمقارنة مع المستخلصات أالخرى. وأظهر

هذا المستخلص أيضا ً أنشطة مضادة لألكسدة بقيمة DPPH- تبلغ 125.0 ميكروغرام/مللتر IC50 وFRAP

و16.95 ملليغرام/ملغDW على التوالي. وكشفت االليضات المستندة إلى نظام رصد ألرض عن عدة مثبطات لـ غلوكوسيداز في الزنجبيل، وهي 7 - ميثوكسي كومارين، وسوبينين، و12 هيدروكسيكوريانولين. وعلى الرغم من البالغ عن وجود هذه المركبات في نباتات أخرى، فإن وجود هذه المركبات في الزنجبيل يجري البالغ عنه ألول مرة في هذه الدراسة. وكان نشاط هذه المركبات مدعوما ً بدراسة حسابية استخدمت في الرسو

الجزيئي للسيليكو.

ونُظهر هذه المركبات ثباتاً من الطاقة المرتبطة بالارتباط عن قيمة الإيغان (<-6.0 kcal/mol) التي تشير إلى صلتها القوية بالإنزيم. وقد شكّل المركب السوكسي الكومارين والسوبين تفاعلين مع ASP 352 وARG 422 من خلال O- رابطة هيدروجين التفاعل بين ألنيونات، على التوالي. وبالمثل، تفاعل السوبين مع ARG 442 من خلال رابطة الهيدروجين. وحدثت هذه التفاعلات في الموقع النشط للتريبات في حين تفاعل 12 هيدروكلوريداً مع ملزم موقع كلوسيركي لالنزيم. وتسلط هذه الدراسة الضوء على إمكانات الريزوم الزنجبيل كعامل مضاد للسكر عن طريق نشاط مثبط لغلوكوسيداز ألفا وآليات مضادة لألكسدة، وهو أمر مفيد في تطوير التريسوتاتيات المانعة للسكر في المستقبل .

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Science in Pharmacy.

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2D	2-dimensional	IL	Interleukin
3D	3-dimensional	JOD	Juvenile onset diabetes
ADG	α-D-glucose	LC	Liquid chromatography
AGI	α-Glucosidase Inhibition Assay	LCMS	Liquid chromatography-mass spectrometry
ATR	Attenuated total reflectance	LDL	Low-density lipoprotein
ANOVA	One-way analysis of variance	MS	Mass spectrometry
BBM	Brush border membrane	MVDA	Multivariate data analysis
DPPH	1,1-diphenyl-2-picrylhydrazyl	NAC	N-acetylcysteine
DNA	Deoxyribonucleic acid	NAD	Not appropriately described
DM	Diabetes mellitus	NHMS	The National Health and Morbidity Surveys
DMSO	Dimethoxysulfoxide	NIDDM	Non-insulin dependent diabetes mellitus
EC_{50}	Effective concentration 50%	NIST14	National Institute of Standards and Technology
ESI	Electrospray interface	NMR	Nuclear Magnetic Resonance
FRAP	Ferric Reduction Activity Potential	NR	Not reported
FTIR	Fourier-Transform Infra-Red	OPLS	Orthogonal Partial Least Squares
GAD	Glutamic acid decarboxylase	PDB	Protein Data Bank
GAD65	Glutamic acid decarboxylase 65-kilodalton isoform	PNPG	ρ-nitrophenyl-α-D-glucopyranoside
GC	Gas chromatography	QTOF	Quadrupole Time-of-Flight
GCMS	Gas chromatography-mass spectrometry	RMSD	Root mean square deviation
GE	Ginger extract	ROS	Reactive oxygen species
GH13	Glycoside hydrolases	SCI	*Saccharomyces cerevisiae* isomaltase
HCT116	Human colorectal carcinoma cell line	SD	Standard deviation
IA-2	Islet Cell Antigen 512	TNBS	2,4,6-trinitrobenzene sulphonic acid

TPTZ	2,4,6-tripyridyl-s-triazine	UV	Ultraviolet
USD	United States Dollar	WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Diabetes mellitus (DM) is a chronic carbohydrate metabolic disorder triggered by either body not effectively using insulin or production of insulin in the pancreas being insufficient to counter higher consumption of sugar (Syihabudin et al., 2018). This causes hyperglycaemia, or increased blood glucose levels, which can harm body systems, including blood vessels and nerves. It affects fundamental biochemical activity in every age group, making it a major global source of disease, mortality, and disability (Dong et al., 2017).

DM is a worldwide epidemic metabolic syndrome (Choudhary et al., 2014; Okur et al., 2017). It affects most of the world's population, and the number of diabetics is rapidly growing worldwide, particularly in developing countries such as Malaysia, Thailand, India, and Indonesia (Ali et al., 2012). According to the Second National Health and Morbidity Survey (Noman et al., 2020), 3.4 million Malaysians had diabetes in 2010, and 425 million people (aged 20 to 79 years) worldwide had diabetes in 2019, as revealed by the International Diabetes Foundation (International Diabetic Foundation, 2017). These figures are projected to rise up to 700 million by 2045 (Zabidi et al., 2021). Furthermore, it was estimated that 374 million people had impaired glucose tolerance and that nearly 21.3 million live births to women were affected by some form of hyperglycaemia in pregnancy (International Diabetic Foundation, 2017). DM was responsible for approximately 5 million deaths worldwide between 20 to 99 years old in 2017. In 2017, the global healthcare expenditure on people with diabetes was estimated to be USD 850 billion (Cho et al., 2018). In Malaysia, the diabetic prevalence rate is about 17.5% or 3.5 million people in 2016 and is expected to double by 2025 (Noman et al., 2020).

To overcome this problem, extensive research has been conducted primarily on developing antidiabetic drugs, either injectable or oral medication. To date, insulins are

considered the most prominent injectable drug for diabetic treatment, which is already available for short-acting, intermediate and long-acting, or a mixture of intermediate or long-acting insulin (Saunders et al., 2022; Hirsch et al., 2020). Oral antidiabetic drugs such as biguanides (metformin), sulphonylureas (gliclazide) and dipeptidyl peptidase-4 inhibitors (alogliptin) are widely used as a medication (Mukhtar et al., 2020). However, according to Montvida et al. (2018), there is a growing proportion of diabetes patients receiving second-line therapy prescriptions for antidiabetic medications, raising concerns about the medications' efficacy. On the other hand, continuous diabetic medications might cause several side effects, such as skin problems (Jedlowski et al., 2019), formation of scars (Ross et al., 2016), liver injuries and gastrointestinal damage due to prologue use of miglitol and acarbose (Usman et al., 2019); and allergy-related problems which are caused by insulin injection (Jacquier et al., 2013). Besides that, medication cost also influences diabetic treatment since antidiabetic drugs are expensive and rising over the year, burdening the low-income population (Gong et al., 2018; Obakiro et al., 2021). Thus, searching for an alternative medicine is vital and traditional treatment via natural products containing bioactive compounds seems a promising solution to cure DM.

Nature has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs have been produced from natural sources that play a vital role in treating diseases (Singh et al., 2017). Moreover, medicinal plants have a great history of use for the benefit of humankind. The World Health Organization revealed that about 80% of the world's inhabitants depend on traditional plant extract therapies (Sofowora et al., 2013). Nevertheless, medicinal plants play an important role in traditional healthcare systems for treating many diseases (Kumar Gupta & Sharma, 2014).

Although treatment using phytochemicals extracted from traditional medicinal plants is widely applied in traditional medication and practices such as Ayurveda, Unani and Traditional Chinese Medicine systems, this treatment faces several significant problems, including lack of scientific evidence, self-medication and prescribed by an unqualified practitioner (Chattopadhyay, 2020). These drawbacks hinder the application of traditional medication, and most of the consumers blindly follow the unprofessional testimonies or a "trial and error" style in pivotal on the medication

(Bhamra et al., 2017, Rao et al., 2016, Rudra et al., 2017; Srinivasan and Sugumar, 2017). Thus, several researches have been conducted to evaluate the effectiveness of the extracted phytochemicals from medicinal plants to various treatments, combined with the latest quantitative and qualitative analyses to prove the claims.

Singh (2011) reported that about 800 plants have been identified as a potential antidiabetic effect, including ginger. Ginger (local Malay name: *halia*) is well known to have many medicinal, dietary and ethnomedicinal characteristics as a significant plant. It is scientifically known as *Zingiber officinale* Roscoe and it is a member of the Zingiberaceae family (Shahrajabian et al., 2019). It is known as one of the most important plants with countless medicinal, nutritional and ethnomedical characteristics. It is globally used as a spice, flavouring agent, and herbal remedy. Historically, in Indonesian Jamu, Malay, Ayurveda, Siddha, Traditional Chinese Medicine, and many other medicinal systems, it is used to treat a range of illnesses (Tiwari and Rao 2002; Grzanna et al. 2005) such as diabetes, nausea, vomiting, asthma, cough, palpitations, swelling, dyspepsia, loss of appetite, constipation, indigestion and pain.

Ginger originated in South-East Asia and is the most widely used spice globally. It is a pungent, aromatic spice that adds a unique flavour and taste to our food. The ginger plant has a thick, branched rhizome (underground stem) with a brown outer layer and yellow centre that has a spicy, citrusy aroma. Rhizome of ginger is like the roots of other plants that grow underground. In botanical term, however, ginger is considered as stem instead of root. This perennial herb is cultivated mainly as a spice and a condiment. Ginger is widely used as a culinary ingredient due to its medicinal benefits and distinct aroma and taste. In addition, ginger is mentioned in various ancient and historical Chinese, Indian, and Middle Eastern texts. It has always been appreciated for its scent, culinary and, above all, separate medicinal properties. Ginger is not generally discovered to be an allergic food.

Previous research showed that the ginger improved insulin sensitivity and declined fasting glucose concentrations in induced diabetic rat (Wang et al., 2022, Rehman et al., 2019; Saravanan et al., 2017). Momoh et al. (2022) observed the antidiabetic properties of ginger towards induced diabetic Wistar rats through reduction of the blood sugar level, total cholesterol, triglyceride and low-density lipoprotein

cholesterol in blood plasma. Ginger has demonstrated significant protective impacts on complications of the liver, kidney, eye, and neural system of induced diabetic rats (Li et al., 2012). The finding reported by Wei et al. (2017) recommended the ginger as one of the dietary supplements for diabetic patients due to its antidiabetic activity property.

The use of ginger as the anti-diabetic agent should also be supported by a scientific evidence of the presence of bioactive compounds that can be used as the targeted metabolites during its quality control. Ginger contains various compounds that differ considerably between the types of plants and the areas where they are cultivated. It is known that there are more than 50 active constituents in ginger, usually separated into volatile and non-volatile compounds (Wang et al., 2019). Hydrocarbons, mostly monoterpenoid hydrocarbons and sesquiterpene, include the ginger's volatile compound which act as distinct flavour and taste. On the other side, gingerols, shogaols, paradols, and zingerone are non-volatile compounds. Zingerone is not naturally present in fresh ginger but is a by-product of thermal degradation of gingerol and shogaol generated during ginger drying (Ahmad et al., 2015). Ginger is also found to contain quercetin, catechin and rutin (Ghasemzadeh et al., 2011)

1.2 PROBLEM STATEMENTS

Despites numerous reports on anti-diabetic activity of ginger, the active constituent's metabolite profile is not sufficiently addressed in the literature. Hence, this present study was designed to identify the bioactive compounds found in the local ginger. Many compounds have been reported in various literatures, however none of them considered as compounds' synergistic and antagonistic effects when presented as an intact extract (Vaou et al., 2022). Thus, the compound clarification can be determined through the application of modern metabolomics approach. LCMS-based metabolomics was applied as the analytical instruments to identify the bioactive compounds. Metabolomics is one effective approach that has lately been widely used to profile the metabolites in medicinal plants. This technique is useful for complete examination of a wide range of plant metabolites in a short period of time. It facilitates the mapping of metabolites that have been shown to have bioactivity (He et al. 2018; Liu et al. 2020) as well as those that have yet to be identified. This strategy requires the use of sensitive

and accurate analytical instruments capable of detecting a wide range of chemicals. One of the widely used analytical instrument is LCMS. Currently, this technique has been applied to predict the antidiabetic activity of bioactive compounds from several plants, such as *Paederia foetida* L. (Tiwari and Rao 2002), *Psychotria malayana* Jack leaf (Nipun et al. 2021), and *Salacca zalacca* fruit (Saleh et al. 2021).

The relationship between these chemicals and bioactivity can be evaluated utilising docking techniques *in silico*, thereby verifying the results of metabolomics. This method virtually evaluated the interaction of the putative bioactive compounds (ligands) from medicinal plants and targeted protein (Maheshwari et al. 2021). This interaction generates specific binding energy of ligands-protein complex (Afriza et al. 2018). Lower binding energy indicated better stability of the ligand-protein complex (Woods et al. 2014). This technique is proven (Syihabudin et al. 2018; Torres et al. 2019) to speed up the screening process of bioactive compounds. It has been applied to evaluate the molecular interaction between α-glucosidase and bioactive compounds present in the extracts obtained from the bark of *Ceiba pentandtra* L. (Syihabudin et al., 2018), *Vicia faba* L. (Choudhary et al., 2018), *Vinca rosea* leaf (Qamar, 2022), *Agrimonia asiatica* (Kashchenko and Olennikov, 2020) and *Curculigo latifolia* stem (Zabidi et al., 2021).

1.3 RESEARCH HYPOTHESIS

1. *Z. officinale* rhizome extract will show α-glucosidase inhibitory and anti-oxidant activities.
2. LC-MS-based metabolomics can identify the α-glucosidase inhibitors and antioxidants in *Z. officinale* rhizome extract.
3. The protein-ligand interactions of α-glucosidase inhibitors from *Z. officinale* rhizome extract to the enzyme can be observed through *in silico* docking study.

1.4 RESEARCH QUESTIONS

1. What is the α-glucosidase inhibitory and anti-oxidant activities of the *Z. officinale* rhizome extract?
2. What is the α-glucosidase inhibitors and antioxidants in the ginger rhizome extract?
3. How do the α-glucosidase inhibitors from the ginger rhizome interact to the enzyme?

1.5 RESEARCH OBJECTIVES

1. To evaluate the α-glucosidase inhibition and antioxidant activity of *Z. officinale* rhizome extracts.
2. To identify the α-glucosidase inhibitors in *Z. officinale* rhizome extract using LC-MS-based metabolomics.
3. To elucidate the protein-ligand interactions of α-glucosidase inhibitors from *Z. officinale* rhizome extract using *Saccharomyces cerevisiae* isomaltose crystal structure.

1.6 SIGNIFICANCE OF THE STUDY

This study has emphasized the anti-oxidant and antidiabetic properties of Z. officinale rhizome extract. The primary function of α-glucosidase inhibitors is to block α-glucosidase, which prevents carbohydrates from being absorbed from the small intestine thus lowers blood glucose levels and aids in the control of postprandial hyperglycemia. Moreover, using LCMS-QTOF techniques, the α-glucosidase inhibitors from this plant extract have been discovered. Although the existence of these compounds has been reported in other plants, the presence of these compounds in ginger is being reported for the first time in this study, as well as the α-glucosidase inhibitory and anti-oxidant activities of some compounds. The impact of this study can lead to a decrease in mortality rate especially in type 2 diabetic patient by lowering the blood glucose levels, thus increasing the rate of the population for the future generation.

Moreover, the in silico molecular docking study revealed the protein-ligand interaction of these compounds to the α-glucosidase. This study provides more scientific evidence on the ability of ginger in diabetic therapy.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Zingiber officinale* BOTANY

Medicinal plants are often referred to as "Chemical Goldmines" because to their extensive repository of metabolites (Padalia, 2013). The metabolites derived from plants possess significant importance and find utility in several pharmacological applications. Throughout the course of human civilization, the reliance of individuals on plants for their healthcare requirements has been evident (Joy et al., 1998).

Ginger, officially identified as *Zingiber officinale* Roscoe and classified under the family Zingiberaceae, holds significant botanical significance due to its diverse ethnomedical, therapeutic, and nutritional properties. Consequently, it is extensively utilised as a taste enhancer, culinary spice, and herbal medicine (Britannica, 2024). *Zingiber officinale,* commonly known as ginger, has been historically utilised in various traditional medicinal practices including Traditional Chinese Medicine, Jamu, Ayurveda, Arabian, Africans, Caribbean, and others (Kumari, Kumar & Solankey, 2020). Its therapeutic applications encompass a wide range of ailments such as diabetes, nausea, vomiting, asthma, cough, palpitation, inflammation, dyspepsia, loss of appetite, constipation, indigestion, and pain (Grzanna et al., 2005).

The Zingiberaceae family is recognised as one of the most extensive families within the order Zingiberales, and it holds the distinction of being the largest family among monocotyledonous plants (Benedict et al., 2016). The occurrence of this phenomenon is predominantly observed in tropical regions, encompassing around 52 taxa and 1400 species (Ferdous et al., 2022). The highest concentration of this phenomenon is found in the Indo-Malayan region of Asia, namely in India, where it is represented by 22 genera and 178 species (Dhanik et al., 2017). The Zingiberaceae family is a notable group that holds substantial potential for economic opportunities. Several species of this plant family exhibit the capacity to generate important commodities such as spices, dyes, perfumes, and pharmaceuticals, while others fulfil

ornamental purposes. The bioactive compounds of *Z. officinales* are mentioned in Ayurveda and other traditional therapeutic practices (Sharifi-Rad et al., 2017). A plethora of scholarly articles have been published on the biological attributes of Zingiberaceae extracts, which include antibacterial, antioxidant, anticancer, and immunostimulatory properties (Ballester et al., 2023). The aforementioned extracts are recognised for their composition of various essential oils, including terpenes, alcohols, ketones, flavonoids, carotenoids, gingeroles, and phytoestrogens (Habsah et al., 2000). Ginger is classified as an herbaceous rhizomatous perennial plant that can attain a maximum height of 90 cm when cultivated. The rhizomes exhibit fragrant properties and possess a thick, lobed structure that is pale yellowish in colour. These rhizomes bear simple, alternate, distichous leaves that are thin, oblong, and lanceolate in shape (Pakrashi & Pakrashi, 2003). Based on Meenu and Jebasingh (2019), the herb exhibits the growth of several lateral branches in clusters, which commence desiccation upon reaching maturity. As stated by Xizhen, Jinfeng, and Xia (2016), The leaves exhibit elongated morphology, measuring approximately 2 to 3 centimetres in width, and feature bases that are enveloped by sheaths. The leaf blade gradually narrows towards an apex. The inflorescence is characterized by solitary, lateral, radical pedunculate oblong cylindrical spikes (Aleem et al., 2020). According to Mishra (2012), flowers exhibit characteristics of rarity, tiny size, a superior calyx with three teeth, and an opening that splits on one side. The corolla consists of three subequal segments that are oblong to lanceolate in shape and connate, with a greenish coloration.

2.2 ETHNOMEDICINAL USES OF *Zingiber officinale*

Zingiber officinale, often known as ginger, is a plant of considerable significance due to its extensive ethnomedicinal and nutritional properties. As a result, it is widely used on a global scale as a spice, flavouring agent, and herbal cure. *Zingiber officinale*, commonly known as ginger, has been utilised in various traditional medicinal systems, including Ayurveda, Siddha, Chinese, Arabian, Africans, Caribbean, and others, for the treatment of diverse ailments such as nausea, vomiting, asthma, cough, palpitation, inflammation, dyspepsia, loss of appetite, constipation, and indigestion (Kumar et al., 2011).

Zingiber officinale, often known as ginger, is frequently employed in the practice of traditional medicine within the Asian and Chinese traditional medicine frameworks. Table 2.1 presents a comprehensive compilation of the various conventional medicinal applications attributed to *Z. officinale*. Ginger is widely used as a culinary ingredient across several global cuisines. The utilisation of ginger rhizome has been prevalent in Ayurvedic and traditional Chinese medicinal practices for addressing various health conditions, notably gastrointestinal disorders such as motion sickness and pregnancy-related nausea and vomiting, abdominal spasms, as well as respiratory and rheumatic ailments. Ginger is commonly employed as a home medicine for many gastrointestinal ailments, such as dyspepsia, flatulence, stomach discomfort, and nausea. According to Kunnumakkara et al. (2009), herbalists have suggested the use of this substance for its potential carminative, diaphoretic, antispasmodic, expectorant, peripheral circulatory stimulant, and astringent properties.

Figure 2.1 The Plant of *Zingiber officinale.*

Table 2.1 Traditional and modern uses of *Zingiber officinale*.

Indications	Plant part used	Herbal preparation	Prescription and dosage form	Reference
Osteoarthritis	Rhizomes	NAD	255 mg capsule extracted from 2,500–4,000 mg of dried ginger rhizomes	(Altman & Marcussen, 2001)
	NAD	NAD	Ginger extract in Nanostructure Lipid Carrier (NLC) rubbed three times a day for 12 weeks	(Amorndoljai et al., 2015)
	NAD	NAD	NAD	(Rahmani et al., 2014)
Nausea	NAD	NAD	1-gram ginger capsule	(Seidi et al., 2017)
	NAD	NAD	NAD	(Ernst & Pittler, 2000)
	NAD	Fresh ginger juice	NAD	(Haniadka et al., 2013)
Myalgia and soreness	Rhizomes	NAD	Ginger capsule	(Black et al., 2010)
	NAD	NAD	2-gram ginger capsule	(Black & O'Connor, 2010)
Diabetes	Fresh rhizomes	NAD	1-gram ginger in a capsule	(Khandouzi et al., 2015)
Dyspepsia	NAD	NAD	NAD	(Haniadka et al., 2013)
Dysmenorrhea	NAD	NAD	Oral ginger	(Chen et al., 2016)
	Ginger root	NAD	500 mg capsule of ginger root powder	(Rahnama et al., 2012)
Lipid profile management	NAD	NAD	3-gram ginger capsule	(Alizadeh-Navaei et al., 2008)
Antioxidants	Leaves and rhizomes	NAD	NAD	(Butt & Sultan, 2011; Ujang et al., 2015)

Note. NAD: Not appropriately described.

2.3 PHYTOCHEMISTRY OF *Zingiber officinale*

Zingiber officinale, commonly known as ginger, has numerous bioactive components, including phenolic and terpene chemicals (Prasad & Tyagi, 2015). Some examples of phenolic compounds include gingerol, paradols, and shogaol. On the other hand, examples of terpene compounds include zingiberene, β-bisabolene, α-farnesene, β-sesquiphellandrene, and α-curcumene.

Table 2.2 Reported Phytoconstituents of *Zingiber officinale*

Category	Examples	References
Phenolic Compounds	Gingerol, Paradols, Shogaol	(Liu et al., 2019; Prasad & Tyagi, 2015)
Terpene Compounds	Zingiberene, β-bisabolene, α-farnesene, β-sesquiphellandrene, α-curcumene	(Tyler, 1994; Langner, 1998; Prasad & Tyagi, 2015)
Volatile Oils	Zingiberene, β-sesquiphellandrene, α-terpinene, α-terpineol, terpinolene, γ-terpinolene, cineole, β-eudesmol, nerol, trans-nerolidol, 4-isopropylbenzyl alcohol, ,7-dimethylocta-1,6-dien-3-ol, 3,7-dimethyloct-6-en-1-yn-3-ol, 3-methylhexan-2-ol, cis-piperitol, borneol, elemol, myrtenol, citronellol, geraniol, muurolol, 2-methoxy-1,7,7-trimethylbicyclo-heptane, 1-isopropyl-4-methylcyclohex-3-enol, 2-tetradecanol, trans-4-isopropyl-1-methyl-2-cyclohexen-1-ol, cis-4-isopropyl-1-methyl-2-cyclohexen-1-ol, 2-heptanol	(Tan, 2011)
Others	Polysaccharides, Lipids, Organic Acids, Vitamins, Minerals, Raw Fibres, Amino Acids, Sugars, Inorganic Elements	(Hsiang et al., 2013; Prasad & Tyagi, 2015)

Zingiber officinale, often known as ginger, has been found to contain over 400 distinct phytoconstituents. These phytoconstituents can be broadly classified into three main groups: volatile oils, gingerol, and diarylheptanoids (Zhang et al., 2012; Prasad & Tyagi, 2015; Liu et al., 2019). In addition to the aforementioned primary categories, ginger has been found to contain various additional chemicals, including polysaccharides, lipids, organic acids, vitamins, minerals, and raw fibres (Hsiang et al., 2013; Prasad & Tyagi, 2015). The elements can also be categorised into aromatic and

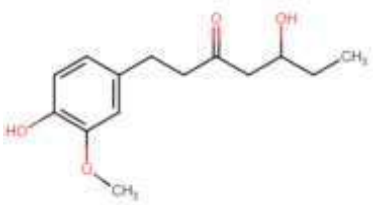
pungent components. Bisabolene and zingiberene are among the aromatic elements, as documented by Tyler (1994). Additionally, gingerols and shogaols are recognised as examples of pungent ingredients (Figure 2.2).

The initial classification pertains to volatile oils, alternatively referred to as essential oils. According to Dagli et al. (2015), essential oils are characterised as secondary metabolites found in plants, comprising terpenic hydrocarbons and oxygenated derivatives including aldehydes, ketones, epoxides, alcohols, and esters. Ginger essential oils mostly consist of terpene molecules, including zingiberene and β-sesquiphellandrene (Langner, 1998). The distinctive olfactory characteristic of gingeris attributed to the presence of volatile oils. Nevertheless, the chemical composition of ginger volatile oils varies depending on the time of harvest (Zhang et al., 2012; Liu et al., 2019; Mahboubi, 2019). According to a study conducted by Onyenekwe and Hashimoto in 1999, it was shown that dried ginger rhizomes have greater quantities of essential oil and β-zingiberene when compared to fresh ginger rhizomes. Some examples of other volatile oils present in ginger are *α*-terpinene, *α*-terpineol, terpinolene, *γ*-terpinolene, cineole, *β*-eudesmol, nerol, *trans*-nerolidol, 4-isopropylbenzyl alcohol, ,7-dimethylocta-1,6-dien-3-ol, 3,7-dimethyloct-6-en-1-yn- 3-ol, 3-methylhexan-2-ol, cis-piperitol, borneol, elemol, myrtenol, citronellol, geraniol, muurolol, 2-methoxy-1,7,7-trimethylbicyclo-heptane, 1-isopropyl-4-methylcyclohex-3-enol, 2-tetradecanol, trans-4-isopropyl-1-methyl-2-cyclohexen-1-ol, cis-4-isopropyl-1-methyl-2-cyclohexen-1-ol, 2-heptanol (Tan, 2011).

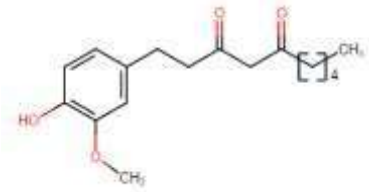
The subsequent group to be discussed is gingerol, as depicted in Figure 2.2. The pungency of *Zingiber officinale* is derived from the chemical component known as gingerol. Gingerol is a compound composed of many constituents that possess the functional group 3-methoxy-4-hydroxyphenyl (Zhang et al., 2012; Liu et al., 2019). According to Mao et al. (2019), it has been observed that gingerols have the ability to undergo a conversion into equivalent shogaols when subjected to heat treatment or long-term storage. Furthermore, it has been noted that shogaols, in turn, can undergo a transformation into paradols through a process of hydrogenation. The various fatty acid chains connected to this particular functional group are what determine the modifications (Zhang et al., 2012; Liu et al., 2019). The third category comprises diarylheptanoids, which are characterised as a class of compounds with 1,7-

disubstituted phenyl groups and heptane skeletons within their fundamental structure (Zhang et al., 2012; Liu et al., 2019). Diarylheptanoids, alternatively referred to as diphenylheptanoids, can manifest in two distinct forms: macrocyclic or open-chain diarylheptanoids (Ganapathy et al., 2019). In addition, *Zingiber officinale* also contains minor ingredients, including amino acids, sugars, organic acids, and inorganic elements.

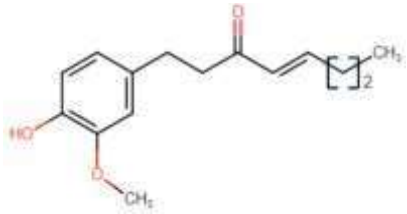
The *Zingiber officinale* plant contains a diverse range of components that exhibit distinct bioactivities. Zingerone, gingerol, zingiberene, gingerols, and shogaols have been identified as the primary bioactive constituents found in ginger, exhibiting notable anti-diabetic and antioxidant properties. In their research, Akhani et al. (2005) examined the anti-diabetic efficacy of galanolactone and gingerol, which were both extracted from *Z. officinale*. The findings of the study indicated a notable decrease in fasting glucose levels and an increase in insulin levels among non-insulin dependent type diabetic mellitus rats that received treatment with galanolactone and gingerol derived from *Zingiber officinale*. Another research study conducted by Al-Amin et al. (2006) similarly yielded comparable findings, wherein diabetic rats fed with ginger exhibited a significant decrease of 52% in serum glucose levels compared to diabetic rats in the control group over a period of 7 weeks.



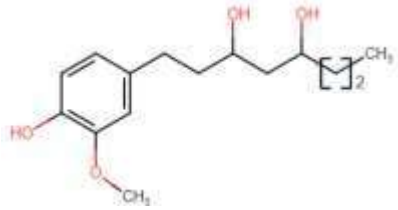
3-Gingerol



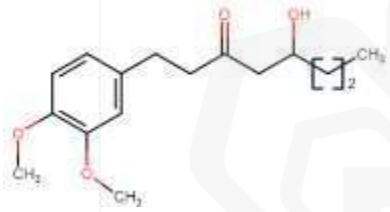
6-Gingerdione



4-Shogaol

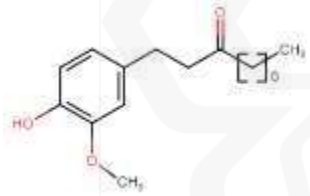


4-Gingerdiol

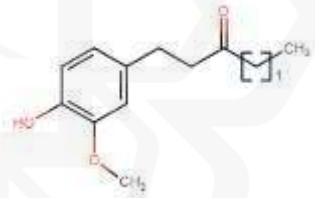


4-Gingeryl methyl ether

6-Zingerine



Zingerone



Paradol

Figure 2.2 Some examples of gingerol derivatives isolated from *Zingiber officinale.*

Zingiber officinale has also been investigated for its antimicrobial properties. According to Ali Hasan (2012), many compounds derived from ginger extract, including gingerol, zingiberene, β-bisabolene, shogaol, and β-sesquiphellandrene, have demonstrated significant inhibitory effects against Gram-positive bacteria and the fungus strain *Candida albicans*. In a different study, Moon et al. (2018) found that several chemicals, including terpinene, isoborneol, and citral, significantly inhibited the growth of *Aspergillus flavus*. The anti-inflammatory properties of zingerone have been substantiated through the observation that ginger effectively ameliorated colitis produced by 2,4,6-trinitrobenzene sulphonic acid (TNBS) by modulating the activity of

NF-κB and the IL-1β signalling pathway (Hsiang, 2013). The effects of ginger extracts on apoptosis, proliferation, and differentiation have been shown in the normal-appearing colonic mucosa. According to Citronberg (2013), it has been observed that gingerols have the ability to decrease the proliferation in normal-appearing colorectal epithelial crypts and trigger apoptosis. This observation demonstrates the potential of ginger extract in the domain of cytotoxicity. In addition, it has been observed that certain components of ginger, namely 6-shogaol and 6-paradol, exhibit neuroprotective properties. Studies have indicated that these chemicals effectively inhibit neuronal cell death and mitigate hippocampal synapse damage in mice (Huh et al., 2018). There have been reports indicating that the use of ginger also demonstrates cardiovascular protection. Akinyemi et al. (2016) have confirmed the presence of substances in ginger such as catechin, quercetin, kaempferol, luteolin, and curcumin. These compounds have been found to inhibit the activity of adenosine deaminase, leading to an increase in adenosine levels. Adenosine is known for its vasodilatory properties and its ability to inhibit platelet aggregation (Akinyemi et al., 2016b). This intervention is expected to yield antihypertensive advantages. In addition, ginger has been subjected to testing in order to evaluate its potential inhibitory effects on adipogenesis and lipid buildup. Previous studies have reported that zingerone exhibits a significant inhibitory impact on adipogenesis and lipid formation, leading to a reduction in fat mass in mice subjectedto experimentation (Pulbutr et al., 2011; Li et al., 2023). There have been suggestions that ginger possesses properties that may exhibit anti-nausea and anti-emetic effects. Jinet al. (2014) conducted a study that observed the inhibitory effects of three pungent chemicals found in ginger, namely 6-shogaol, 6-gingerol, and zingerone, on serotonin (5-HT) receptor activity. The investigated compounds demonstrated non-competitive antagonist activity against the serotonin receptor, resulting in the manifestation of anti-nausea and anti-emetic properties.

2.4 PHARMACOLOGICAL ACTIVITIES

Ginger is a botanical ingredient that possesses both culinary and medicinal properties, exhibiting considerable biological and therapeutic prospects. The plant under consideration, which is native to India, has been utilised in Ayurvedic and traditional Chinese medicinal practices for numerous centuries to address a diverse array of health conditions (Mbaveng & Kuete, 2017). *Zingiber officinale* has been documented to

possess various pharmacological properties, including anticancer (Ghasemzadeh et al., 2012; Park et al., 2014), antiemetic (Ryan et al., 2012; Ullah et al., 2015), anti-inflammatory (Mahluji et al., 2013; Ezzat et al., 2018), antinociceptive (Vendruscolo et al., 2006; Hasanein & Gomar, 2014), antioxidant (Ghasemzadeh, 2010; Tohmah et al., 2017), antiarthritic (Murugesan, 2020), cardiovascular disease effects (Akinyemi et al., 2014; Wang et al., 2017), and effects on the gastrointestinal tract (Mathiyazhagan & Gothandam, 2021; Murukesan & Kesavan, 2022).

2.4.1 Antioxidant Activity

Antioxidants have the ability to counteract the detrimental effects of free radicals, which have the potential to induce cellular harm and contribute to the development of various ailments, including cancer, skin disorders, and age-related conditions. In a study conducted by Heeba et al. (2014), it was observed that curcumin, a bioactive constituent found in both ginger and turmeric, exhibited antioxidant and anti-inflammatory properties. The administration of curcumin resulted in the induction of haemoglobin, a crucial factor in the prevention of vascular inflammation and the preservation of endothelial cells from oxidative stress. Antioxidant activity serves to impede the presence of reactive oxygen species (ROS), including superoxide, peroxides, hydroxyl radicals, singlet oxygen, and alpha-oxygen, with the aim of averting potential harm to DNA, which is linked to the development of cancer, cardiac illness, and age-related ailments. According to previous studies (Dikalov, 2011; Yeh et al., 2014), it was found that ginger extracts, specifically gingerol, shaogouls, and curcumin, exhibited notable efficacy in terms of scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. The findings of this study indicate that the aqueous extract of ginger rhizomes exhibited more efficacy compared to the ethanolic extract. This is supported by the lower EC_{50} values observed for the aqueous extract, which were measured at 11.95 ± 0.05 and 8.96 ± 0.08 (g/100 g). In contrast, the EC_{50} values for the ethanolic extract were higher, measuring at 8.44 ± 0.16 and 6.96 ± 0.13 (g/100 g).

2.4.2 Anticancer Activity

The results of the study by Cheng et al. (2011) show that steamed ginger extract has superior chemopreventive and chemotherapeutic properties in relation to cervical cancer. The processed ginger extract had a more pronounced antiproliferative effect on the development of human Hela carcinoma cells compared to both fresh and dry ginger. We found that cells grew much slower when they were exposed to 100 μg/ml of steamed ginger for 24 hours and 48 hours, compared to fresh ginger (31.45 ± 2.86%) and dry ginger (47.77 ± 1.51%). The differences were 70.92 ± 1.18% and 89.48%, respectively. The polyphenol derived from ginger has been characterised as having anticancer properties and potential for mitigating degenerative illnesses. According to a study conducted by Danciu et al. (2015), it was shown that the ethanolic extract of Curcuma rhizome exhibited a greater impact on the proliferation and death of B164A5 murine melanoma cells compared to the ethanolic extract of ginger rhizome.

2.4.3 Anti-Inflammatory and Analgesic Activity

When tissues are subject to injury or infection, inflammation is a crucial immune system response that is essential for promoting survival. The inflammatory response plays a crucial role in the restoration of tissue homeostasis. The anti-inflammatory qualities of ginger have been recognised for a significant period of time and were first documented as early as 1993, when ginger's ability to scavenge O_2 was published. In their study, Penna et al. (2003) investigated the impact of the hydroalcoholic extract derived from ginger rhizome on rat paw edema produced by carrageenan. The rat paw edoema caused by carrageenan, compound 48/80, or serotonin was lowered by an intraperitoneal dose of alcoholic ginger extract. With dosages of 0.6 and 1.8 mg/site, the extract successfully suppressed 48/80-induced rat cutaneous edema. Multiple lines of evidence have been presented, primarily in diverse animal models of inflammation, with limited representation in human subjects or human cell studies. The gingerols have been identified as the primary constituents associated with the pharmacological effects of the crude medicine. Gingerol has been identified as the predominant phenolic metabolite, as reported by Alharbi et al. (2022). Among the several homologs of gingerol, 6-gingerol has been found to be the most prevalent, as documented by Loung et al.

(2019). Researchers Sang et al. (2009) found that shaogols were more effective at stopping H-1299 human lung cancer cells and HCT-116 human colon cancer cells than gingerols. This is similar to what Ali et al. (2008), Alizadeh-Navaei et al. (2008), and Amorndoljai et al. (2015) found. Numerous in vivo investigations employing animal models have extensively documented the anti-inflammatory properties of ginger and its constituent compounds. Funk et al. (2016) looked at how ginger can reduce inflammation in lipopolysaccharide (LPS), which is a main part of the outer cell wall of Gram-negative bacteria. The researchers generated rheumatoid arthritis in female Lewis rats as part of their evaluation. According to a study by Podlogar and Verspohl (2012), the pungent-enriched oily extract had stronger inhibitory effects on lipopolysaccharide-induced IL-8 production than the ginger spissum extract. Nevertheless, it has been documented that the substances 6-gingerol and 6-shaogols exhibited no significant impact on the production of IL-8. This lack of effect may be attributed to the potential variability in the extractant or extraction methods employed during the preparation of the extracts. The anti-inflammatory properties of ginger are commonly attributed to its gingerols. However, Funk et al. (2016) conducted experiments that demonstrated the joint-protective effects of both the essential oils and gingerols in an animal model of arthritis.

2.4.4 Benefits in Gastrointestinal Tract (GI Tract)

Ginger is highly regarded for its therapeutic attributes, specifically as a carminative and stimulant for the gastrointestinal tract (Moghaddasi & Kashani, 2012). Ginger is recognised for its sialagogue properties and is frequently employed in traditional medicine for the treatment of digestive issues, as documented by Malu et al. (2009). Ginger has been investigated using animal models to elucidate the mechanisms underlying its gastric stimulating activity. Multiple studies have demonstrated the efficacy of ginger in the treatment of nausea induced by conditions such as seasickness, morning sickness, and chemotherapy. A study conducted by Hickok et al. (2007) examined the impact of incorporating ginger into a conventional antiemetic medicine on the severity of post-chemotherapy nausea in a limited sample of cancer patients undergoing chemotherapy. The findings revealed that the inclusion of ginger resulted in a further reduction in the intensity of nausea experienced by these individuals. A

single-blind clinical trial study was conducted to assess the impact of ginger on pregnancy-related nausea and vomiting. The study included a patient group with a mean gestational age of 13 ± 3 weeks. Over a span of four days, the group of individuals who consumed ginger exhibited a significantly greater rate of improvement compared to the group that received a placebo, with a respective improvement rate of 85% and 56%. The study conducted by Ozgoli et al. (2009) found that the reduction in episodes of vomiting was considerably higher among individuals who consumed ginger comparedto those who were administered a placebo. Specifically, the ginger users saw a 50% decrease in vomiting instances, whereas the ladies who received the placebo only experienced a 9% decrease. In their study, Prakash and Srinivasan (2013) examined theimpact of ginger on the absorption of minerals, specifically iron, calcium, and zinc, overa period of 8 weeks. The findings of the study revealed an increased uptake of these elements inside the intestinal segments. According to the Hu et al. (2011) study, patientswith dyspepsia had a faster rate of stomach emptying after receiving ginger versus a placebo. The median half-emptying time after ginger was 12.3 minutes (range: 8.5–17.0minutes), whereas after placebo, it was 16.1 minutes (range: 8.3-22.6 minutes). The useof ginger by Wistar rats has been observed to cause changes in the fluidity and permeability of the brush border membrane (BBM) in the intestines. According to Kieliszek et al. (2020), there was an observed augmentation in the absorptive area of the small intestine, subsequently leading to a decrease in cholesterol levels in both the jejunum and ileum. According to Srinivasan (2017), it has been observed that the use of ginger in one's diet has the potential to enhance the activity of specific digestive enzymes located in the brush border membrane (BBM) of the small intestine mucosa. These enzymes include glycyl glycine dipeptidase, leucine aminopeptidase, and gamma-glutamyl transpeptidase. The study conducted by El-Abhar (2008) demonstrated that ginger extract (GE) has the capacity to mitigate the severity of ulcerative colitis in rats with ulcers. The effectiveness of GE was found to be comparable to that of sulfasalazine, particularly at the highest dosage level.

Table 2.3 *In vitro* and *in vivo* studies supporting pharmacological activities of *Zingiber officinale*.

Pharmacological assay	Extraction method	Observations	Bioactive compounds	References
Antioxidant activity				
Amylase and α-glucosidase inhibition activity	Homogenized, centrifuged then vacuum-filtered through Whatman No. 1 filter paper	• Ginger extracts tested with α-Glucosidase caused 10% reduction in the production of glucose • 15% reduction in glucose production due to the ginger extract (Amylase)	NR	(Abeysekara et al., 2007)
Hepatic enzymes inhibition activity	Homogenized in ethanol, kept on water bath, centrifuged then evaporated at low temperature	• Administration of ginger extracts dose dependently decrease serum transaminases and ALP activities by enhancing hepatic antioxidant activity	• Polyphenols	(Ajith et al., 2007)
Erythrocyte CuZn-SOD activity	NR	• At day 64, enzymatic antioxidant activities, including CuZn-SOD, CAT and GPx, of the ginger extract group were significantly higher compared to those of the placebo group	• 6-gingerol	(Danwilai et al., 2017)

Anti-Microbial activity				
Agar-well diffusion assay	Soxhlet extraction with methanol and n-hexane	• The inhibition activity by the extracts were more effective towards Gram-positive bacteria in comparison to the Gram-negative bacteria • Growth of *candeda albicans* fungi were also inhibited by the extracts of *Zingiber officinale*	• Gingerol • Zingiberene • β-bisabolene • α-famesene • Shogaol • β-sesquiphellandrene • α-curcumene • γ-terpinene	(Ali Hasan, 2012)
Inhibitory effects on growth and aflatoxin production of *Aspergillus flavus*	NR	• γ-terpinene, isoborneol, and citral exhibited strong anti-fungal activity at 1000 lg/mL • γ-terpinene and citral markedly reduced the expression of most of the 11 genes involved in aflatoxin biosynthesis by A. flavus	• Isoborneol • Citral	(Moon et al., 2018)
Anti-Inflammatory Activity				
Synthesis of thromboxane-B_2, prostaglandin-E_2, and cholesterol and triglyceride levels in the serum of normal rats	Homogenized in NaCl, filtered then centrifuged	• High oral doses of ginger were effective in reducing the synthesis of PGE_2 and TXB_2 levels in serum • Significant reduction in levels of cholesterol was observed in the rats given high dose of ginger	• NR	(Thomson et al., 2002)

Modulation of nuclear factor-κB activity and interleukin-1β signalling pathway	Ground to fine powder, extracted by mixing with ethanol then lyophilized	• Ginger improved 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis via modulation of NF-κB activity and IL-1β signalling pathway	• Zingerone	(Hsiang et al., 2013)
Cytotoxicity				
Effects of ginger on apoptosis, proliferation, and differentiation in the normal-appearing colonic mucosa	NR	• Ginger extract reduced the proliferation in the crypts of normal-appearing colorectal epithelium and increase apoptosis and differentiation relative to proliferation especially in the differentiation zone of crypts	• Gingerols	(Citronberg et al., 2013)
COX-1 and NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) inhibition activity	NR	• Ginger significantly lowered COX-1 protein expression in participants at increased risk for colorectal cancer but not in those at normal risk for CRC • Ginger did not alter 15-PGDH protein expression in either increased or normal-risk participants	• NR	(Jiang et al., 2013)

Neuroprotection

Hippocampal neuronal cells protective activity	Heated and extracted with 70% ethanol	• Fermented ginger inhibited neuronal cell loss and synapse disruption in the mouse hippocampus by observation through immunohistochemistry	• 6-shogaol • 6-paradol	(Huh et al., 2018)

Cardiovascular Protection

Modulation of platelet ectonucleotidase activity	NR	• Turmeric and ginger rhizomes caused an increase in adenosine diphosphate and adenosine monophosphate hydrolysis and inhibited adenosine deaminase activity • Increased the level of adenosine, a potent vasodilator and inhibitor of platelet aggregation thus exerting antihypertensive benefits	• Gallic acid • Catechin • Caffeic acid • Epicatechin • Rutin • Quercitrin • Quercetin • Kaempferol • Luteolin • Curcumin	(Akinyemi et al., 2016)
Hypolipidemic and insulin sensitizing effects	Maceration in rotary shaker, extracted with 70% ethanol, filtered then lyophilized	• Ginger extract improved lipid profile and attenuated the increase of plasma levels of glucose, insulin and leptin in high fat diet rats • Ginger extract caused higher liver expression of peroxisome proliferator- activated receptors α, PPARγ and glucose transporter 2 and an enhancement of plasma adiponectin levels	• NR	(De Las Heras et al., 2017)

Anti-obesity Activity				
Adipogenesis and lipid accumulation inhibitory activity	NR	• Gingerone A had the most potent inhibitory effect on adipogenesis and lipid accumulation in 3T3-L1 cells among ginger components tested at a single concentration (40 μM) • Gingerone A diminished diet-induced obesity by reducing fat mass in mice	• Gingerone A	(Suk et al., 2017)
Antidiabetic Activity				
Antihyperglycemic activity	Homogenized in sterile NaCl, filtered then centrifuged	• 52% reduction of serum glucose levels of the ginger-treated diabetic rats in comparison to the control diabetic rats after 7 weeks of treatment • After 7 weeks, the urine protein levels of the ginger-treated diabetic rats were approximately equal to the level in normal rats	• NR	(Al-Amin et al., 2006)
5-HT receptors inhibition activity	Extracted with methanol using a Soxhlet extractor	• Significant decrease in fasting glucose and increase in insulin levels in non-insulin dependent type diabetes mellitus rats which treated by *Zingiber officinale*	• Galanolactone • Gingerol	(Akhani et al., 2005)

Anti-nausea and Anti-emetic Activities

Inhibition of serotonin (5-HT) receptor activity	Finely grounded, extracted with water, filtered then freeze dried	• Pungent constituents of the ginger, 6-shogaol, 6-gingerol, and zingerone inhibited 5-HT responses in a dose dependent manner • The tested ginger constituents exerted its effect as non-competitive antagonist	• Zingerone • 6-gingerol • 6-shogaol	(Jin et al., 2014)

Note. NR: Not Reported

2.5 DIABETES MELLITUS

2.5.1 Definition and Epidemiology

Diabetes mellitus (DM) is widely recognised as a prevalent metabolic disorder characterised by the presence of hyperglycemia, a chronic elevation of blood sugar levels. This condition is closely linked to the disruption of normal body metabolism, specifically affecting the breakdown of carbohydrates, fats, and proteins. Diabetes mellitus (DM) is commonly recognised as a substantial health concern, leading to notable rates of illness and death as a result of problems in the microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (ischemic heart disease, stroke, and peripheral vascular disease) systems.

The World Health Organisation (WHO) has provided a definition of diabetes mellitus (DM) as a metabolic illness characterised by hyperglycemia as its primary clinical manifestation (Mohan & Nandhakumar, 2014). Diabetes mellitus (DM) is a medical disorder that arises from impaired insulin secretion and/or insulin action, or a combination of both factors (American Diabetes Association, 2010). Insulin is the endocrine hormone that plays a crucial role in regulating and stabilising elevated levels of blood glucose within the physiological range of 80 to 126 mg/dl. The exclusive

transfer of monosaccharides, such as glucose and fructose, from the intestinal lumen to the circulation is seen. Prior to absorption in the colon, it is necessary to hydrolyze complex starches, oligosaccharides, and disaccharides into their constituent monosaccharides. The process of digestion is aided by enteric enzymes, such as pancreatic α-amylase, as well as α-glucosidases that are located on the brush edge of the intestinal cells (Van de Laar et al., 2005).

The incidence of diabetes mellitus (DM) is seeing a notable escalation on a global scale, impacting various regions across the world. Many developing countries, including Malaysia, have observed a significant rise in the number of reported cases in recent times. The phenomenon of rapid globalisation has been accompanied by a significant increase in the prevalence of metabolic disorders among Malaysian residents, which can be attributed to their sedentary lifestyle and consumption of unhealthy food (Noor et al., 2017).

According to Loh and Hadira (2011), the global prevalence of diabetes was around 170 million individuals in the year 2000. Projections indicate that this figure is expected to rise to 366 million by the year 2030. The World Health Organisation (WHO) conducted a comprehensive statistical analysis in 2014 to examine the prevalence of diabetes mellitus (DM) among persons aged 18 years and older. The investigation revealed that the prevalence of DM has increased from 4.7% in 1980 to 8.5% in 2014. In addition, it is concerning to note that the incidence of diabetes mellitus (DM) is increasing at a rapid rate throughout the middle-class and low-income populations of developing nations, such as Malaysia. The National Health and Morbidity Surveys (NHMS) conducted in Malaysia have revealed a consistent rise in the reported prevalence of diabetes mellitus (DM) among adults over the years. Specifically, the surveys conducted in 1996, 2006, 2011, and 2015 indicated prevalence rates of 6.9%, 11.6%, 15.2%, and 17.5%, respectively (Tee and Yap, 2017). This data demonstrates a significant increase of more than double in DM prevalence within a span of two decades.

2.5.2 Types of Diabetes Mellitus

2.5.2.1 Type 1 Diabetes Mellitus

Frequently, this condition is identified in individuals who are children or young adults, leading to its prior designation as juvenile onset diabetes (JOD). This condition is commonly referred to as insulin-dependent diabetes mellitus. Type 1 diabetes mellitus (DM) is characterised by a clinical presentation in which individuals exhibit a significant reliance on exogenous insulin administration for glycemic control. Type 1 diabetes mellitus (DM) can be categorised into two subtypes: immune mediated DM and idiopathic DM (Mohan & Nandhakumar, 2014). The former is characterised by the cellular-mediated autoimmune destruction of β-cells in the pancreas, while the latter lacks a clear aetiology. The immune-mediated form of diabetes mellitus (DM) can result in a patient experiencing a lack of insulin. Several markers that can be identified in the phenomena encompass islet cell autoantibodies, autoantibodies targeting GAD (GAD65), autoantibodies against insulin, and autoantibodies directed at the tyrosine phosphatases Islet Autoimmunity (IA)-2 and IA-2b (Ying et al., 2014). The condition is frequently correlated with the coexistence of other ailments such as autoimmune hepatitis, Grave's disease, pernicious anaemia, Addison's disease, and Hashimoto's thyroiditis (Mohan & Nandhakumar, 2014). Conversely, idiopathic type 1 diabetes is observed in patients who exhibit persistent insulin deficiency.

2.5.2.2 Type 2 Diabetes Mellitus

It is alternatively referred to as non-insulin dependent diabetes mellitus (NIDDM) or maturity onset diabetes in obese and non-obese individuals. Type 2 diabetes is characterised by the highest incidence of diabetes mellitus and primarily affects older adults, with a particular emphasis on obese adolescent children (Mohan & Nandhakumar, 2014). Patients with type 2 diabetes do not experience autoimmune destruction of β cells. The majority of patients in this study exhibit obesity, which has been identified as the primary factor contributing to the onset of diabetes metabolic disease. However, it is worth noting that in certain instances, individuals with a normal

body mass index but elevated amounts of visceral fat are also susceptible to developing type 2 DM (American Diabetes Association, 2010).

2.6 *IN VITRO* BIOASSAY TO SCREEN ANTI-DIABETIC EFFECTS OF *Zingiber officinale*

The *in vitro* bioassay is a significant analytical technique characterised by its high capacity, cost-effectiveness, and ability to yield speedy findings. This approach holds potential for several applications, including pre-screening, screening, monitoring, and secondary testing. Bioassays are used to screen initial samples in order to pre-screen and select materials for secondary testing, thereby assessing the potential bioactivity of the desired or prospective substances. Several pre-screening bioactivity assays were conducted in this investigation, which encompassed the following:

2.6.1 Antioxidant Activity

Oxidative stress is characterised by an inherent disparity between the overall presence of reactive oxygen species or free radicals in the body and the body's capacity to effectively neutralise or eliminate these reactive intermediates, as well as repair the ensuing damage through the neutralising effects of antioxidants. Free radicals are typically generated as a result of routine physiological processes and biochemical reactions within the human body. These reactive molecules have been extensively linked to various health conditions, including cancer, ageing, hair loss, inflammation, immunosuppression, diabetes, atherosclerosis, and ischemic heart disease, as well as neurodegenerative disorders like Parkinson's and Alzheimer's disease (Surveswaran et al., 2007). Hence, it can be inferred that oxidative stress plays a significant role in the development and pathogenesis of numerous issues associated with chronic diseases. Oxidative stress can also arise as a consequence of hyperglycemia, occurring during the glucose oxidation process (Maritim et al., 2003). The presence of high blood sugar levels in individuals with diabetes contributes to the process of lipid peroxidation in low-density lipoprotein (LDL) through a route that is dependent on the presence of superoxide. This, in turn, leads to a significant rise in the concentration of free radicals

within the body (Giacco et al., 2010). Due to this rationale, the assessment of antioxidant properties has emerged as a prevalent method for evaluating the biological activity of plant extracts (Clarke et al., 2013). Numerous botanical species have served as valuable sources of significant antioxidants, including polyphenols. These compounds exhibit their efficacy through mechanisms such as the adsorption and neutralisation of free radicals, the quenching of singlet and triplet oxygen, and the decomposition of peroxide (Anderson et al., 2001).

2.6.1.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging (DPPH Assay)

Various antioxidant assays are employed to evaluate the antioxidant activity of plant extracts. Among these, the most commonly employed method is the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, which is rapid, standardised, and uses a colorimetric approach to measure antioxidant scavenging ability. Typically, this methodology is employed for the evaluation of plant activity (Clarke et al., 2013; Djeridane et al., 2006). The fundamental principle underlying the assay involves a reduction reaction wherein a hydrogen (H) atom is transferred from the scavenger molecule, which is an antioxidant. This leads to the reduction of DPPH to DPPH2, causing a shift in colour from purple to yellow and a simultaneous decrease in absorbance at a wavelength of 517 nm. The DPPH assay is characterised by its seeming simplicity, which can be attributed to the stability of its radical (Mishra et al., 2012).

2.6.1.2 Ferric Reduction Activity Potential (FRAP) Assay

The FRAP assay employs a redox reaction in which reductants, specifically antioxidants, are utilised in a calorimetric approach that involves a reduced oxidant, iron (III) (Fe). The process involves the reduction of the ferric tripyridyltriazine complex to form ferrous (2,4,6-tripyridyl-s-triazine). The observed outcome of the reaction is the transformation of the ferric (III) ion, which is initially colourless, into the ferrous (II) ion, which exhibits a blue hue. This change in colour was seen during the process of measuring the absorbance at a wavelength of 593nm. The spectrophotometric measurements are associated with the electron-donating capacity

of the antioxidant contained in the test substance. Hence, the FRAP assay is regarded as a pioneering technique for evaluating antioxidant capacity (Benzie and Strain, 1996).

2.6.2 α-Glucosidase Inhibitory Activity Assay

Hyperglycemia is a metabolic disorder characterised by an increased concentration of glucose in the bloodstream. Hyperglycemia can be facilitated by various enzymatic pathways, with particular emphasis on the α-glucosidase enzyme, which is primarily found in the intestines. The α-glucosidase enzyme is of significant importance in the process of carbohydrate digestion, as it functions by breaking down oligosaccharides into monosaccharides. The catalysis of the final step in the carbohydrate digestion process, which ultimately results in postprandial hyperglycemia, is considered a responsible action. The enzyme is specifically responsible for the hydrolysis of 1,4-α-glucosidic linkages. The inhibitors of the enzyme engage in competition with the oligosaccharides for the binding site, hence falling into the category of typical competitive inhibitors. According to Sulaiman et al. (2015), α-glucosidase inhibitors have been identified as a promising therapeutic option for effectively managing postprandial hyperglycemia in individuals with type-2 diabetes mellitus. α-Glucosidase inhibitors are a class of oral anti-diabetic medications that exert their therapeutic effect by impeding the enzymatic breakdown of carbohydrates, including starch and table sugar, during digestion. The rate of carbohydrate digestion is decreased by inhibition of this particular enzyme. The absorption of glucose is reduced due to the incomplete breakdown of carbohydrates into glucose molecules. One notable benefit of α-glucosidase inhibitors in comparison to alternative antidiabetic medications is their reduced incidence of adverse effects. The gastrointestinal adverse effects of this medication are primarily non-systemic in nature when compared to the gastrointestinal side effects of other anti-diabetic medications. The localised nature of its action, along with its limited absorption, serves to restrict the occurrence of systemic adverse effects. The adverse effects are typically confined to flatulence, stomach pain, and diarrhoea resulting from the bacterial fermentation of undigested carbohydrates (Rhabasa-Lhoret & Chiasson et al., 2004). According to Aller et al. (2011), it has been noted that acarbose, voglibose, and miglitol (Figure 2.3) possess sugar moieties, that necessitate

complex and time-consuming synthetic procedures. Additionally, these compounds have been associated with significant gastrointestinal adverse effects, as documented in the literature. Hence, it is imperative to explore secure alternatives that exhibit α-glucosidase inhibitory activity, devoid of any adverse reactions or effects, as the development of α-glucosidase inhibitor drugs has remained stagnant since the introduction of acarbose, voglibose, and miglitol, with no noteworthy advancements observed thus far. Currently, there is a significant research emphasis on the discovery of powerful α-glucosidase inhibitors derived from natural sources that do not rely on sugar as their base. This focus is driven by the presence of numerous chemicals in medicinal plants and their potential for exhibiting beneficial biological actions (Ghani, 2015). Therefore, the researchers utilised the α-glucosidase inhibitory assay to assess the inhibitory potential of the plant extracts. This assay involved measuring the release of p-nitrophenol from p-nitrophenyl-α-D-glucopyranoside (PNPG) and monitoring the resulting change in absorbance at a wavelength of 400 nm (Javadi et al., 2014).

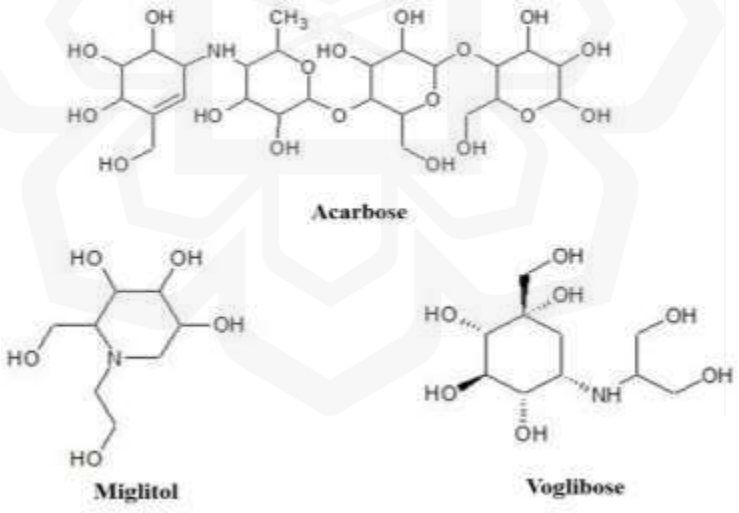


Figure 2.3 α-Glucosidase Inhibiting Drugs.

2.7 IMPORTANCE OF PHYTOCHEMICAL PROFILING

The analysis of phytochemicals plays a crucial role in comprehending the chemical makeup of molecules obtained from plants and their possible implications for human

health. Phytochemicals refer to bioactive compounds that exist naturally in plants, comprising a diverse array of molecules including polyphenols, flavonoids, alkaloids, and terpenoids. These molecules play a significant role in the wide range of colours, flavours, and fragrances found in plants, and they have been associated with numerous health-enhancing attributes. The process of phytochemical profiling not only facilitates the understanding of the biological properties of these substances but also furnishes significant data for the advancement of functional meals, nutritional supplements, and pharmaceutical products. The integration of plant metabolomics and molecular docking techniques in phytochemical profiling contributes to an enhanced comprehension of the intricate chemical composition of plants and the potential biological properties shown by their components. These methodologies offer significant insights into the identification of bioactive chemicals, comprehension of their interactions with target biomolecules, and eventual utilisation of plants' potential benefits for diverse purposes, such as medicine and nutrition.

2.7.1 Plant Metabolomics

Metabolomics serves as a comprehensive approach for investigating the whole range of metabolites associated with various biological functions. It helps us to comprehend the metabolic pathways in the systems of cells, tissues, organs, or organisms (Perumal et al., 2015). These analytes exhibit a wide range of physicochemical features and can vary in terms of their abundance levels. The objective of metabolomics is to comprehensively examine the complete set of metabolites present in a given sample, employing both qualitative and quantitative methods. Consequently, it is necessary to employ various analytical instruments to achieve this goal. It is recommended to utilise a variety of analytical equipment in order to expand our analytical capabilities, as no one equipment is capable of analysing all types of substances (Quansah & Karikari, 2016). In previous years, the prevailing analytical instruments utilised for metabolomics research were Fourier-Transform Infra-Red (FT-IR) and Nuclear Magnetic Resonance (NMR) spectroscopy. These techniques were employed in conjunction with chemometrics tools to establish correlations between bioactivity and the nature of plants, properties of extracts, and the sensitivity of the instruments. Both instruments have the capability to identify functional groups and detect a diverse array

of chemicals, which play a vital role in bioactivity (Wiklund et al., 2008; Ahmed et al., 2010). The use of attenuated total reflectance (ATR) in Fourier-transform infrared spectroscopy (FT-IR) is an efficient, expeditious, and resilient technique. It offers the ability to verify the presence of certain bonds and associated functional groups within extracts. This information could potentially facilitate the formulation of a predictive model for determining the phytoconstituent group(s) likely to be present in the examined extract. One of the notable benefits of Attenuated Total Reflection (ATR) is its ability to eliminate the need for sample preparation, enabling the measurement of various conforming samples, including all plant components. In addition to this, it also serves to reduce spectral variation among sample operators (Allwood et al., 2008).

Therefore, the integration of FT-IR analysis with multi-variate data analysis will yield a distinctive pattern for the plant under investigation, enabling its standardisation and facilitating future research endeavours involving the same plant (Mattoli et al., 2006). Consequently, in this research endeavour, Fourier-transform infrared spectroscopy (FT-IR) was employed to establish a fingerprinting model for the plant under investigation. Therefore, the undertaking of comprehensive metabolomics investigations poses a significant challenge for the field of analytical chemistry. In particular, mass spectrometry exhibits immense potential as a valuable instrument for facilitating such investigations.

Metabolomics necessitates the utilisation of specialised methodologies for the production of samples, separation techniques, and the analysis of mass spectrometry. The primary focus of this study is on metabolic fingerprinting, a technique that involves the analysis of all detectable analytes present in a particular sample. The classification of the samples' nature and the identification of metabolites with differential expression follow this. These metabolites are then used to identify the classes to which the samples belong. The majority of studies in the field of metabolomics involve the integration of many analytical instruments in order to detect and identify a comprehensive range of chemical markers. This approach aims to provide a comprehensive database that can be used for future reference (Shyur & Yang, 2008). Hence, the integration of chromatography and mass spectrometry (MS) has been widely employed in the field of metabolomics profiling. In essence, a mass spectrometer produces a multitude of ions derived from the examined material.

Subsequently, it segregates these ions based on their distinct mass-to-charge ratio (m/z) and subsequently documents the relative prevalence of each ion category. One significant benefit of mass spectrometry is its ability to accurately identify unknown peaks, even in the absence of prior knowledge regarding their precise chemical structure, with the same level of reliability as known target analytes. Hence, the integration of mass spectrometry and chromatography techniques facilitates the identification of analytes in a sample containing diverse metabolites exhibiting distinct features.

Gas chromatography (GC) and liquid chromatography (LC) are commonly employed in analytical research for the purpose of separating analytes in a given sample. By combining these techniques with mass spectrometry, it becomes possible to detect and identify analytes with a high degree of precision and accuracy based on their mass-to-charge ratio (m/z) (Allwood et al., 2008). In the realm of analytical instrumentation, gas chromatography mass spectrometry (GC-MS) is a commonly employed technique that involves the integration of a gas chromatograph (GC) with a mass spectrometer (MS). The gas chromatography-mass spectrometry (GC-MS) method is highly effective in the field of plant metabolite profiling. It is particularly well-suited for the investigation of several low molecular weight compounds that possess appropriate volatility and thermal stability, allowing for their simultaneous detection. In order to enhance the quality of the acquired spectra, it may be necessary to perform chemical modification, specifically derivatization, as a preliminary step in sample preparation. This process serves to mitigate undesired adsorption effects that could otherwise impact the accuracy and reliability of the analysis.

Compound identification will be conducted through the comparison of mass spectra and retention times. This will be done by comparing the obtained results with either commercially available reference compounds or by utilising the commercially available database library, specifically the National Institute of Standards and Technology (NIST14). Additionally, the identification process will involve comparing the obtained data with information found in literature sources such as the studies conducted by Allwood et al. (2008) and Javadi et al. (2014). The investigation of medicinal plants has garnered significant attention from researchers in their quest to

identify pharmacologically active plant species, a pursuit that dates back to the early stages of modern scientific inquiry.

The primary objective of plant studies is the identification of new bioactive chemicals, which can then be utilised in the field of drug discovery and development (Newman and Cragg, 2012). A wide array of pharmacologically valuable medications, obtained from natural sources, are currently given for the treatment and mitigation of many human ailments. In recent times, there has been a surge in the popularity of herbal and traditional medicines, primarily driven by the escalating expenses associated with mainstream medicine and the adverse consequences it can have on human health. Nevertheless, herbal medicines fail to garner widespread acceptance or capture the attention of the majority of the general public and contemporary medical professionals (Wang, 2012). The primary cause of this disease can be attributed to the insufficiency of scientific content in the formulation of allopathic medications. The understanding and acceptance of the holistic approach to traditional medicinal practices among modern medicine practitioners have been limited.

2.7.2 Molecular Docking

The application of molecular docking methodologies has surfaced as a noteworthy accomplishment in the domain of pharmaceutical research and development. This technology can be applied to simulate the interaction between a molecule and protein at the subatomic scale, allowing us to ascertain and explain the behaviour of small molecules that complement the binding site of the protein of interest. This facilitates the acquisition of knowledge regarding fundamental biological mechanisms. The docking approach comprises two fundamental stages. The initial stage entails the anticipation of the structure, location, and orientation of the ligand inside the binding sites.

The subsequent stage entails the assessment of the binding affinity (Taha et al., 2015). The conventional depiction of protein function entails the establishment of a complex via the interaction between the substrate and the protein. The functionality of proteins can be diminished or altered when there is a disturbance in the process of substrate-protein interaction or when the formation of protein complexes is hindered.

The aforementioned phenomenon can lead to a reduction or impairment of protein functionality (Taha et al., 2015). In the setting of type 2 diabetes mellitus (DM), oral hypoglycemic medicines, such as acarbose and miglitol, primarily act on the α-glucosidase enzyme, which is predominately situated in the proximal segment of the small intestine. The pharmaceutical substances demonstrate competitive inhibition of the enzyme located at the brush edge of the small intestine. This enzyme is responsible for the hydrolysis of oligosaccharides and disaccharides into monosaccharides, enabling their absorption into the systemic circulation. As a result, the medications hinder the process of absorbing carbohydrates in the intestines, leading to a delay (Marques et al., 2015).

There are several elements that influence inhibition mechanisms, including the attributes of the inhibitor and the characteristics of the protein being studied. The activity of allosteric inhibitors is mediated through either mixed or non-competitive binding mechanisms. The application of molecular docking analysis provides significant insights into the affinity of interactions between ligands and proteins, as well as the mode and process by which ligands bind (Lu et al., 2014; Du et al., 2016). The understanding of the molecular mechanisms underlying α-glucosidase inhibitors has facilitated the identification of novel hypoglycemic medicines that are characterised by improved selectivity and reduced toxicity. Currently, there is a significant emphasis on the identification of naturally occurring compounds that possess the ability to block the α-glucosidase enzyme. Despite numerous reports on the antidiabetic benefits of various natural substances, there remains a lack of comprehensive understanding of their underlying mechanisms of action. Hence, the present investigation employed AutoDock tools 1.5.6 (Scripps Research Institute, USA) to examine the compounds derived from medicinal plants. The primary objective was to ascertain the potential molecular target(s) and provide additional evidence for the antidiabetic properties of the identified compounds based on enzyme/receptor protein metabolomics (Phosrithong and Ungwitayatorn, 2010).

AutoDock is computer software that uses precalculated grids of affinity potentials and employs various search algorithms to identify an appropriate binding location for a ligand on a certain macromolecule. The aforementioned study by Phosrithong and Ungwitayatorn (2010) demonstrates that the programme effectively

maintains the rigidity of the macromolecule while simultaneously permitting torsional flexibility for the ligand. The α-glucosidase enzyme derived from *Saccharomyces cerevisiae*, which is acquired from yeast maltase, belongs to the glycoside hydrolases (GH13) family. The MAL12 gene encodes a protein that is involved in the hydrolysis of non-reducing α-D-glucose residues connected at position 1,4. This hydrolysis process results in the release of α-D-glucose, as indicated by the enzyme classification number EC 3.2.1.20. The enzyme has been extensively utilised in enzymatic bioassays for the purpose of identifying novel α-glucosidase inhibitors that exhibit unique biological properties, mostly due to its widespread accessibility and convenient manipulability (Ferreira et al., 2010). The current studies on the docking of bioactive chemicals provide persuasive evidence that these newly developed structures demonstrate a high level of binding affinity with the allosteric site of the C-terminal domain of the α-glucosidase protein. This binding affinity surpasses that of the substrate.

As a result, these substances can be classified as mixed inhibitors. The existence of allosteric sites in α-glucosidase and similar enzymes, including α-amylase, has been supported by a multitude of theoretical and experimental studies. Brindis et al. (2011) discovered (Z)-3-butilidenephthalide as an allosteric non-competitive inhibitor in their study. The chemical exhibited a significant anti-hyperglycemic effect by substantially reducing the activity of intestinal and yeast α-glucosidases, both in experimental models and in laboratory settings. The research conducted by Porto et al. (2012) presented findings that suggest the involvement of N-acetylcysteine (NAC), a widely recognised pharmaceutical drug, as a recently identified allosteric chaperone for lysosomal α-glucosidase. The argument presented by the authors suggests that NAC and its derivatives possess the ability to bind reversibly to allosteric sites, which are situated at a considerable distance from the catalytic site. The researchers propose a hypothesis that suggests these substances exercise their effects by interfering with the conformational dynamics of α-glucosidase. This interference ultimately leads to the destabilisation of the protein, rendering it incapable of functioning properly. Liu et al. (2013) presented a study that elucidated the mechanism of action of xanthone inhibitors, namely 1,3,7-tridroxyxanthone and 1,3-dihydroxybenzoxanthone, in their ability to inhibit yeast α-glucosidase. Taha et al., (2015) conducted a study wherein a composition containing flavonoids and indanes was patented based on its demonstrated efficacy in

inhibiting lipase, α-amylase, and α-glucosidase activity in animal subjects. The idea proposed by the authors suggests that both quercitrin and quercitrin-3-O acetate possess inhibitory properties against α-glucosidase by interacting with allosteric regions on the enzyme.

Liu et al., (2013) ran a computational analysis and reported that ‘diboside A’ has a significant affinity for two allosteric binding sites on α-amylase, the enzyme responsible for starch breakdown. This suggest that the computational approach will produce insightful information on the molecular-level interactions that occur between proteins and ligands.

CHAPTER THREE

MATERIALS AND METHOD

3.1 CHEMICALS

All organic solvents and materials used were of analytical quality. The chemicals, reagents, standards and enzymes used in this research study are as follow : 2,4,6-tripyridyl-s-triazine (TPTZ) Sigma Aldrich (St. Louis, MO, USA); *ρ*-Nitrophenyl-*ρ*-D-glucopyranosidase (PNPG) Sigma Aldrich (St. Louis, MO, USA); quercetin Sigma Aldrich (St. Louis, MO, USA); xanthine Sigma Aldrich (St. Louis, MO, USA); palmitic acid Sigma Aldrich (St. Louis, MO, USA); heptadecanoic acid Sigma Aldrich (St. Louis, MO, USA); methoxyamine hydrochloride (Purity > 97%); N-methyl-N-(trimethylsilyl)-trifluoroacetamide (Purity > 97%) Sigma Aldrich (St. Louis, MO, USA); 1,1-diphenyl-2-picrylhydrazil (DPPH) Merck (Darmstadt, Germany); glycine Nacalai Tesque (Kyoto, Japan); potassium dihydrogen phosphate HmbG Chemicals, Germany; dimethoxysulfoxide (DMSO) HmbG Chemicals, Germany; α-glucosidase (yeast maltase) Megazyme, Ireland; n-hexane; ethyl acetate; methanol; and pyridine was obtained from Megazyme, Ireland.

3.2 APPARATUS

The equipments and instruments employed to accomplish the objectives of this research study consisted of a vortex apparatus (Janke and Kunkel, Staufen, Germany), a Sonicator (Elma, S30H, South Orange, New Jersey, USA), a rotary evaporator (Buchi®, Flawil, Switzerland), a freeze drier (Crist, Alpha 1-4 LD, Newtown, Wem Shropshire, UK), a spectrophotometer Infinite 200 PRO (Tecan, Männedorf, Switzerland), a micropipette (Eppendorf, Hamburg, Germany), an FTIR-ATR instrument (Perkin Elmer, USA), a GC-MS system (Agilent, Santa Clara, United States), and finally an LC-MS-QTOF system (Agilent Technologies, Santa Clara Calif).

3.3 SAMPLE COLLECTION AND EXTRACTION

The ginger root was collected from a farm in Bukit Tinggi, Karak, Pahang, Malaysia. Approximately 100 g of the peeled raw root was thinly sliced and dried in an oven at 40^{o}C for 6 hours. The dried root was then ground into powder using a grinder. A total of 30 ml of solvent was poured to the 10 g of the root powder. Different solvents with different polarity were added, e.g. hexane, ethyl acetate, methanol and water. The mixture was ultrasonicated for 30 minutes with a sonicator (Elma-Hans Schmidbauer GmbH & Co., Singen, Kolpingstrabe, Germany) before being centrifuged for 15 minutes. The extract was filtered using a Whatman filter paper no.1. To obtain the dried extract, the supernatant was vacuum evaporated at 40^{o}C using a rotary evaporator (BUCHI Labortechnik AG, Flawil, Switzerland), and freeze dried. Since the methanol is the active extract, the metabolomics work was performed based on the methanol-aqueous solvents. The solvent was prepared using various concentration of methanolin water (0, 25, 50, 75, and 100%, v/v). The extraction and drying process were done following the above method. All extracts were produced with five replicates and stored at -80^{o}C before further analysis (Alothman et al., 2009; Okwu, 2001; Abdul et al., 2011). However, there is no QC sample run in LC-MS analysis. The percentage yield of extraction was determined following the below equation:

Percentage of yield (%) = Mass of dried extract / Mass of root powder x 100%(1)

3.4 *IN-VITRO* BIOASSAY ACTIVITY ANALYSIS

The biological activity of the plant extracts was analysed by three type of individual experiments which were α-Glucosidase Inhibition Assay (AGI), 2,2-1-Diphenyl-2-picrylhydrazil and (DPPH) Radical Scavenging Activity and Ferric-Reducing Antioxidant Power (FRAP) Assay. All the individual experiment was conducted with five replications with a different type of assay. Both DPPH and FRAP assays are two common types of antioxidant assays that are used to assess the antioxidant activity of plant extracts (Clarke, 2013).

3.4.1 α-Glucosidase Inhibition Assay (AGI)

The AGI activity assay was conducted using the methodology outlined by Javadi et al. (2014), which duplicates the conditions of intestinal fluid. The percentage of α-glucosidase inhibition (AGI) was determined at sample assay concentration of 200µg/mL. The positive control in this study involved the utilisation of quercetin, while the substrate used was nitrophenyl-D-glucopyranosidase (PNPG). Quercetin modulates signaling pathways to enhance antioxidant capacity (Qi et al., 2002). The solutions of quercetin and PNPG were produced by dissolving 2 mg of quercetin in 1 ml of dimethyl sulfoxide (DMSO) and 6 mg of PNPG in 20 ml of a 50 mM phosphate buffer with a pH of 6.5, respectively. DMSO is frequently used in industry and medical studies and it is also be used in lifespan assay as a negative control (Wang et al., 2010).

A glycine solution was made by dissolving 15 mg of glycine in 100 ml of distilled water, followed by a pH adjustment to 10 using sodium hydroxide. A volume of 10 µL of the samples, quercetin solution, and negative control (the solvent of the sample) were added to each well of the 96-well plate. Following that, 100 µL of a 30 mM phosphate buffer and 15 µL of a 0.02 U/µl α-glucosidase type one derived from *Saccharomyces cerevisiae* were introduced into every well. In the interim, the empty space was filled with a volume of 115 µl of the buffer solution devoid of the enzyme. After a 5-minute incubation period at room temperature (24 ± 1 °C), both the sample and blank mixtures were subjected to treatment with 75 µL of PNPG. The process was halted by the introduction of 50 µL of glycine solution following an additional 15 minutes of incubation. The quantification of ρ-nitrophenol produced by PNPG was conducted using a microplate reader (Tecan Nanoquant Infinite M200, Switzerland) at a wavelength of 405 nm, as described by Aykul and Martinez-Hackert (2016). Each sample was analysed in four replicates. The inhibitory activity of each extract was quantified as a percentage using the following formula:

$$\text{Inhibitory activity (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100\% \quad (3)$$

where, $A_{control}$ is the absorbance of the negative control, and A_{sample} is the absorbance of the sample or positive control. The determination of the half-maximal inhibition concentration (IC_{50}) value indicates the concentration of the sample required to reduce

the response by half. It was calculated using a linear regression analysis (Burlingham Widlanski 2003).

3.4.2 2,2-1-Diphenyl-2-picrylhydrazil and (DPPH) Radical Scavenging Activity

The DPPH scavenging experiment was used to test the antioxidant activity following a modified version of the steps described by Pieroni et al. (2002). The preparation of the sample involved the dissolution of a 1mg sample in methanol. Approximately 20 microliters of the sample solution underwent treatment with 80 microliters of a 0.1 millimolar DPPH solution that was produced using methanol as the solvent. A blank sample solution of 100 µL was utilised, together with ascorbic acid serving as the positive control, in order to validate the assay. Following a 10-minute incubation period, the absorbance at a wavelength of 517 nm was quantified. The DPPH radical scavenging activity was determined using the following equation:

$$\text{Inhibition (\%)} = [(A_0 - A_1)/ A_0] \times 100 \quad (2)$$

where, A_0 and A_1 corresponds to the absorbances at 517 nm of the radical (DPP^+) in the absence and presence of antioxidant, respectively (Patrick 2009).

3.4.3 Ferric-Reducing Antioxidant Power (FRAP) Assay

We used the ferric reducing antioxidant power (FRAP) assay to find out how much the crude extract could reduce, following the steps laid out by Zhang et al. (2021) with a few small changes. The necessary reagents for the FRAP assay were produced separately. A buffer solution with a pH of 3.6 was made using acetic acid. A total of 455.40 mg of acetic acid was weighed and dissolved in 50 mL of distilled water. Subsequently, 3.97 mL of glacial acetic acid was added to the solution, which was then diluted to a final volume of 250 mL. This resulted in a concentration of 300 mM per litre of acetic acid buffer. A quantity of 156.20 mg of TPTZ was dissolved in distilled

water, followed by the addition of 0.17 mL of concentrated hydrochloric acid. The resulting solution was then diluted to a final volume of 100 mL, yielding a concentration of 10 mM per litre of TPTZ solution. A sample of $FeCl_3 \cdot 6H_2O$ weighing 270.03 mg was dissolved in water and then diluted to a final volume of 50 mL, resulting in a solution with a concentration of 20 mM $FeCl_3 \cdot 6H_2O$. Subsequently, a total of 10 µL ofthe crude sample extract was meticulously combined with 180 µL of FRAP reagent, which consisted of a mixture of 300 mM acetate buffer at pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ in a volumetric ratio of 10:1:1. The resulting mixturewas then subjected to incubation at a temperature of 37°C. Following a 2-hour incubation period in a light-restricted environment, the absorbance of the solution was measured at a wavelength of 593 nm. The relationship between the absorbance value and the antioxidant capacity of the sample is direct, indicating that higher absorbance values correspond to greater antioxidant capacity.

3.5 LC-MS MEASUREMENT

The identification of metabolites in the *Z. officinale* extracts was accomplished through the use of liquid chromatography-mass spectrometry (LC-MS), following the methodology outlined by Nipun et al. (2021) with slight modifications. The samples underwent analysis on an Agilent 1290 Infinity and 6550 iFunnel Q-TOF LC/MS system that was equipped with an electrospray interface (ESI) operating in positive ionisation mode. The equipment used for this analysis was manufactured by Agilent Technologies, located in Santa Clara, California. A total of 2 mg of each extract was dissolved in 200 µL of methanol, subsequently filtered, and then placed into glass vials that were already inserted. The samples were introduced into a Phenomenax Kinetex C18 core-shell technology column with dimensions of 250 x 4.6 mm and a particle size of 5µm. The samples were subjected to elution using a gradient system, starting with a mixture of 5% methanol in water with 0.1% formic acid and transitioning to pure methanol over a period of 10 minutes.

Following this, the elution was continued using pure methanol for an additional 10 minutes, with a flow rate of 0.7 mL/min. The entire elution process lasted for a total of 30 minutes. All eluted metabolites were identified using a Q-TOF LC/MS instrument

operated in electrospray ionisation (ESI) under positive ionisation mode. The mass spectrometry (MS/MS) data was acquired within the m/z range of 100 to 1700 at a scan rate of 1 spectrum per scan. The collision energy ramp utilised for acquiring the MS/MS spectra ranged from 30 to 35 eV. The Q-TOF LC-MS data obtained were analysed using ACD/Spec Manager v.12.00, a software developed by Advanced Chemistry Development, Inc., located in ACD/Labs Toronto, Canada. The raw files in the (*.xms) format were subsequently transformed into the netCDF (*.cdf) format utilising the ACD/Spec manager.

The Q-TOF LC-MS data underwent pre-processing utilising Mzmine software (VTT Technical Research Centre, Finland) (Pluskal et al., 2010) to perform peak filtering, peak identification, peak matching, retention time correction, and peak filing. The pre-processed data was then saved in CSV format.

3.6 MOLECULAR DOCKING

The crystallographic structure of yeast α-glucosidase was acquired from the Protein Data Bank (PDB). In this study, a crystallographic structure of *Saccharomyces cerevisiae* isomaltase (SCI) with a resolution of 1.6 Å (PDB code: 3A4A) was used as the receptor for molecular docking analysis. The 3-dimensional (3D) structures of the positive control compound quercetin, as well as ADG and the probable compounds discovered by LCMS-based metabolomics, were acquired from reputable sources such as Pubchem (National Centre for Biotechnology Information, USA) and Chemspider (Raleigh, North Carolina, United States).

The water molecule was eliminated from the protein structure using AutoDockTools (version 1.5.6) as described by Sanner in 1999. The homology model of the enzyme was constructed prior to conducting molecular dockings. This model was generated using the PDB2PQR Server, version 2.0.0 (Kumar et al., 2022), and was designed to replicate the assay conditions, including a pH of 6.5, in order to accurately simulate the AGI activity experiment. The parameter for the docking grid box was configured to encompass a volume of 126 Å x 126 Å x 126 Å and was positioned at the

coordinates x, y, and z of 21.272, -0.751, and 18.633, respectively, with regard to the protein.

The Gasteiger charges were incorporated into the ligands, and the rotatable bonds inside the ligand were allocated using Auto Dock Tools, allowing all torsions to freely rotate. The docking procedure was conducted via Auto Dock (version 1.5.6) and employing the Lamarkian genetic algorithm, as described by Morris et al. (1998). In the study conducted by Yan et al. (2014), a population size of 150 and energy assessments of 2500000 (medium) were utilised in order to create 50 ligand docked conformations. The conformation with the lowest binding energy was chosen. The protein-ligand complex was visualised using a two-dimensional (2D) representation, whereas the docked compounds were depicted in a three-dimensional (3D) format. The 2D diagram was generated using Biovia Discovery Studio (San Diego, USA), while the 3D superimposed complex diagram was produced using PyMOLTM 1.7.4.5 (Schrödinger, LLC, New York, USA).

3.7 STATISTICAL ANALYSIS

The data analysis was conducted using GraphPad Prism software version 6.0. The data were provided as the mean ± standard deviation (SD) of five replicates. The researchers utilised the one-way analysis of variance (ANOVA) with the Tukey post hoc test to evaluate the statistical significance of the differences observed among the samples. The confidence level was established to be greater than 95%, with a p-value of less than 0.05 indicating a statistically significant difference between the samples. The pre-processed data for multivariate data analysis were imported into the SIMCA P^+ 14.0 programme, developed by Umetrics in Umea, Sweden. The study employed the Orthogonal Partial Least Squares (OPLS) model with the UV scaling approach. The model was fitted and validated using a permutation test. The utilisation of score scatter and loading plots was employed in order to analyse sample discrimination and find the m/z that exhibited a positive association with AGI activity, respectively.

3.8 FLOWCHART OF RESEARCH METHODOLOGY

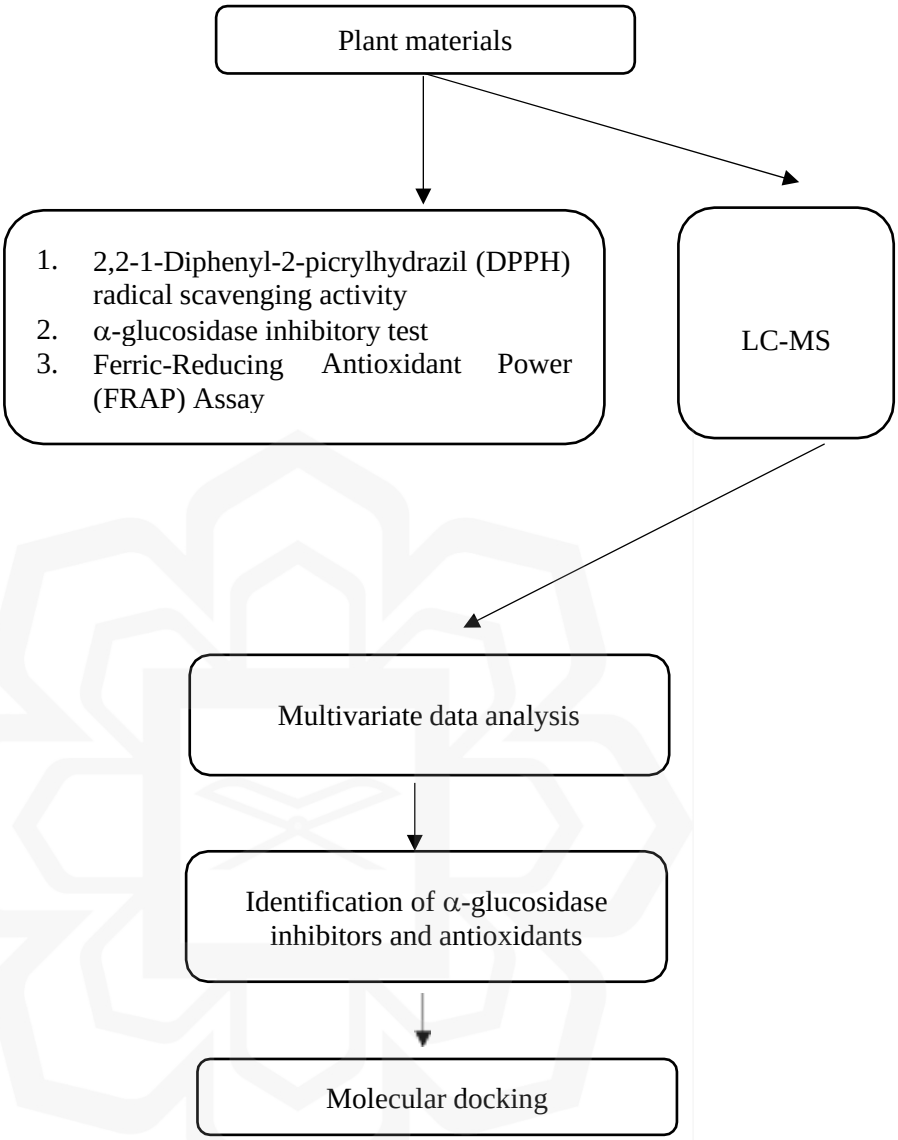


Figure 3.1 Flowchart on Research Methodology

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 α-GLUCOSIDASE INHIBITORY ACTIVITY OF DIFFERENT POLARITY EXTRACT OF GINGER

In this study, solvents of different polarities were used in the extraction process for screening alpha-glucosidase inhibition activity. The % inhibition is displayed in Table 4.1. Among the different polarity extract of ginger, only methanol showed above 50% inhibition. Therefore, the metabolomic work was performed using methanolic based extract.

Table 4.1 α-Glucosidase Inhibitory activity of different extract of ginger of different polarity at 60 µg/mL

Different polarity extract of ginger	% inhibition
n-hexane	11.6
Ethyl acetate	42.3
Methanol	60.0
Water	0.0

4.2 EXTRACTION YIELD, α-GLUCOSIDASE INHIBITORY AND ANTI-OXIDANT ACTIVITIES OF THE GINGER EXTRACT

The α-glucosidase catalyses starch and glycogen digestion in the body into glucose (Nisar et al., 2022). The inhibition of this enzyme facilitates the maintenance of circulating glucose levels by decreasing the rate of blood sugar absorption, and thus is referred to as antidiabetic properties (Nisar et al., 2022). Table 4.1 lists the percentage of α-glucosidase inhibitory (AGI) activity of different ginger rhizome extracts. The AGI activity followed the methanol concentration within the extraction solvent. There were

significant different ($p > 0.5$) between methanol concentration and AGI activitiy sample assay concentration of 200µg/mL. Highest concentration of methanol extracts (100%) showed highest AGI percentage (60.7%) compared to 75% methanol extracts (45.7%). 0% of methanol extracts exhibit the lowest (20.5%) inhibition against the enzyme, and no significant difference ($p > 0.05$) was observed to that of 25% and 50% methanol extract. The percent of AGI increases from the lowest activity (20.5 % inhibition) at 0% methanol concentration to the highest activity (60.7 % inhibition) at 100% methanol concentration. Fatmawati et al., (2023) found that the analysis of α-glucosidase activity showed that methanol extracts outperformed ethyl acetate, dichloromethane, and n-hexane extracts in terms of inhibitory activities. Methanol solubilised the bioactive compounds of the extracts that resulted in the inhibition of α-glucosidase. However, the AGI-IC_{50} value can be determined only for the methanol extract, which was 185.2 µg/mL. The IC_{50} of other extracts could not be determined because they did not achieve % AGI activity greater than or equal to 50% at the plant extract concentrations higher than 2 mg/mL.

In this study, two antioxidant tests were used, the DPPH inhibitory activity and the ferric reducing ability of plasma (FRAP) tests. Antioxidants are compounds with property that can inhibit or reduce the oxidation process of free radicals (Irawan et al., 2022). The fundamental mechanism of DPPH method is the ability of the antioxidant compounds to scavenge free radicals, while FRAP test is the ability to reduce Fe^{3+} into Fe^{2+} (Sethi et al., 2020). The IC_{50} value for DPPH activity inhibition of the ginger rhizome extracts ranged from 125.0 to 223.4 µg/mL. The highest DPPH inhibition activity was shown by the 75% methanol extract with the IC_{50} value of 125.0 µg/mL. Under the condition that water was available, the trend of DDPH inhibition antioxidant activity reduced as the methanol content in the solvent decreased. When compared to other methanolic extracts, the 100% methanol extract had the lowest DPPH inhibitory efficacy, suggesting the importance of water in extracting antioxidant chemicals. Likewise, the water extract showed lower DPPH inhibitory activity (IC_{50} value= 223.4 µg/mL) compared to the methanolic extracts, indicating the necessity of methanol to extract the antioxidant compounds. The strength of the antioxidant activity is categorized to be very strong if the IC_{50} value is 50 ppm, strong at 50-100 ppm, moderate at 101-150 ppm, and weak at >150 ppm (Irawan et al., 2022). Therefore, the

extract possessing the highest DPPH inhibitory activity, the 75% methanolic extract, is categorized in the moderate activity (IC_{50} value of 125.0 µg/mL).

The FRAP value ranged from 5.86 to 14.04 mmol TE/mgDW. The trend of this activity was similar to the DPPH inhibition activity. The highest FRAP value was exhibited by the 75 and 100% methanolic extracts. While the water extract exhibited the minimum FRAP value of 5.86 mmol TE/mgDW. The reduction in the FRAP value occurred subsequent to the decrease of the concentration of methanol in the extraction solvent.

Table 4.2 α-Glucosidase Inhibitory (AGI) and antioxidant activities, and the extraction yield of different ginger rhizome extracts.

Methanol concentration (%) used in the sample extraction	**AGI (% of inhibition) at sample assay concentration of 200µg/mL**	**AGI (IC_{50}, µg/ml)**	**DPPH (IC_{50}, µg/ml)**	**FRAP (**mmol TE/mgDW**)**	**Extraction Yield (%)**
100	60.7 ± 5.22^{a}	185.2 ± 4.50	161.7 ± 0.20^{b}	$12.66 \pm 2.01^{a,b}$	$9.2 \pm 0.40^{b,c}$
75	45.7 ± 2.79^{b}	ND	125.0 ± 0.10^{e}	14.04 ± 3.26^{a}	$13.3 \pm 0.62^{a,b}$
50	28.5 ± 5.38^{c}	ND	138.7 ± 0.46^{d}	6.62 ± 2.11^{c}	7.7 ± 0.60^{c}
25	25.5 ± 3.62^{c}	ND	159.8 ± 0.39^{c}	$7.88 \pm 0.50^{b,c}$	14.6 ± 1.82^{a}
0 (water)	20.5 ± 4.54^{c}	ND	223.4 ± 0.7^{a}	5.86 ± 0.60^{c}	$11.2 \pm 3.49^{a,b,c}$

Note. -The data presented as mean ± SD, n=3
-The values share the same letter are significantly different ($p<0.05$) as measured by one-way -Anova and Tukey's comparison test
-ND= not determined, could not achieve % AGI activity greater than or equal to 50% at the plant extract concentrations higher than 2 mg/mL

4.3 MULTIVARIATE DATA ANALYSIS

Multivariate data analysis (MVDA) technique is employed to determine the bioactive compounds extracted from ginger, whereby develop orthogonal partial least square model (OPLS). The LC-MS signals (x-variables) was correlated to the AGI activity (y-variable). The retention time and mass to charge ratio (m/z) obtained from LC-MS were applied as the x-variables. Figure 4.1 indicates that the OPLS model was successfully generated two OPLS components, with the total variation explained (R^2Y-cum), and the total predicted variation (Q^2Y-cum) of 0.77 and 0.50, respectively. The difference between R^2Y-cum and Q^2Y-cum in the Figure 4.1 was 0.27 which is less than 0.3, indicating the validity of this model (Eriksson et al., 2003). The highest variation represented by OPLS component 1 (71.2 %), followed by OPLS component 2 (5.6 %). The validity of the calibration model was further verified using permutation, exhibiting the R^2Y and Q^2Y intercept values of less than 0.4 and 0.05, respectively, which are under acceptable range according to Erickson et al. (2003).

Figure 4.1 Summary of Fit of Established Orthogonal Partial Least Square (OPLS) Model for the Ginger Rhizome Extract

Figure 4.2A shows a score scatter plot for the OPLS model as the extracts were separated along the OPLS components 1 and 2. Following its AGI activity, the extracts are well divided along the OPLS component 1. The methanol 100 and 75% extracts were placed on the positive side of the OPLS component 1, whereas the less active extracts were situated on the negative side.

Figure 4.2B displays the loading scatter plot for the correlation of m/z (x-variables) to AGI activity (y-variable). The m/z values closed to the AGI activity dot had a positive correlation to the bioactivity. However, only three ions (m/z 177.0545, 284.2344 and 384.4100) could be identified after comparison with the database and references. Three putative compounds were identified from Multivariate Data Analysis Software (Simca) as 7-methoxycoumarin, supinine and 12-hydroxycorynoline as shown in Table 4.2.

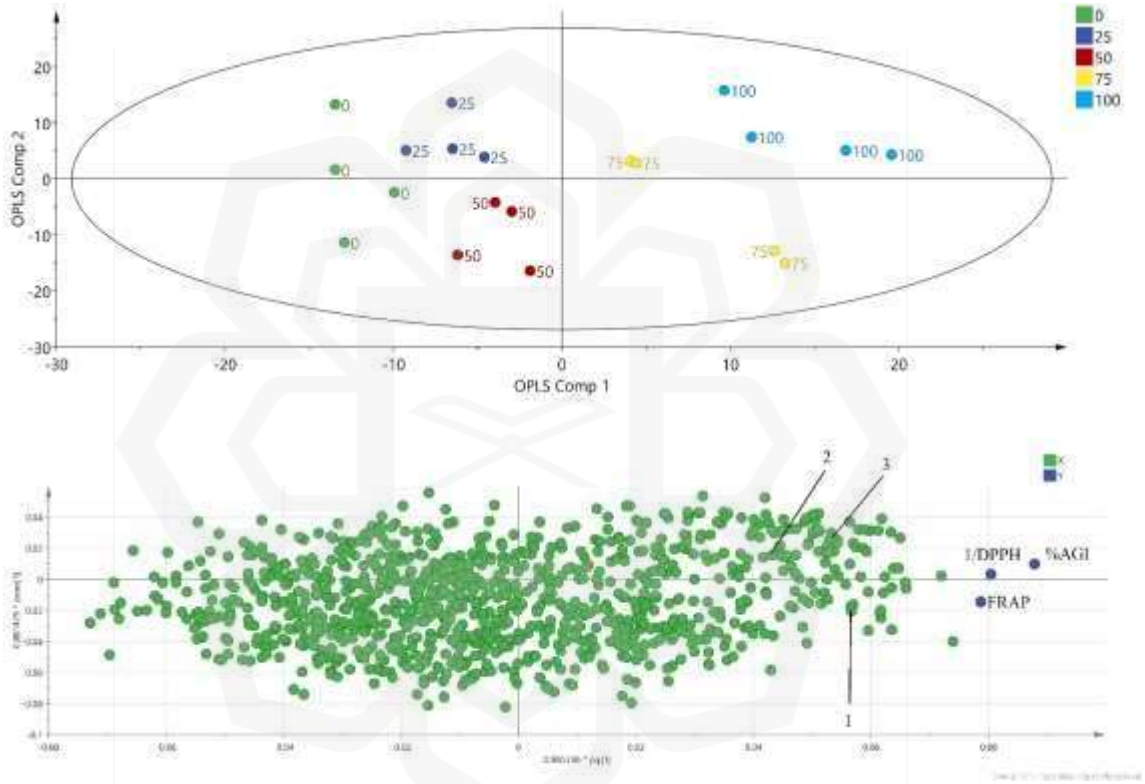


Figure 4.2 (A) Score Scatter Plot of OPLS Model for *Z. officinale* Rhizome Extracted with Methanol 100, 75, 50, 25, and 0%, (B) The Loading Scatter Plot of the OPLS Model, 1= m/z 177; 2= m/z 284; 3= m/z 384.

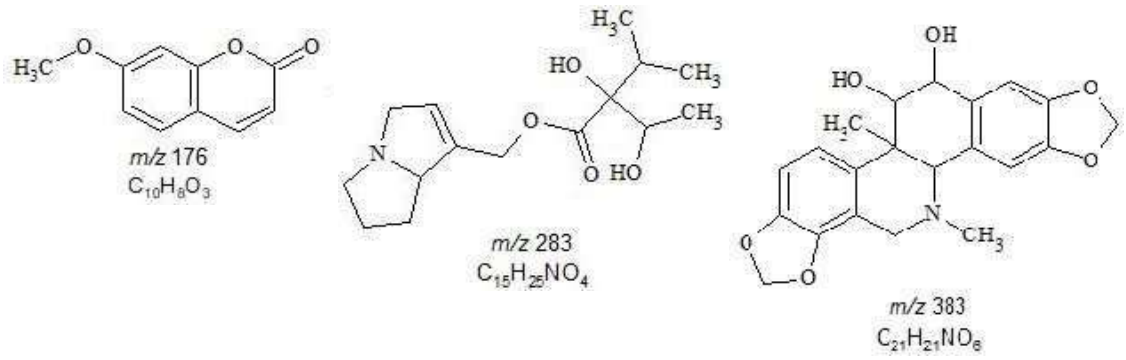
4.4 IDENTIFICATION OF THE PUTATIVE COMPOUNDS RELATED TO THE α-GLUCOSIDASE INHIBITORY ACTIVITY

To identify the putative compounds related to the α-glucosidase inhibitory activity as mentioned in the section 2.2, the m/z spectrogram of fragmented ions of each compound

resulted from the LC-MS/MS analysis with a positive ionization was analysed. Table 4.2 shows MS^2 fragment ion putative of each compound. Only three putative metabolites identified from the ginger rhizome extract which are 7-methoxycoumarin, Supinine and 12-hydroxycorynoline. While the chemical structure of each compound is shown in the Figure 4.3. The fragmentation pathway of these compounds is shown in the Appendix A1 and A2.

Table 4.3 Putative metabolites identified in the ginger rhizome extract via LC-MS/MS analysis with positive ionization.

Compound	**m/z** **$[M + H]^+$**	**MS^2 Fragment Ions**	**Putative metabolites**	**Reference**
1	177.0545 ($C_{10}H_9O_3$)	$[M – H_4O]^+$ at *m/z* 156, $[M – C_3H_3]^+$ at *m/z* 137, $[M – C_3H_2O]^+$ at *m/z* 122	7-methoxycoumarin	Ang et al., (2019), Tine et al., (2017)
2	284.2344 ($C_{15}H_{26}NO$)	$[M – CH_2]^+$ at *m/z* 270, $[M – C_4H_8O]^+$ at *m/z* 211, $[M – CH_4O_4]^+$ at *m/z* 203	Supinine	Kopp et al., (2020), Arshad et al., (2020)
3	384.4100 ($C_{21}H_{22}NO_6$)	$[M – H_4O]^+$ at *m/z* 363, $[M – C_2H_3]^+$ at *m/z* 356, $[M – CH_4O]^+$ at *m/z* 351, $[M – C_3H_3O]^+$ at *m/z* 328, $[M – C_{13}H_{14}NO_2]^+$ at *m/z* 167	12-hydroxycorynoline	Niu et al., (2011), Dong et al., (2015)



1 **2** **3**

Figure 4.3 Chemical Structures of the Putative Compounds 1, 2, and 3

Compound **1** belongs to the classes of coumarin with the substitution of methoxy group at C-7, also known as herniarin (Güvenalp et al., 2017). Protonation of compound **1** at carbonyl functional group formed the parent ion $[M+H]^+$ with m/z 178. Then, consequent removal of H_4O^+, C_3H_3 and C_3H_2O from the parent ion generated daughter ions with m/z 156, 137 and 122, respectively.

Compound **2** is a pyrrolizidine alkaloid. It was protonated at nitrogen in pyrrolizidine ring to produce the parent ion $[M+H]^+$ having an m/z 284. Addition of hydrogen in the pyrrolizidine ring cause the ring opening reaction and form straight imines functional group before removal of CH_2 from the parent ion, resulting daughter ion having an m/z 270. Then, the fragmentation of parent ion continued with the removal of C_4H_8O and CH_4O_4, formed other daughter ions with m/z 211 and 203, respectively.

Compound **3** is one of derivatives for benzophenanthridine alkaloid that is corynoline substituted by hydroxyl group at C-12. Protonation of this compound via addition of hydrogen at nitrogen in the quinoline ring formed parent ion $[M+H]^+$ with m/z 284. Then, a series of ring restructure reaction followed by removal of H_4O generate a daughter ion having m/z 363. Subsequent removal of other organic compounds, specifically C_2H_3, CH_4O, C_3H_3O and $C_{13}H_{14}NO_2$ from the first daughter ion produced another daughter ion with m/z 356, 351, 328 and 167, respectively.

4.5 MOLECULAR DOCKING

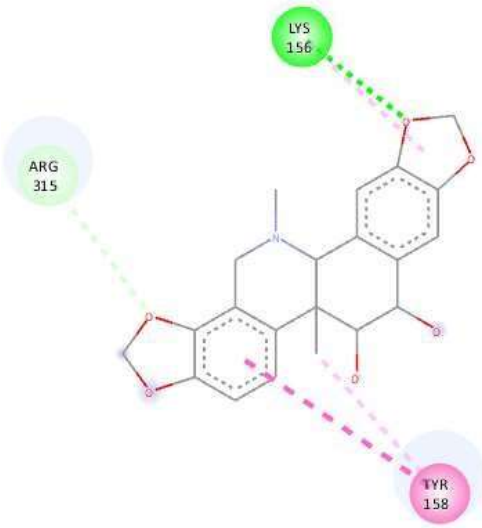
Analysis of molecular docking was carried out to investigate the interaction between protein and ligand at a molecular level with the aid of the computational technique. To validate the docking parameters, the co-crystallized control ligand, alpha-D-glucose (ADG) and quercetin were employed as the controls. In this study, the complex with low binding energy is regarded as the strong-docked complex. The re-docked ADG was discovered to bind with 3A4A in the same way that its crystallographic configuration did. The root mean square deviation (RMSD) of the re-docked ADG was determined to be 0.595 Å, suggesting that the docking parameters employed were able to regenerate the crystallized conformation. The docking parameters are considered suitable if the RMSD value of the redocked ligand with reference to the crystallized one is less than 1.5 A^{o} (Wei et al., 2008). As shown in Table 4.3, the ADG and quercetin had a binding energy of -6.0 and -8.4 kcal/mol, respectively. ADG formed interaction through hydrogen bond with ASP 352, ARG 442, ARG 213, and ASH 69 in the active site of the enzyme (see Figure 4.4 and 4.5), with bond distance less than 3.00 A^{o}. While quercetin exhibited interaction with ASP 307, GLH 277, and ASP 215 through hydrogen bond; dipole-ions interactions with ASP 352 (π -anion) and ARG 442 (π -cation); and dipole-dipole interaction with PHE 303 (π-π-T-shaped). The interactions took place in the active site of the enzyme (see Figure 4.4. and 4.5) which is in line with the report by Yamomoto et al. (2010).

Table 4.3 points out that 12-hydroxylcorynoline had higher binding affinity (lower binding energy, -10.5 kcal/mol) compared to the control ligand (-6.0 kcal/mol), followed by supinine and 7-methocxycoumarin. The lower value of binding energy indicates the stronger binding affinity of protein-ligands, thus more stable complex is formed (Iman et al., 2015 and Zabidi et al., 2021). The carbonyl moiety of 7-methoxycoumarin formed hydrogen bond with ARG 442; while the lactone ring formed π-anion interaction with ASP 352, and π-π-T-shaped interaction with TYR 158 and PHE 178. The benzene ring formed π-π-T-shaped interaction with TYR 158. Meanwhile, ARG 315 interacted with methoxy moiety via alkyl interaction. Next, dioxolane ring of 1,3-benzodioxole moiety of 12-hydroxylcorynoline interacted with LYS 156 through hydrogen bond and π-alkyl interaction. TYR158 formed π-π T-shaped interaction with the benzene ring of 1,3-benzodioxole moiety and π-alkyl with the methyl of

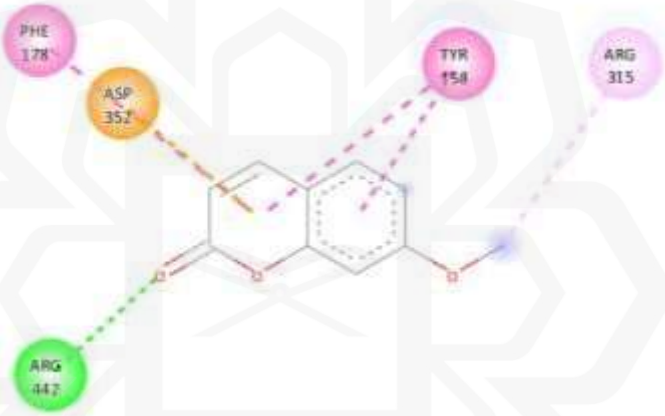
decahydroquinoline ring. While ARG 315 interacted through carbon and hydrogen bonding with dioxolane ring of 1,3-benzodioxole moiety. Supinine interacted with the highest number of amino acids from α-glucoside. The carbonyl moiety of this compound interacted with GLH 277, forming a hydrogen bond. ARG 442 formed two hydrogen bonds with hydroxy and ether of isopropylbutanoate moiety. The GLU 411 and PHE 303 residues interacted with unsaturated pyrrolizide ring of supinine via carbon hydrogen bond and π-alkyl interaction, respectively. While PHE 178 residue interacted with the ethyl of the isopropylbutanoate moiety via π-sigma and π-alkyl interactions. The HID 112 and TYR 72 interacted through π-alkyl interaction with the ethyl of isopropylbutanoate moiety. While HID 351 and TYR 72 residues exhibited a π-alkyl interaction with terminal methyl moiety of supinine. Lastly, the PHE 303 residue interacted with pyrrolizide ring by π-alkyl interaction. 7-Methoxycoumarin andsupinine interacted at the catalytic site, while 12-hydroxylcorynoline interacted at the allosteric site of the enzyme (see Figure 4.5).

Table 4.4 Data for molecular docking of compounds **1**, **2** and **3** with 3A4A

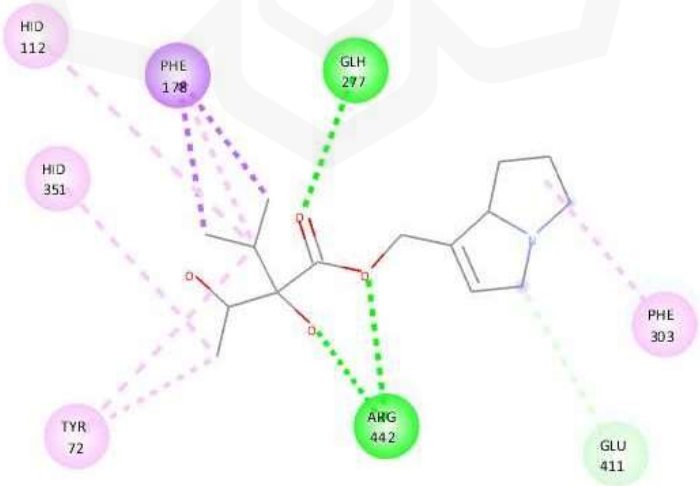
No	Ligand	Binding energy (ΔG) (kcal/mol)	Interaction with proteins	Bond distance (A°)
1	7-methoxycoumarin	-6.3	-hydrogen bond (ARG 442) -π -anion (ASP 352) -π-π-T-shaped (TYR 158, PHE 178) -alkyl (ARG 315)	2.54 4.65 5.03, 5.53 4.07
2	Supinine	-7.6	-hydrogen bond (ARG 442, GLH 277) -carbon hydrogen bond (GLU 411) -π -sigma (PHE 178) -π -alkyl (HID 112, HID 351, TYR 72, PHE 303)	2.72, 1.88 3.41 4.40 3.96, 5.02, 4.76, 2.78
3	12-hydroxylcorynoline	-10.5	-hydrogen bond (LYS 156) -π -alkyl (LYS 156) -alkyl (TYR 158) π-π-T-shaped (TYR 158) -carbon hydrogen bond (ARG 315)	4.22 5.09 2.33 5.24 3.64
4	Positive control, quercetin	-8.4	-hydrogen bond (ASP 307, GLH 277, ASP 215) -π -anion (ASP 352) -π -cation (ARG 442) -π-π-T-shaped (PHE 303)	1.98, 2.02, 2.49 4.29 3.75 4.97
5	Control ligand (ADG)	-6.0	-Hydrogen bond (ASP 352, ARG 442, ARG 213, ASH 69)	1.80, 1.89,2.99, 2.22



12-hydroxycorynoline



7-methoxycoumarin



Supinine

Figure 4.4 2D Binding Interaction of Docked Compounds with α-Glucoside (3A4A).

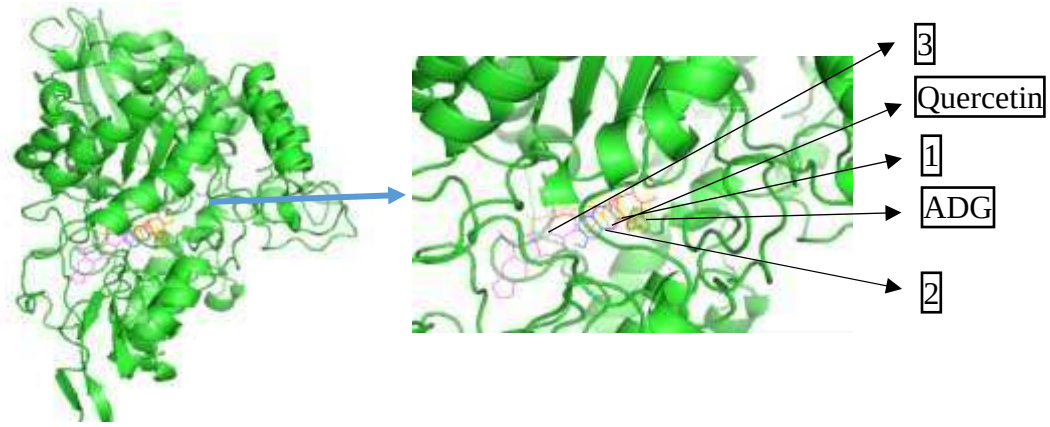


Figure 4.5 3D Superimposed of ADG, Quercetin, and All Docked Compounds (**1**,**2**,**3**). Compound **1** and **2** are Located at the Catalytic Site of the Enzyme. While Compound **3** is Situated at the Allosteric Site of the Enzyme.

4.6 DISCUSSIONS

Ginger contains phytochemicals responsible for bioactivity (Sharma, 2017, Zubair et al., 2020, Bhowmikwmik et al., 2021). However, to extract these compounds from their source, a workable extractant is required to maximize the extraction of bioactive compounds and minimize residues. According to Ghasemzadeh et al. (2011), the polarity of the extractant plays an important role in extracting the compound following the principle of like and dislike during extraction. In this study, the activity of AGI increased with an increasing ratio of methanol in the extractant. The increase in AGI activity can be related to the profile of phytochemicals extracted from ginger in the extract.

In this study, some putative compounds possessing AGI activity have been identified. Although the presence of these compounds has been documented in a variety of plants, it is herein reported for the first time that these compounds were found in the ginger through this investigation. 7-Methoxycoumarin has been isolated from aerial parts of *Artemisia dracunculus* L., and possess AGI activity (Güvenalp et al., 2017). Research studies have demonstrated the antioxidant activity of methoxycoumarins (Asif, 2015; Kang & Hyun, 2020; Lu et al., 2023), indicating their potential to scavenge free radicals and protect cells from oxidative stress. Many coumarins are well known as

an anti-diabetic agent (Li et al., 2017), thus, it is in line with the finding of the current study. Another putative compound is a pyrrolizidine alkaloid, namely supinine. Arshad et al. (2021) reported the presence of this compound in ethanolic extract of *Heliotropium crispum* Desf. It also possesses the AGI activity. Supinine has been reported to exhibit antioxidant activity (Kumar et al., 2022). Lastly, 12-hydroxycorynoline had been isolated from *Corydalis bungeana* Turcz., one of traditional Chinese medicinal herb. Further work showed that this compound contributed to the anti-inflammatory activity (Dong et al., 2015; Yang et al., 2016; and Niu et al., 2011). However, this compound had not been reported to have anti-diabetic and anti-oxidant activities, although its analogues and derivatives, corynoline and acetylcorynaline have been demonstrated to possess strong antioxidant properties(Yang et al., 2016). Hence, the AGI and anti-oxidant activities of these compounds arebeing observed in the current study for the first time.

Antioxidants play a crucial role in counteracting oxidative stress by scavenging and neutralizing reactive oxygen species (ROS), thereby protecting cells and tissues from damage. Several studies have explored the potential benefits of antioxidants in diabetes management. For example, a study by Maritim et al. (2003) discussed the role of oxidative stress in diabetes-related complications and emphasized the importance of antioxidants in mitigating these effects. Additionally, a review by Brownlee (2005) highlighted the role of oxidative stress in insulin resistance and beta-cell dysfunction in type 2 diabetes, underscoring the potential of antioxidants as therapeutic agents. Oxidative stress arises when there is an imbalance between the ROS and the body's ability to neutralize them with antioxidants. In diabetes, persistent hyperglycemia and other metabolic abnormalities lead to increased ROS production, which can cause cellular damage and contribute to the pathogenesis of diabetes and its complications.

The AGI activity of the putative compounds was confirmed further through the *in silico* molecular docking. It is expected that once the enzyme is inhibited, the enzyme will be unable to break down carbohydrate, leading in lesser glucose absorption and lower blood glucose levels (Zabidi et al., 2021). All docked compounds exhibited the binding energy less than the control ligand, indicating strong binding to the enzyme. Among three docked compounds, two of them interacted with the active site of the enzyme, such as 7-methoxycoumarin and supinine. Both compounds interacted

with some amino acid residue which were reported to be in the active site of the enzyme (Yamamoto et al., 2010). 7-Methoxycoumarine interacted with ASP 352 and ARG 442 via π-anion interaction and hydrogen bond, respectively. While supinine interacted with ARG 442 through hydrogen bond. Interaction to the active site of the enzyme reflects its competitive inhibition to the enzyme (Patrick GL, 2009). While the other docked compounds, 12-hydrocoryline exhibited interactions with the allosteric bindingsite of the enzyme since no interaction of this compound with the active site of the enzyme was observed, suggesting its non-competitive inhibition to the enzyme (Patrick GL, 2009).

As for the fragmentation pathways, the illustration of the compound fragmentation has been shown in Appendix B and explained in 4.5. The fragmentation pathways have been explained in Chapter 4.5. The pictures in Appendix B provide a detailed breakdown of the fragmentation processes, including the loss of small molecules like acetylene and ethanol as well as the processes of protonation, deprotonation, loss of water, and decarboxylation.

CHAPTER FIVE

CONCLUSION

This study exhibited that the methanol extract of the ginger rhizome possessed AGI and antioxidant activities. The percent of AGI increases from the lowest activity (20.5 % inhibition) at 0% methanol concentration to the highest activity (60.7 % inhibition) at 100% methanol concentration. The highest DPPH inhibition activity was shown by the 75% methanol extract with the IC_{50} value of 125.0 µg/mL. LCMS-based metabolomics successfully identified the putative compounds possessing the AGI and the antioxidant activities, namely 7-methoxycoumarin, supinine and 12-hydroxycorynoline. The current study is the first to show that these compounds exist in ginger rhizome. All of the identified compounds were anticipated to have AGI and antioxidant activities. The low binding energy of these compounds to the α-glucosidase through *in silico* docking study supported the AGI activity of each of the putative compounds. These compounds had lower binding energy values than the control ligand, indicating a high affinity for the enzyme. Methoxycoumarine and supine formed interactions with the active site of the enzyme, while 12-hydrocoryline interacts with the enzyme's allosteric binding site. This finding gives valuable scientific information for the advancement of forthcoming nutraceuticals.

CHAPTER SIX

RECOMMENDATIONS

1. Quantitative analysis of the identified compounds should be further evaluated.
2. The metabolomics guided fractionation – isolation is required to identify the bioactive compounds of unknown / novel compounds from this plant.
3. Synergistic and antagonistic effect among the compounds should be further studied.

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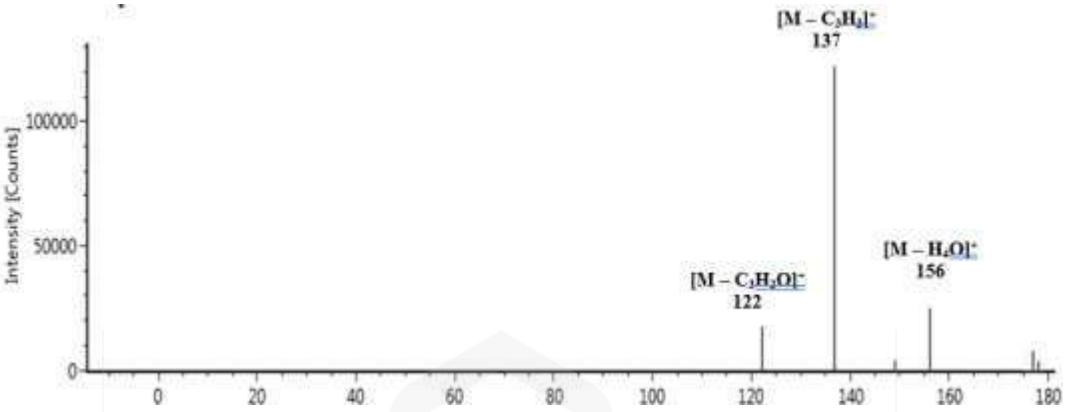
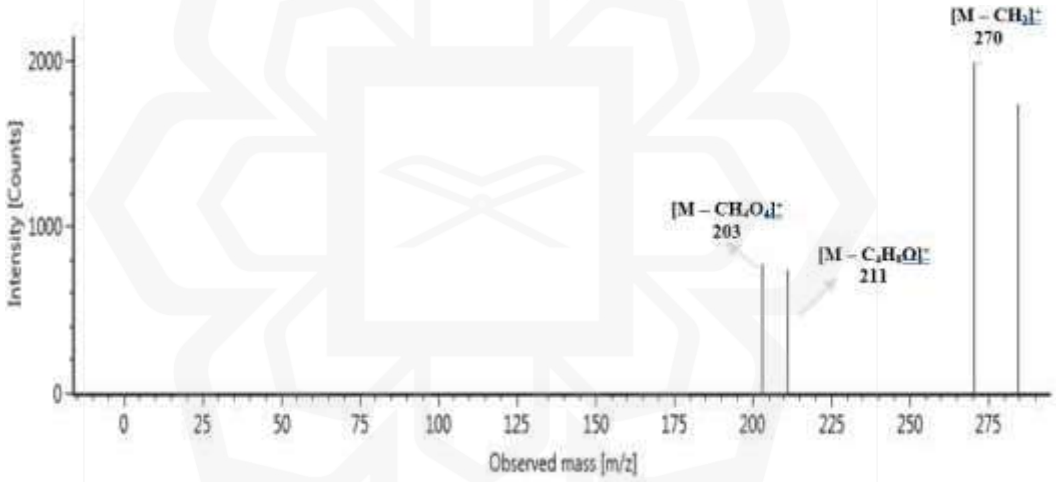
APPENDIX A. LCMSMS SPECTRA

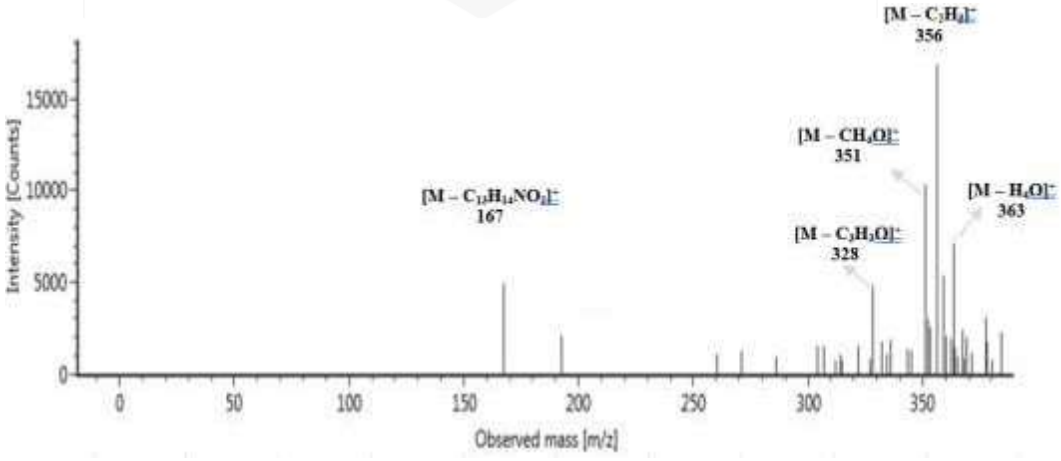
7-methoxycoumarin



Supinine



12-Hydroxycorynoline



APPENDIX B. FRAGMENTATION PATHWAYS

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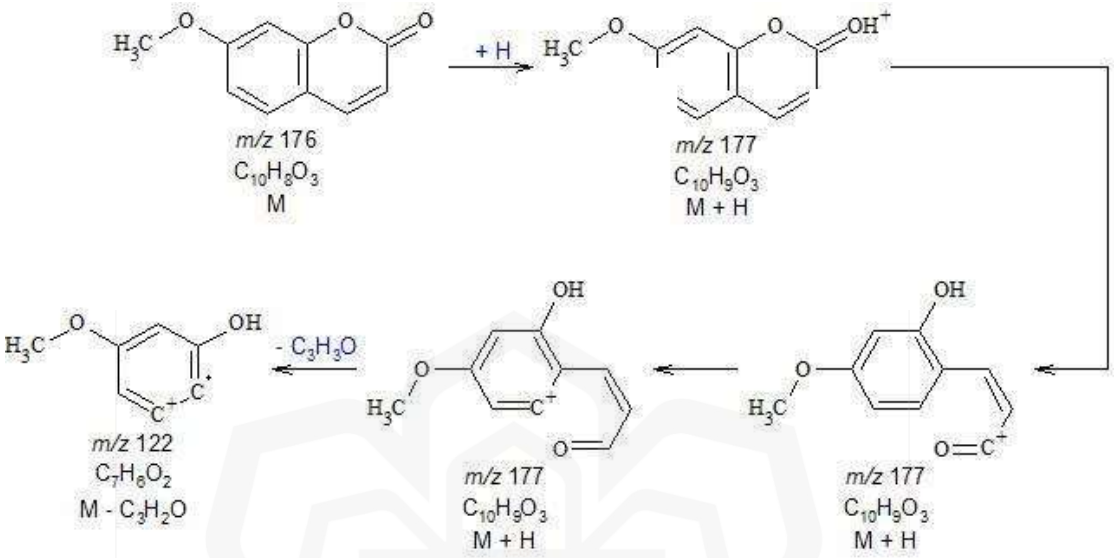

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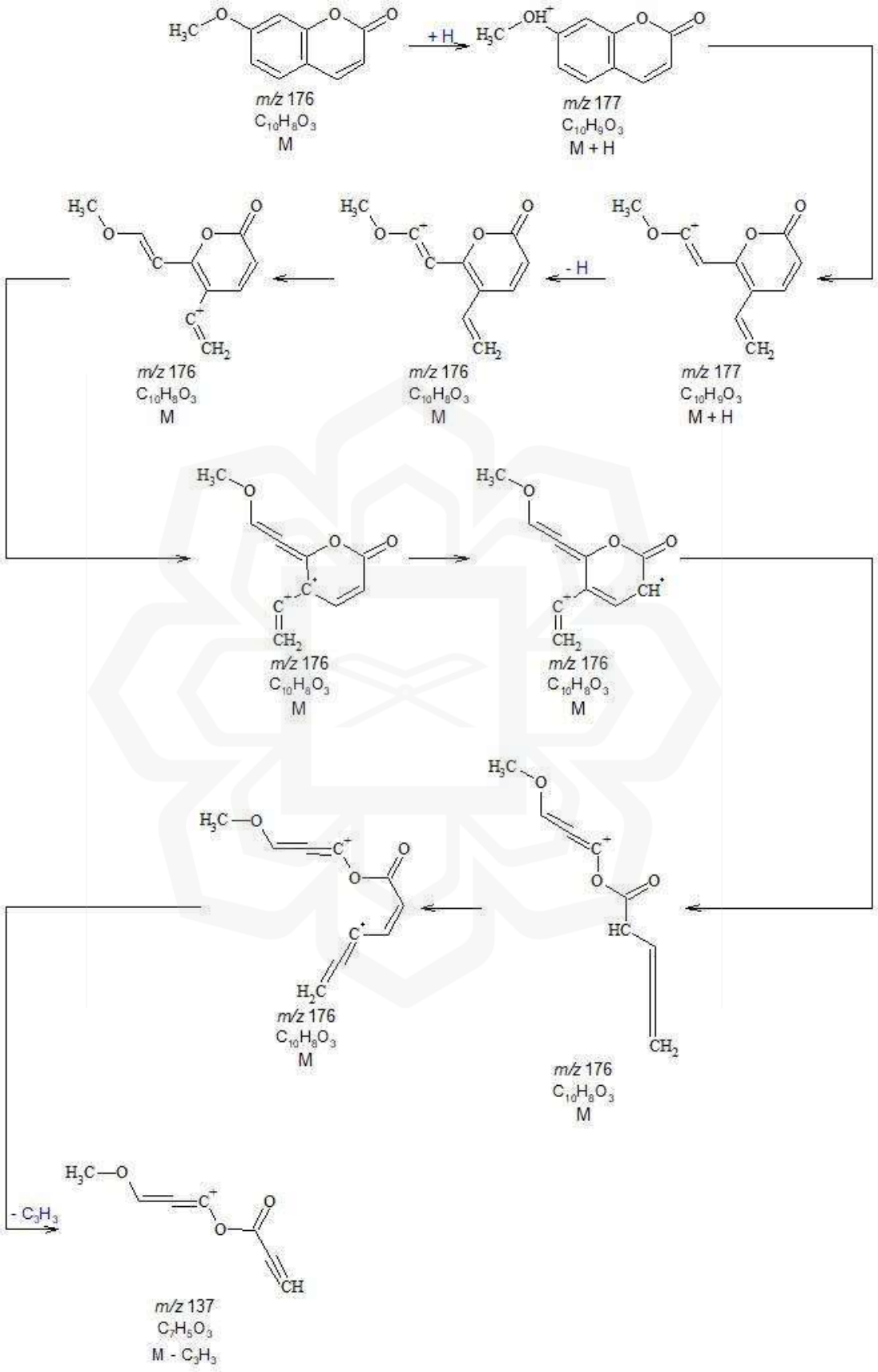

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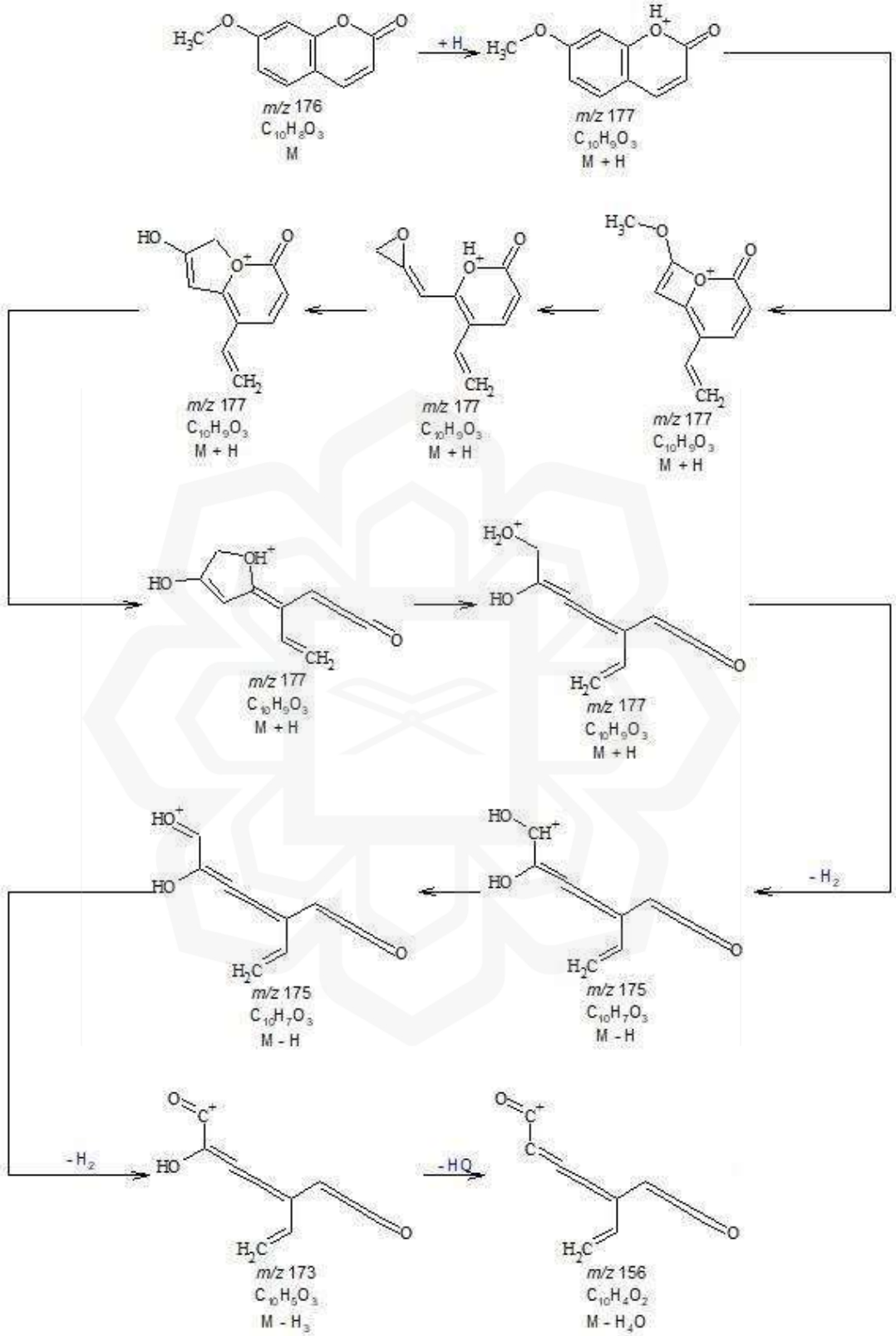

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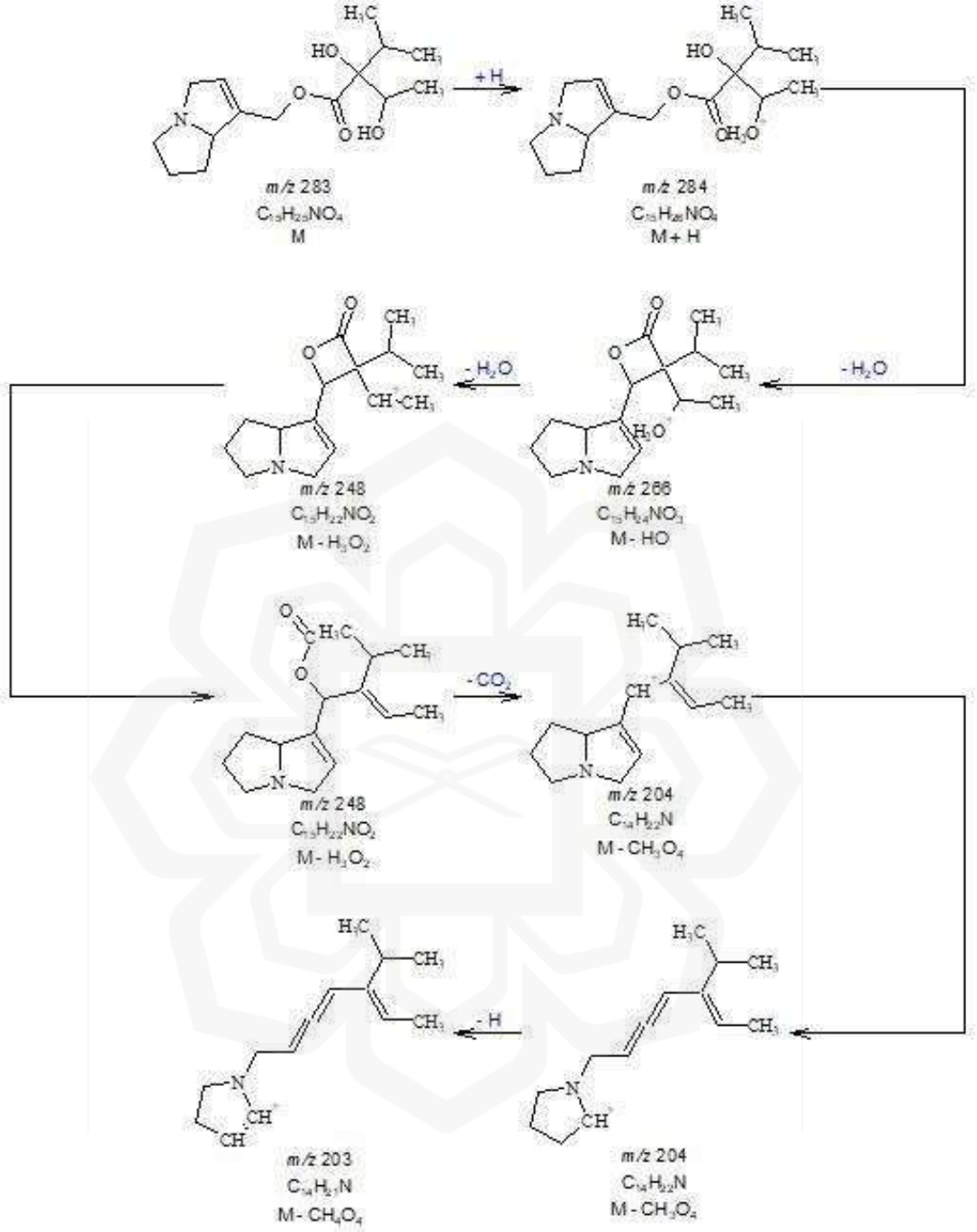

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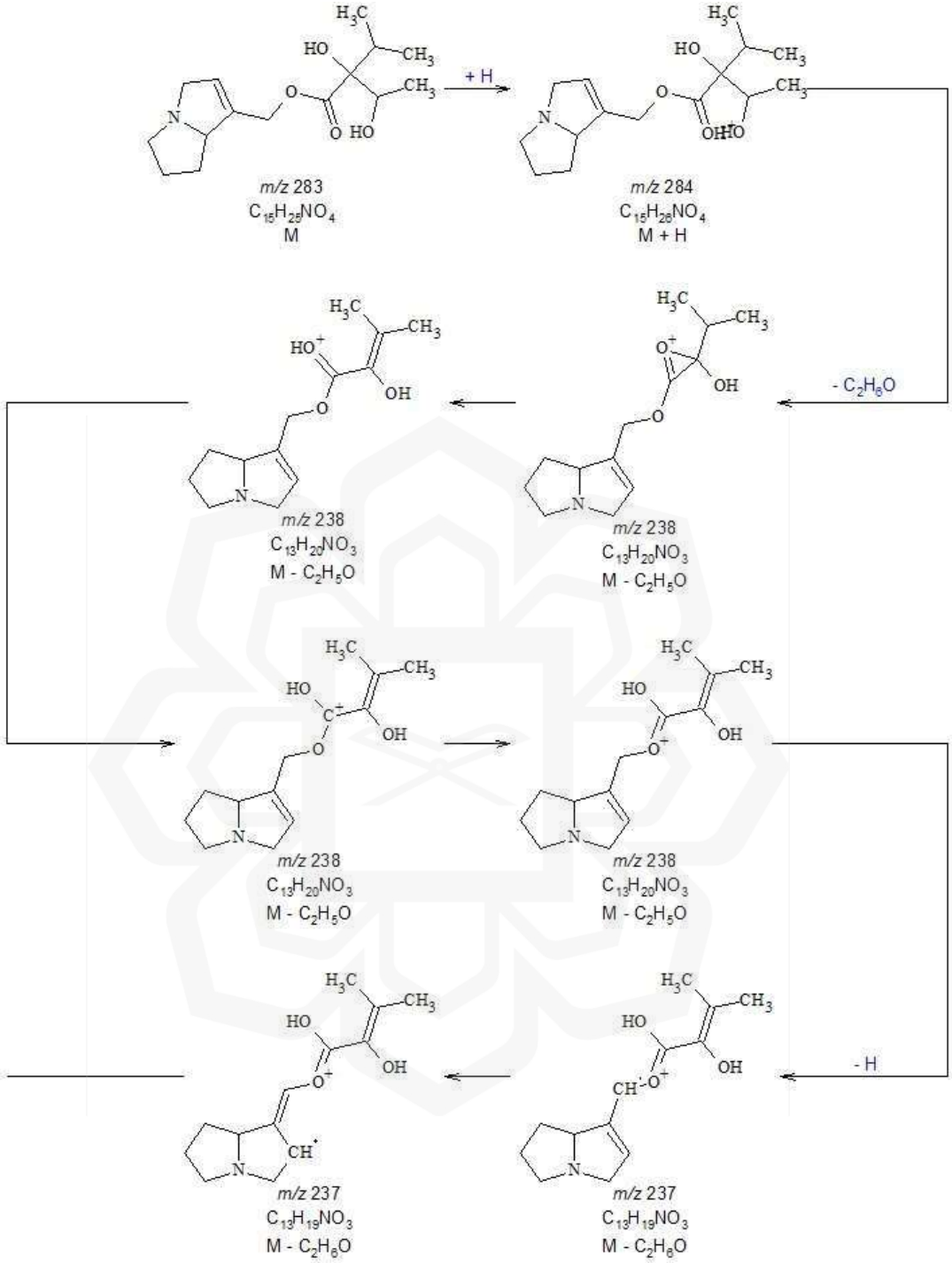

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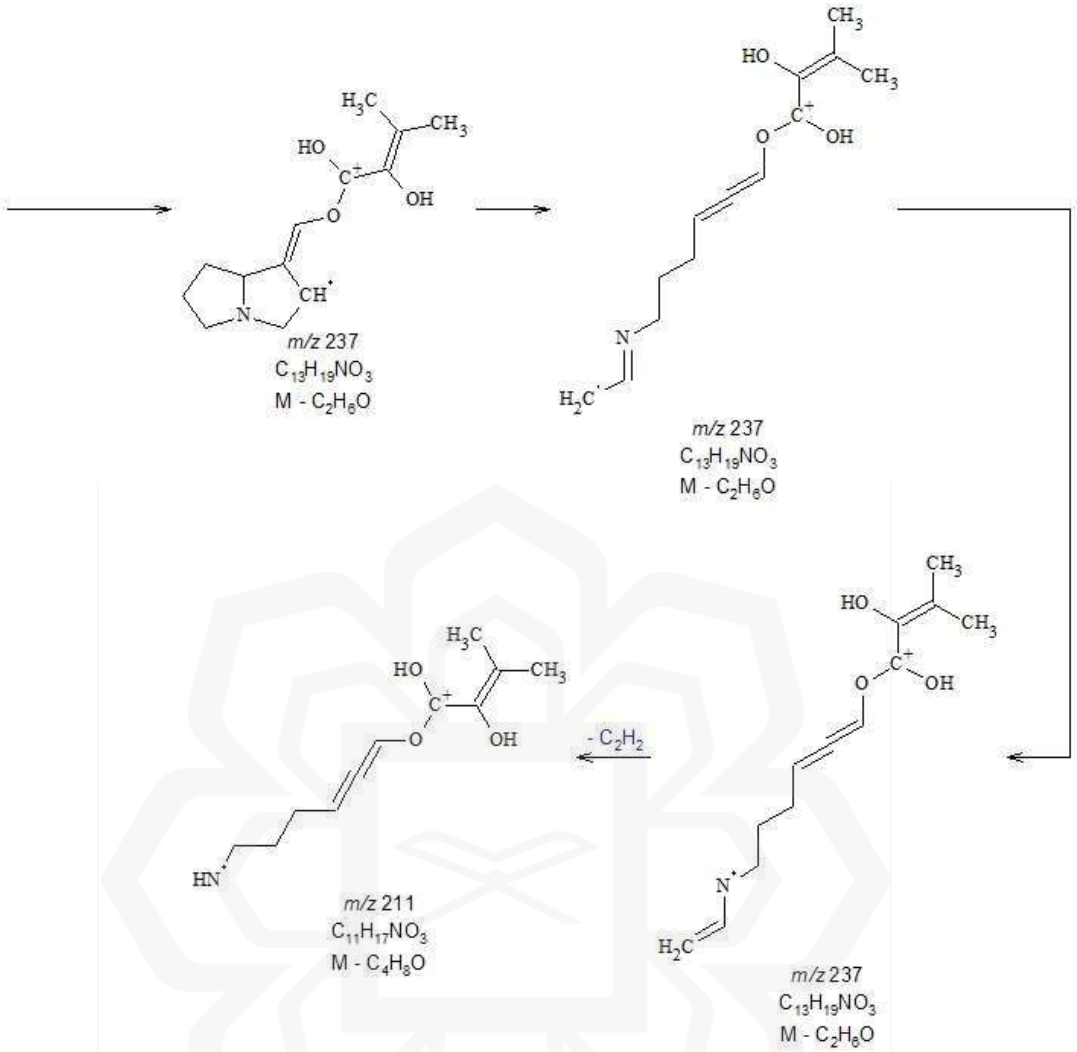
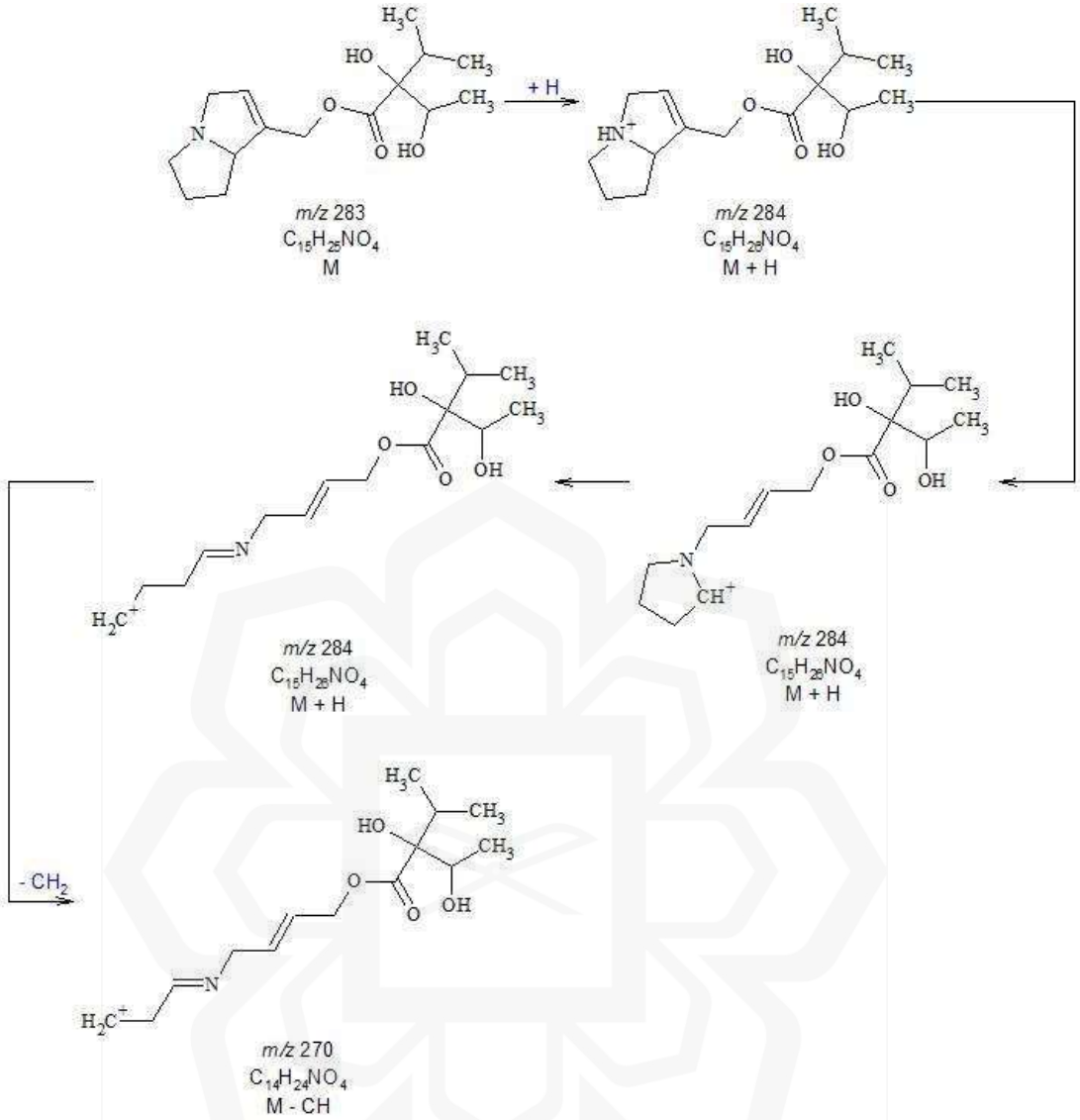


Supinine-270



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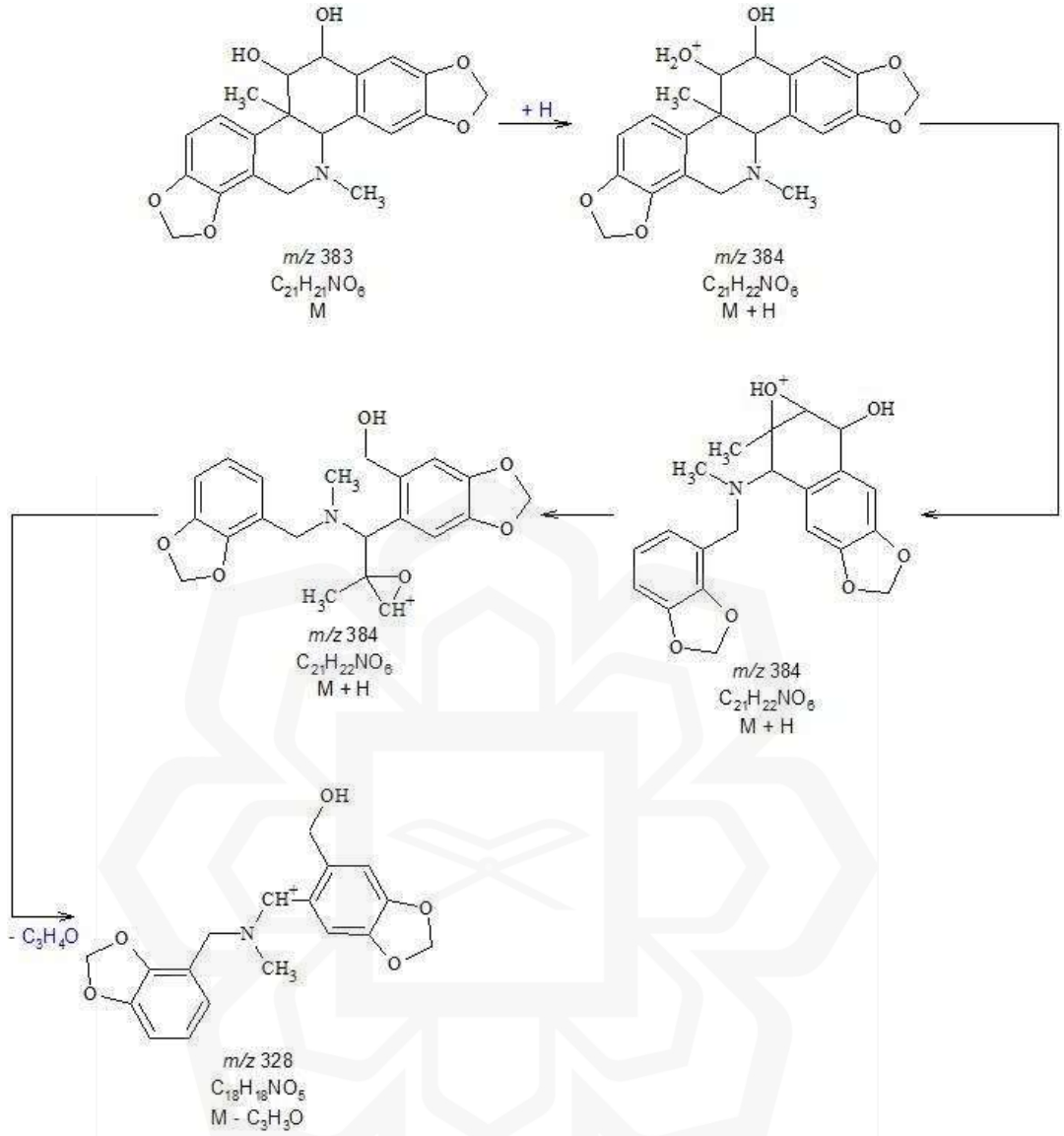

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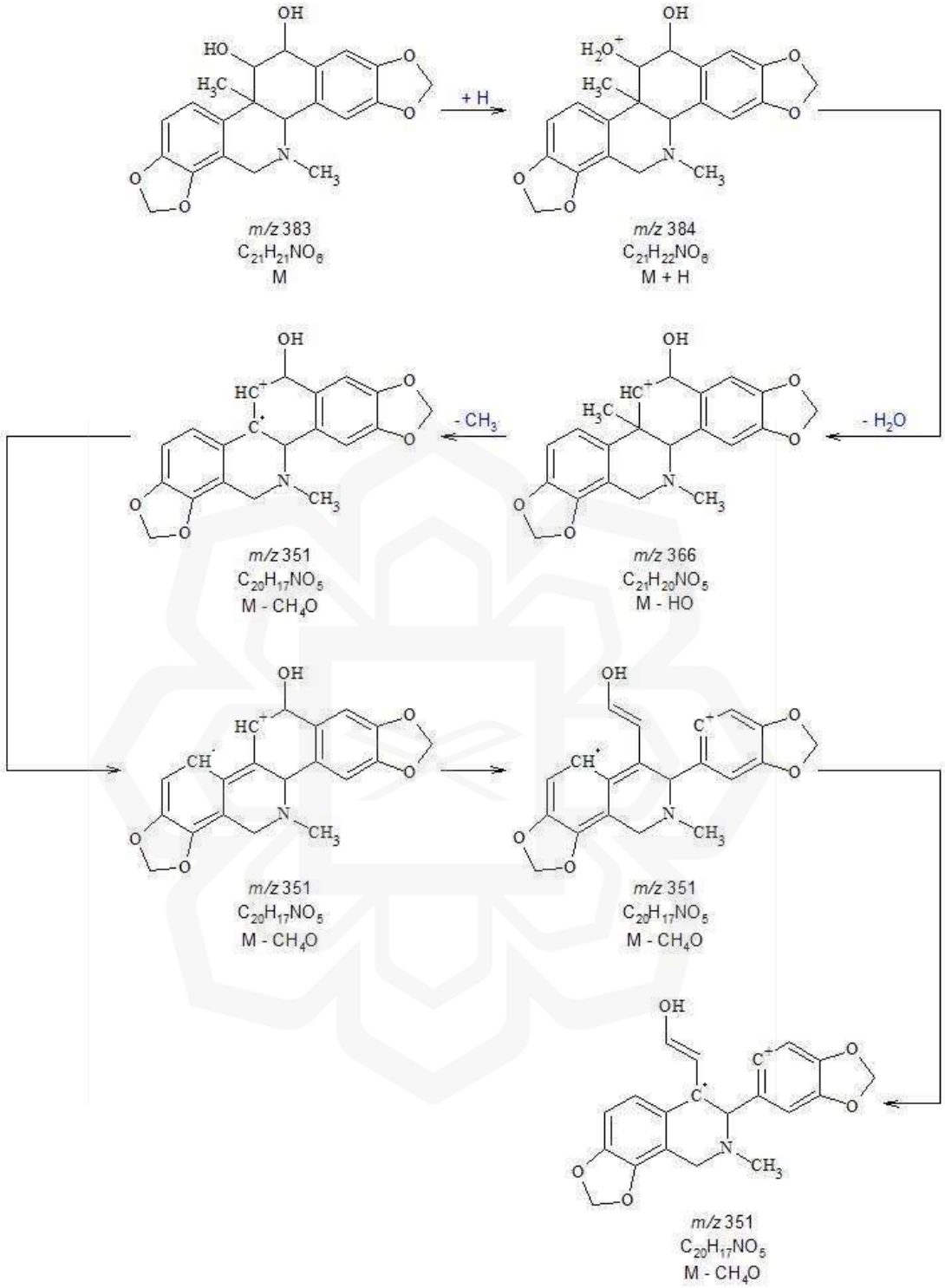

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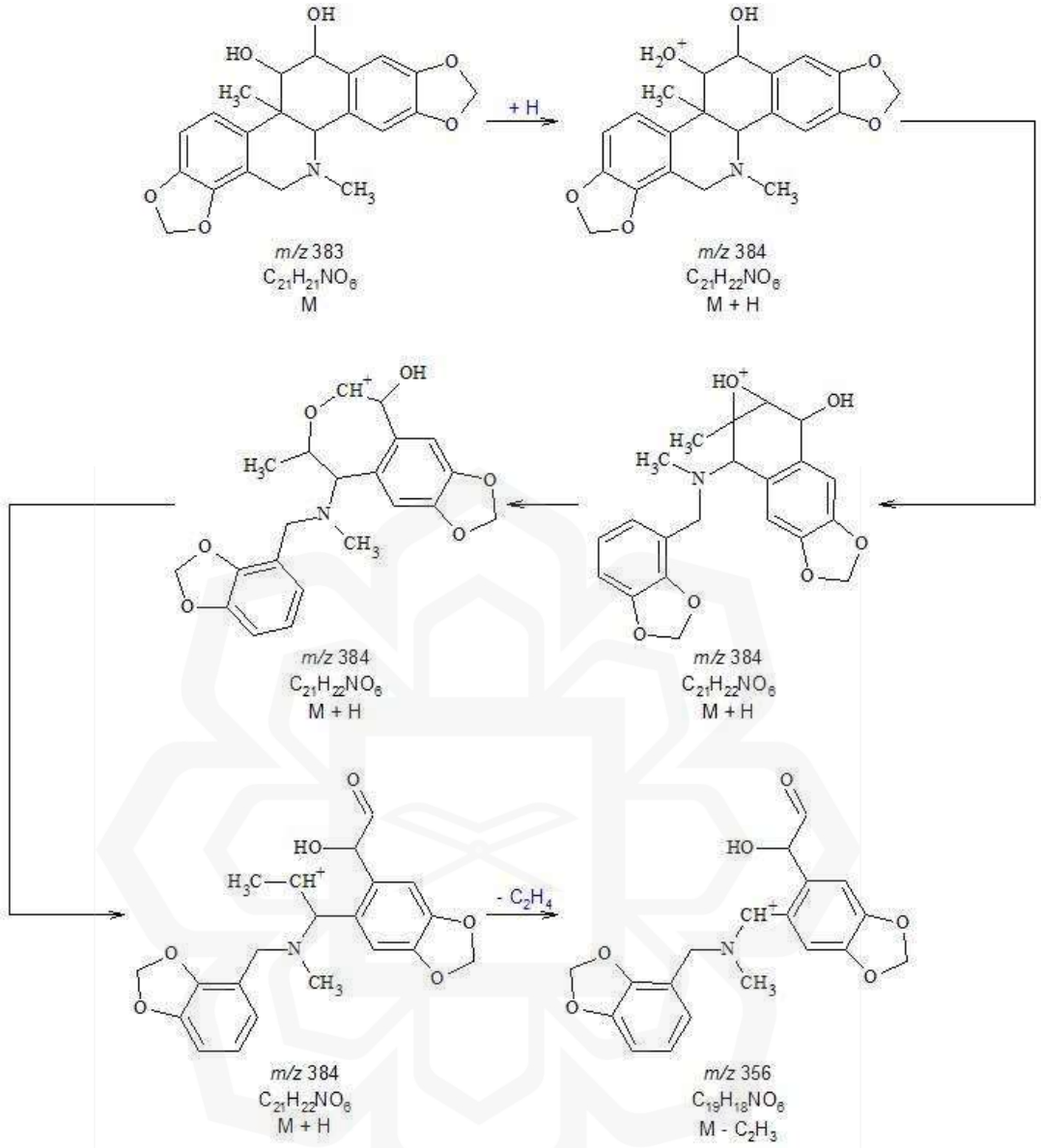

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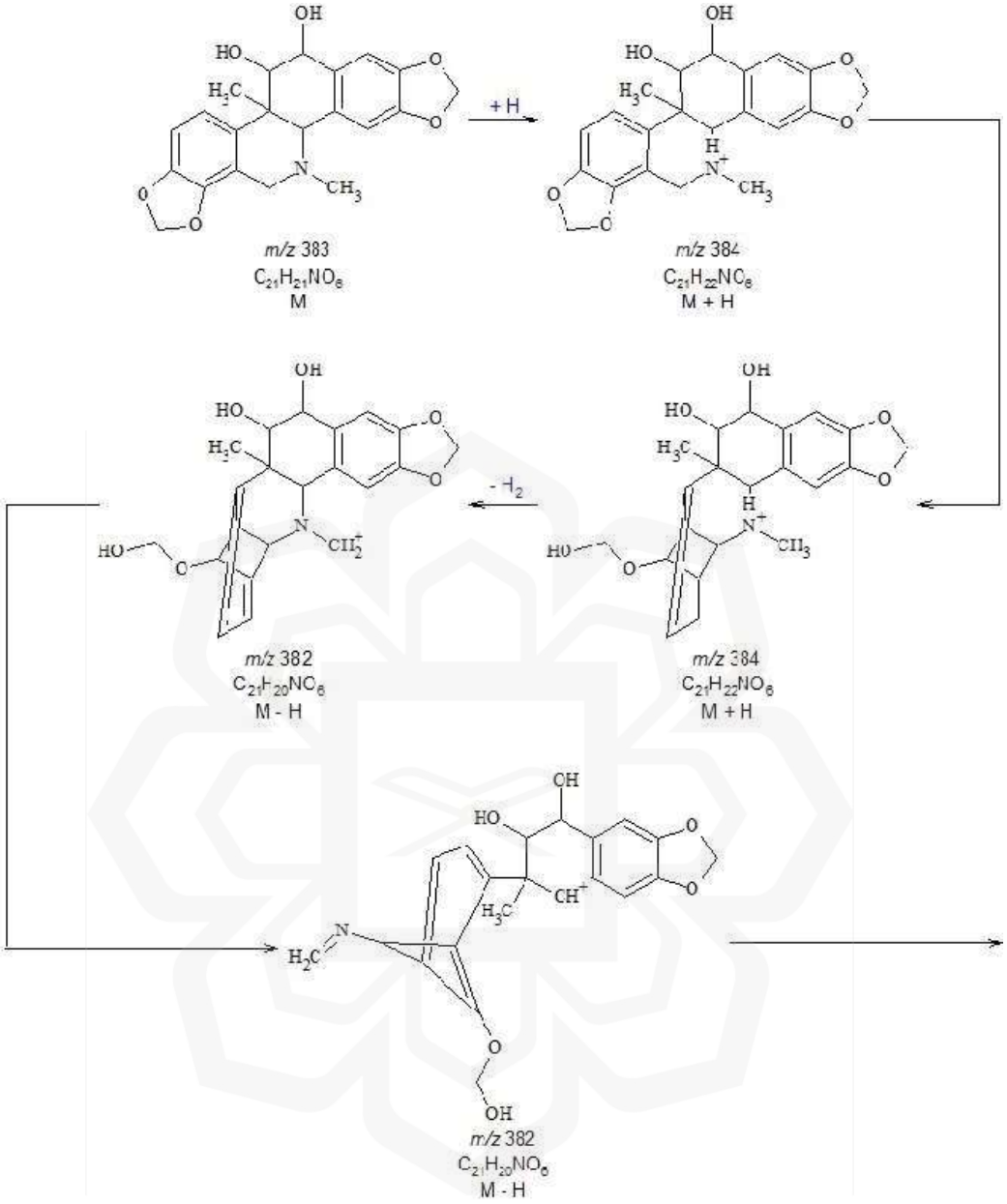

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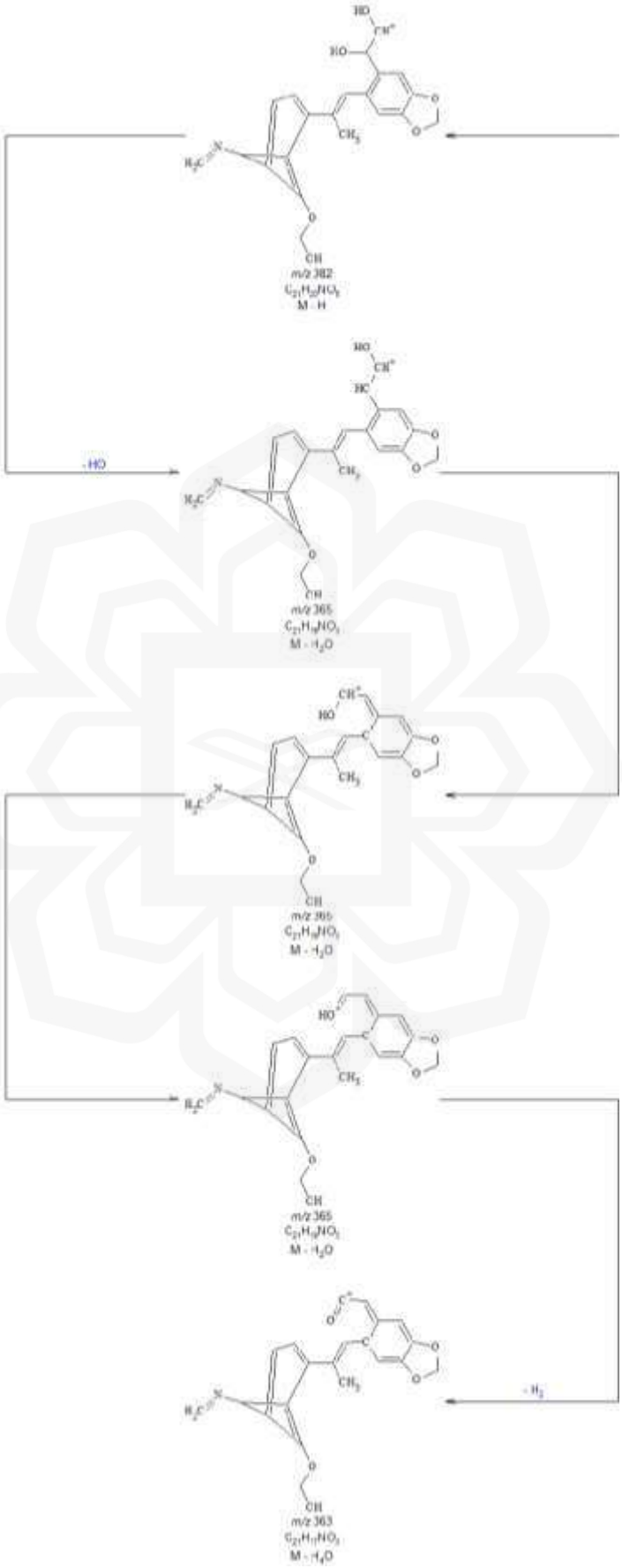

APPENDIX C. THE % INHIBITION OF DIFFERENT METHANOL CONCENTRATION

	100% (1)	100% (2)	100% (3)	100% (4)	100% (5)	75% (1)	75% (2)	75% (3)	75% (4)	75% (5)
Concentration mg/mL	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
% INHIBITION	43.503	38.613	57.506	61.56		46.205	41.555	51.874	51.547	42.363
	45.688	32.787		54.895	64.478	44.543	35.541	44.906	47.189	44.005
	48.792	31. 1	48.279	57.358	62.446	40.83	34.773	47.644	45.079	39.17
	44.263		42.676	58.146	56.351	39.043	33.148	44.743	44.774	41.75
AVERAGE			57.432	57.938	66.723	43.86		48.141	47.938	42.706
Extract (mg)	200.9	208.7	302.4	316.2	298.4	85.3	113.6	124.5	127.1	129.4
STANDARD DEVIATION	2.3364	3.9341	7.4881	2.7539	4.2292	3.2938	3.6719	3.3322	3.1235	2.0081
	5.2244					2.7877				
	50% (1)	**50% (2)**	**50% (3)**	**50% (4)**	**50% (5)**	**25% (1)**	**25% (2)**	**25% (3)**	**25% (4)**	**25% (5)**
Concentration mg/mL	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
% INHIBITION	30.692	23.133	24.864	25.39	38.767	25.722	30.082	23.036	33.505	31.416
	32.504	19.512	22.113	24.169	32.274	26.941	24.134	19.208	32.587	
	29.902		20.854	27.781	33.02	24.084	28.375	18.941	26.95	24.99
	26.192	15.047	27.779	23.282	37.95	18.578	27.897	26.364		26.19
AVERAGE	31.032		22.611	25.78	34.687	25.583	27.53	20.395	28.535	
Extract (mg)	68.7	74.1	73.7	60.4	116.3	117.7	154.7	152.8	121.9	69.4
STANDARD DEVIATION	2.6539	4.05	3.0791	1.9519	3.3286	3.693	2.5072	3.5222	3.5496	3.4169
	5.378					3.6239				

APPENDIX D. TROLOX DATA

Concentration of trolox (µg/mL)	R1	R2	R3	AVERAGE	Concentration	Absorbance
200	0.8594	0.869	0.845	0.8578	200	0.8578
100	0.5542	0.5688	0.5646	0.562533	100	0.562533
50	0.2439	0.2505	0.2484	0.2476	50	0.2476
25	0.2149	0.2078	0.217	0.213233	25	0.213233
12.5	0.1777	0.1785	0.1765	0.177567	12.5	0.177567
6.25	0.1581	0.1576	0.1585	0.158067	6.25	0.158067
3.125	0.1296	0.1176	0.1217	0.122967	3.125	0.122967
1.5625	0.138	0.1483	0.149	0.1451	1.5625	0.1451

APPENDIX E. LIST OF PUBLICATIONS

1. Maniam, N., Khatib, A., Ahmed, Q. U., Ibrahim, Z., Syed Mohamad, S. N. A., Nipun, T. S., & Humaryanto. (2024). Identification of putative α-glucosidase inhibitors and antioxidants in *Zingiber officinale* rhizome using LCMS-based metabolomics and in silico molecular docking. *Natural Product Research*, 1–6. https://doi.org/10.1080/14786419.2024.2369224

2. Maniam, N. Identification of alpha-glucosidase inhibitors and antioxidant activity in *Zingiber officinale* rhizome using LC-MS based metabolomics and molecular docking. KOP Research Symposium, November 2-3,2022.