Chemical Study of *Etlingera elatior* (Zingiberaceae) - Rhizome and Its *α*-Glucosidase and *α*-Amylase Inhibitory Activities

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Thesis Title	Chemical Study of *Etlingera elatior* (Zingiberaceae) - Rhizome and Its α-Glucosidase and α-Amylase Inhibitory Activities
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I hereby certify that this work has not already been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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ABSTRACT

Etlingera elatior is widely cultivated in Southeast Asia and used as a common spice for various foods and beverages. It can be eaten raw or cooked as vegetable as food. Especially it has been utilized as traditional medicines in many recipes. Several pharmacological activities including antimicrobial, antifungal, antioxidant, anti-diabetes, cytotoxic, tyrosinase inhibition and hepatoprotective activities of this plant have been reported. The purpose of this study was to evaluate the potential of *E. elatior* rhizome to inhibit *α*-glucosidase and *α*-amylase enzymes for anti-diabetes treatment. The results of this study showed that crude ethanol extract at concentration of 25μg/mL exhibited *α*-glucosidase and *α*-amylase inhibitory activities. All partitions from crude ethanol extract including hexane, dichloromethane, ethyl acetate, *n*-butanol and water fractions also showed *α*-glucosidase and *α*-amylase inhibitory activities but in different extent ranging from 28.36-99.79% and 35.91-58.13%, respectively. IC_{50} of crude ethanol extract against *α*-glucosidase was at 1.22 μg/mL compared with acarbose (positive drug) 139.85 μg/mL and for *α*-amylase inhibitory activity, crude ethanol extract showed IC_{50} at 68.13 and that of acarbose at 5.67 μg/mL. The enzyme kinetic inhibitory study of crude ethanol extract revealed competitive inhibition mode against *α*-glucosidase while acarbose showed a non-competitive inhibition mode.

Phytochemical screening of crude and partitioned fractions demonstrated the presence of phenolic and flavonoid in all extracts. Tannin was not detected in hexane extract and terpeniod was not found in ethyl acetate and water extracts. Ethyl acetate, *n*-butanol and dichloromethane extracts demonstrated to have good scavengers against DPPH with IC_{50} values of 12.60, 24.77 and 26.61 μg/mL, respectively, compared to ascorbic acid (standard) with IC_{50} value of 4.22±0.42 μg/mL.

Furthermore dichloromethane, *n*-butanol and ethyl acetate extracts revealed good anti-oxidation profile by ferric reducing antioxidant power assay (FRAP) reported as quercetin equivalent in 1.51, 2.18 and 3.28 mg/g sample, respectively. Good antioxidant activity may be due to the presence of tannin, phenolic and flavonoid in the extracts. The purification processes are utilized for isolation of pure compounds from the n-hexane extract and the results revealed that residue of EH2.3.3.8.3 compose of a mixture of *β*-amyrin and fixed oil whereas residue of EH3.4.4.3 comprised of *β*-sitosterol and stigmasterol. The mixture of *β*-amyrin and fixed oil showed IC_{50} of *α*-glucosidase and *α*-amylase inhibitory values at 12.11 and 43.29 μg/mL, respectively. The mixture of *β*-sitosterol and stigmasterol exhibited *α*-glucosidase inhibitory values at 193.5 μg/mL. There results demonstrated the potential utilization of *E. elatior* rhizome for diabetes treatment. However, more detail of the active compounds need to be further investigated.

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LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
δ	Chemical shift in ppm
CC	Column chromatography
$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Dichloromethane
CHCl3	Chloroform
cm^{-1}	Wave number
^{13}C NMR	Carbon-13 nuclear magnetic resonance
d	Doublet
dd	Doublet of doublet
ddd	Doublets doublets of doublets (for NMR spectra)
dt	Doublet of triplet (for NMR spectra)
DMSO	Dimethylsulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EtOAc	Ethyl acetate
EtOH	Ethanol
FRAP	Ferric reducing antioxidant power
HCl	Hydrochloric acid
^{1}H NMR	Proton-1 nuclear magnetic resonance
Hz	Hertz
HPLC	High-performance liquid chromatography
g	Gram
IC_{50}	Medium inhibition concentration
IR	Infrared radiation
J	Coupling constant
Kg	Kilogram
L	Liter
M	Molar
MeOH	Methanol

mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
nm	Nanometer
NMR	Nuclear magnetic resonance
ppm	Part per million
s	Singlet
SD	Standard deviation
t	Triplet (for NMR signals)
T2DM	Type 2 diabetes mellitus
TLC	Thin layer chromatography
UV	Ultraviolet
UV-VIS	Ultraviolet-visible
µ	Micro
µg	Microgram
µL	Microlitre
µM	Micromolar
λ_{max}	Maximum wavelength (nm)
°C	Degree Celsius
%	Percentage

CHAPTER 1

INTRODUCTION

1.1. Introduction

Currently, people are very concerned about the high mortality by chronic diseases, especially cardiovascular diseases and diabetes. In side of these, diabetes mellitus (DM) is one of chronic disease which is presented by hyperglycemia or high blood sugar (World Health Organization, 2011). This disease is a major health problem and also the one of the major killer in recent time (Chakrabarti and Rajagopalan, 2002). This disease affected about 25% of population around the world (Kavishankar et al., 2011). Moreover, the rate of mortality globally is about 9% reported in 2008 (Andrade-Cetto et al., 2008). The number of diabetes mellitus patients is increasing rapidly along the population growth and affecting all parts of the world (Patel et al., 2012). According to World Health Organization (2013), the diabetic population in 2010 was about 347 million people worldwide (Wild et al., 2004) and in 2011 was about 366 million. It is likely increasing to at least 552 million by 2030 (Whiting et al., 2011). In long term diabetic mellitus patients, high blood sugar levels can damage many parts of organs and lead to multiple health problems such as hypertension, coronary vascular disease, cardiomyopathy, stroke, retinopathy, nephropathy and neuropathy. Moreover, oxidative stress and inflammation action are increasing and making diabetes more complicated and gaining more serious problems (Ceriello and Testa, 2009). The causes of diabetes mellitus could be due to the defect in insulin secretion, insulin action or both (World Health Organization, 2011). α-Glucosidase and α-amylase enzymes located in the brush-border surface of intestine are the enzymes responsible for breaking down carbohydrate, protein and lipid into glucose after food taken and these could be also another cause of diabetes.

α-Amylase or α-1,4-glucan-4-glucanohydrolase (E.C. 3.2.1.1) and α-glucosidase are enzymes that play a role in the human digestion system of carbohydrates. α-Amylase catalyzes the initial step in the hydrolysis of starch to maltose which is eventually degraded to glucose by α-glucosidase (Ponnusamy et al.,

2010). These glucoses are then absorbed through the GI tract to bloodstream. If a person has insulin resistance or impaired insulin secretion and no uptake of glucose or not used by cells for energy, it will be resulting in diabetes mellitus type 2. Now a day, number of diabetes mellitus type 2 patients are reaching up to 90-95% of populations who have diabetes worldwide. There are several methods to treat DM type 2 including regular exercise, blood sugar monitoring, medicine, insulin therapy and healthy eating. However, one new alternative therapeutic approach for diabetic patients, especially those who have type 2 diabetes, is to inhibit carbohydrate hydrolyzing enzymes, such as α-amylase and α-glucosidase to retard the uptake of glucose (Ali et al., 2006; Nickavar and Amin, 2011). Deceleration of starch digestion by inhibition of these two enzymes could give a result in the control of diabetes (Ponnusamy et al., 2010). Since slower digestion and absorption of carbohydrates could decrease postprandial hyperglycemia (Ali et al., 2006; Ortiz-Andrade et al., 2007). Recently, the commercially available inhibitors of α-amylase and α-glucosidase which are currently in clinical used are acarbose, miglitol and voglibose (Bailey, 2003). However, side effects such as liver disorders (Shobana et al., 2009), bloating, flatulence, diarrhea and abdominal distension (Chakrabarti and Rajagopalan, 2002; Kimmel and Inzucchi, 2005) were always observed during the patients using these drugs. They may also increase the incidence of renal tumors, hepatic injury and acute hepatitis (Shobana et al., 2009).

It is known that utilization of alternative medicine especially from natural sources may provide less serious side effects and especially low cost in disease treatment. Therefore, natural products are of interest to many traditional practioners to be used as alternative therapy in diabetes especially in developing countries where most people have limited access to the modern medicine (Bhandari et al., 2008). Traditional medicine has been used throughout the world so far. From the oldest document written 4800 years ago which provided description of not less than 360 plants have been reported to be used in diseases treatment (Mann, 1992). Both traditional herbal medicines and modern phytomedicines have been studied so far. At least 136 plants have been described clearly in previous time in the treatment of diabetes mellitus (Kavishankar et al., 2011). Several natural products which have α-amylase and α-glucosidase inhibitory activities also have been previously reported.

For example, cinnamon powder, bitter melon, fenugreek, neem, and tea were demonstrated to be used successfully in blood glucose reduction (Ponnusamy et al., 2012). One major cause of effectiveness of these plants was to delay the progress of diabetic complications.

Etlighera elatior belongs to the family Zingiberaceae (Jackie et al., 2011). It grows widely in tropical forest of South East Asia (Chan et al., 2011). It is widely cultivated for use as food flavouring especially as ingredient in curries, as leafy ornamental, as medicine such as fruits for earache, leaves for cleaning wounds and post-partum women. Moreover, leaves of this plant were used to mix with other aromatic herbs for bathing to remove body odour (Chan et al., 2011).

The phytochemical screening and biological activities testing of its inflorescences were previously reported (Chan et al., 2011). Those activities including antioxidant, antibacterial, antifungal, tyrosinase inhibitory, anti-inflammation, antitumor and cytotoxic activities of inflorescences and rhizome extract were published (Habsah et al., 2005). However, non of any report was focused on the activity of extract from *E. elatior* rhizome on carbohydrate digestive enzyme in particular pancreatic α-amylase and α-glucosidase inhibitory activity. We therefore would like to investigate for these activities of the extracts from *E. elatior* rhizome to study for their potential use in diabetic treatment.

The important of utilization of herbal medicines in diseases treatment is the quality of the herbal products. Therefore, the quality control of herbal medicines is one of the major concerns in order to relate to their biological activities. The effectiveness of utilizing herbal products can be varied depending on the genetic variation, growing conditions, timing and method of harvesting (Liang et al., 2004; Kavishankar et al., 2011). In the case of *E. elatior*, quercetin, gallic acid, cyanidin 3-glucoside and catechin were reported to be used as biomarkers for its inflorescences extracts that showed antioxidant activities (Chan et al., 2013). Leave extracts of *E. elatior* showed antibacterial activity, and chlorogenic acid was used as a biomarker in quality determination (Chan et al., 2011). However, non of any literature has been reported of any compounds as it biomarkers for *E. elatior*'s rhizome.

In our study, rhizomes of *E. elatior* was extracted using several methods and their α-amylase and α-glucosidase inhibitory activities were assessed. Active

components were subjected to be purified by using chromatographic processes and characterized by spectroscopic analysis. They were then used for their enzyme inhibitory testing the development for the standardization protocols to control the quality of the extracts in order to achieve the best biologically active herbal medicines for diabetic treatment.

1.2. Objectives

1.2.1. To prepare *Etlingera elatior* rhizome extracts by using different types of solvents.

1.2.2. To evaluate their α-glucosidase and α-amylase inhibitory activities.

1.2.3. To determine the phytochemical content of the extracts.

1.2.4. To purify the chemical compounds from the *E. elatior* extracts.

1.2.5. To evaluate the α-glucosidase and α-amylase inhibitory activities of pure compounds which have been isolated.

CHAPTER 2

LITERATURES REVIEW

2.1. Diabetes

Diabetes mellitus is a group of metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, 2014). Diabetes is recognized as a serious global health problem which normally associated with many organs damage, dysfunction and failure in proper working, especially eyes, kidneys, nerves, heart, blood vessels and ulcers (Jong-Anurakkun et al., 2007). Blood glucose (blood sugar) is an essential indicator for health condition. The normal blood sugar levels in healthy people are in between 70 and 130 mg/dL before meals and less than 180 mg/dL at 2 hours after meals (Andrade-Cetto et al., 2008). If the blood sugar level is higher than 126 mg/dL after fasting or not eating for eight hours, this indicates diabetes (Verdoia et al., 2014). There are many symptoms resulting from hyperglycemia without glucose meter such as unusual produced large amount of urine, great thirsty, huge appetite, weight loss, and blurred vision (Care, 2014).

2.1.1. Mechanism of insulin

It is known that human body needs to break down the food into glucose to be absorbed by the brush border of small intestine into bloodstream for cells using as energy for living. The healthy human requires the blood glucose level to be stable in the bloodstream. Normally the concentration of glucose can be fluctuated in bloodstream according to pancreas production of insulin and glucagon. After eating, the glucose remains in high level in the bloodstream that will stimulate insulin secretion to reduce blood glucose level. On the other hand if the blood glucose is in low level, that will reduce the level of insulin secretion.

In the case the blood glucose is low between meals or during exercise, glucagon will be secreted by the alpha cells of the pancreas stimulate the liver

function store glucose. From those mechanisms will result in the stability of the blood glucose in the bloodstream **(Figure 2.1a)**.

The patients of type 2 diabetes mellitus have high blood glucose level or hyperglycemia, therefore insulin plays important role to reduce the amount of glucose in the bloodstream and facilitate its transfer glucose into the cells. Insulin will bind to special receptors on the surface of cell membranes and will activate glucose transporter (GLUT-4) to allow glucose entering to the cells. The glucose transporters effectively remove glucose from the bloodstream by this pathway. On the contrary if the patients have insulin impairment or dysfunction, insulin does not stimulate with insulin receptor and the GLUT 4 does not allow glucose to go into the cells, thus resulting in high blood glucose level **(Figure 2.1b)**.

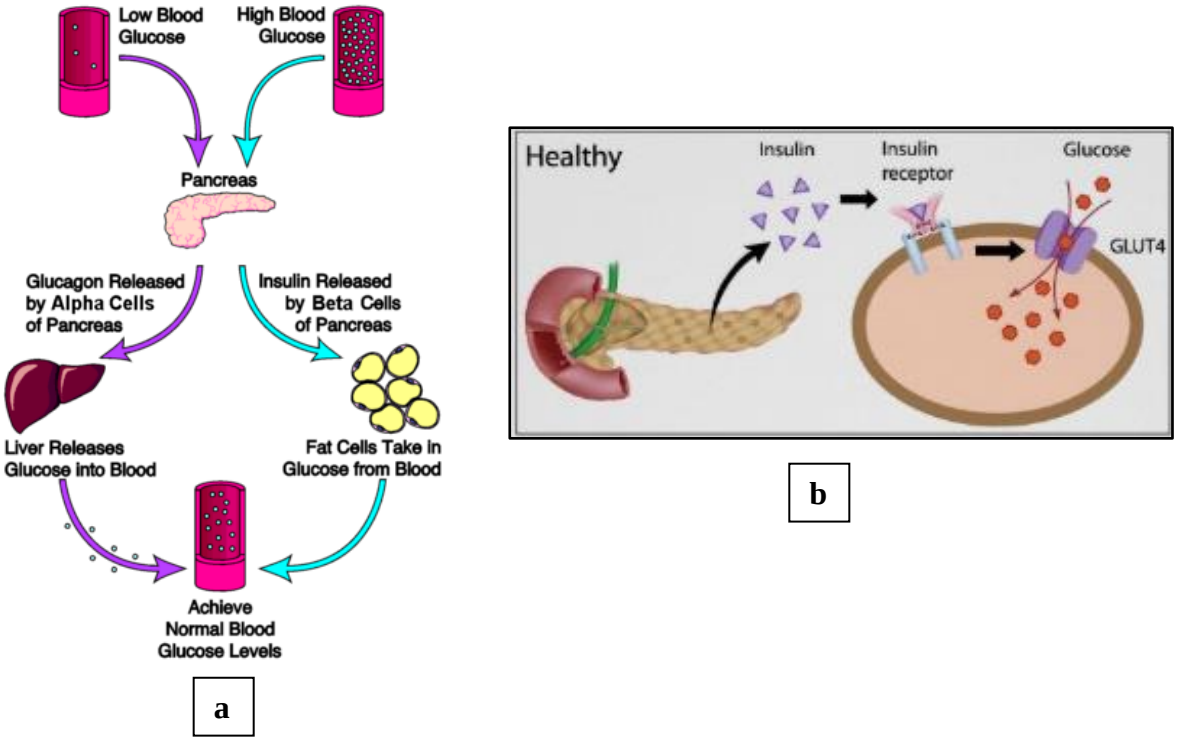


Figure 2.1: (a). The function of pancreas in production of insulin and glucagon (b). the role of insulin on the cells

(From: www.scottsdaleweightloss.com, accessed date: December, 2014)

2.1.2. Types of diabetes

Diabetes can be divided into four main types. Diabetes mellitus type 1 (T1DM) or juvenile-onset diabetes is characterized by insulin production impairment since childhood. It is known as insulin-dependent diabetes since of the body's failure to produce insulin or autoimmune destruction of the β-cells of the pancreas (American Diabetes Association, 2014) **(Figure 2.2)**. This type of diabetes accounts for almost 5-10% and usually occurs with people often in early adulthood or teenage years or at least before forty years old. To manage T1DM, the patients need life long insulin injection.

Diabetes mellitus type 2 (T2DM) is caused by many factors but generally due to are insufficient insulin action. It is known as non-insulin-dependent diabetes mellitus (Scheen, 2003; Fred-Jaiyesimi et al., 2009; Kavishankar et al., 2011) or adult-onset diabetes resulting from insulin resistance **(Figure 2.2)**. Among the four main types of diabetes, diabetes type 2 or diabetes mellitus makes up about 90-95% (American Diabetes Association, 2014) of all cases of diabetes and it is the most complex especially is not easily to manage. People often develop this type of diabetes after 40 years old (48-60 years) (Turner et al., 1998). Nevertheless, since late 1990 this type of diabetes was also found in young people.

Diabetes type 3 is called gestational diabetes (GDM). It occurs mostly in pregnancy women. Degenerate of glucose intolerance during pregnancy occurs normally (Organization, 2013). This type of diabetes occurs when the mother’s body has the glucose intolerance and insufficient of production not enough to stimulate with insulin receptor for transport all of the glucose into the cells resulting in development of diabetes **(Figure 2.2)**.

Other specific types of diabetes are the 4^{th} type of diabetes. This form of diabetes is associated with monogenetic defects in β-cell function. For instance, glucokinase gene mutations to cause genetic defects of β-cell function has effect to insulin secretion and mutations of insulin receptor which will degrade insulin action. Pancreas has been destroyed by exocrine pancreas disease to cause β-cell disfunction related to pancreatitis, trauma, infection, pancreatic-tomy and pancreatic carcinoma.

Besides this, there are many factors to cause this type of diabetes including drugs or chemical-induced such as, pentamidine, nicotinic acid, glucocorticoids, thyroid hormone diazoxide, β-adrenergic agonists, thiazides, dilantin and α-interferon to defect insulin action (Miller et al., 1978). A disease from endocrine gland (endocrinopathies) affect many hormones such as growth hormone, cortisol, glucagon and epinephrine that related to several diseases including acromegaly, Cushing's syndrome, glucagonoma, phaeochromocytoma, hyperthyroidism, and somatostatinoma. Viral infection diseases such as congenital, rubella, cytomegalovirus also damaged β-cell. The impaired immune system is related to genetic syndromes that associated with diabetes.

The control of hyperglycemia is the best way in the management of diabetes type 2 because acute and chronic complications can occur if the blood glucose concentration is not kept in normal levels. Normally, elevated blood glucose level is usually observed after carbohydrate consumption (Ponnusamy et al., 2012).

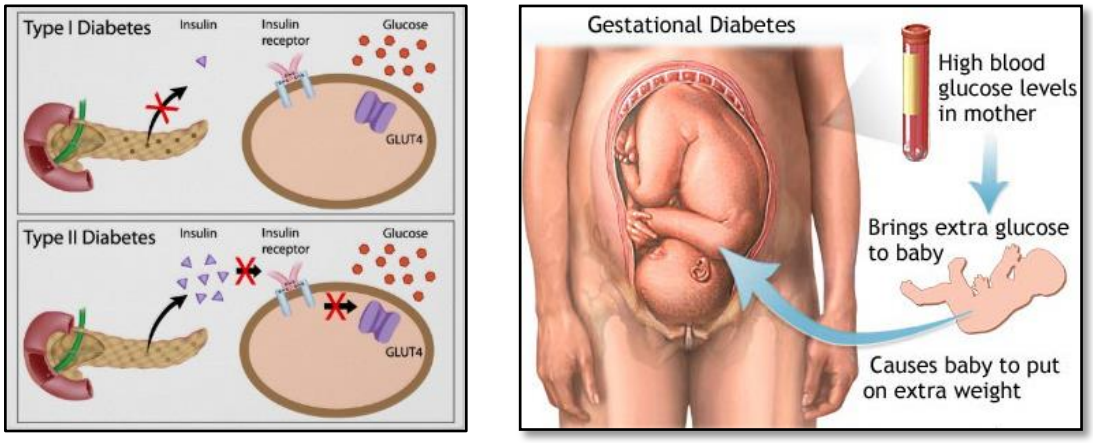


Figure 2.2: The causes of Diabetes mellitus type 1, type 2 and Gestational Diabetes (From: www.scottsdaleweightloss.com, accessed date: December, 2014)

2.1.3. Risk factors in type 2 diabetes

There are many factors that are the risk factor that may lead to type 2 diabetes mellitus including aging, obesity, insufficient energy consumption, alcohol drinking, smoking, etc. They are important risk factors of pathogenesis of type 2 diabetes. Obesity is one of the major risk factors especially related to visceral fat obesity. This

risk factor is found in middle and high aged patients because of patients' lack of exercise so, the muscle mass will be decreased and may resulting in insulin resistance (Ozougwu, 2013).

2.1.4. What are the causes of type 2 diabetes mellitus

Starch belongs to the carbohydrate food in human diets. It consists of a large number of glucose units bond by glycosidic bonds. After carbohydrate food containing starch has been consumed, enzymes in GI tract will break down or hydrolyze starch into the sugars. They are known as α-amylase and α-glucosidase. They are digestive enzymes in the intestinal lumen and in the brush border membrane (Hansawasdi et al., 2000). Starch is then cut into oligosaccharides units by α-amylase that is produced from saliva glands bound with tooth and continue to break down by amylase enzyme from small intestine secretion (Sales et al., 2012). α-Glucosidase in the intestinal are powerful enzyme to catalyze the cleavage of glycosidic bonds to release glucose into bloodstream (Borges de Melo et al., 2006) **(Figure 2.3)**. Upon the presence of glucose in the blood, pancreas produces insulin to stimulate insulin receptor of the cells to be used as energy. When the insulin production is not enough or insulin resistance occur, therefore, no insulin stimulation on insulin receptor resulting in no glucose consumption of cells. Glucoses still remain in the blood vessel cause hyperglycemia. Finally with the high level of glucose in the blood vessel, a person will acquire type 2 DM-diabetic.

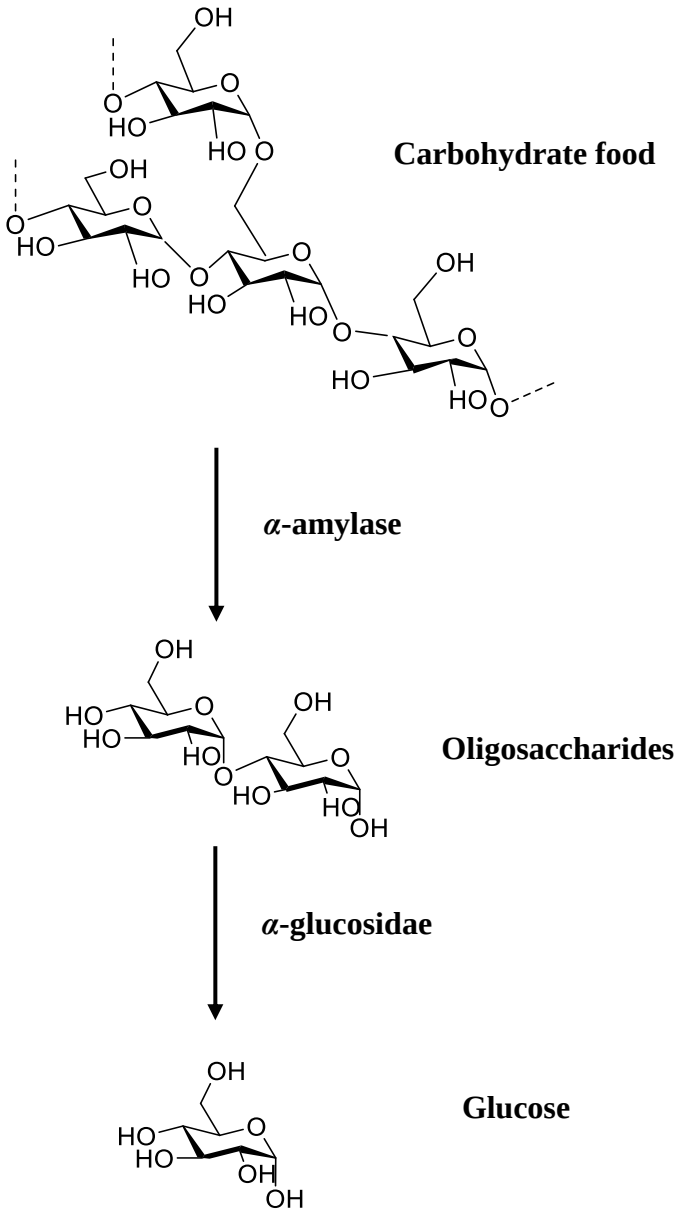


Figure 2.3: *α*-amylase and *α*-aglucosidase digestive enzymes break down or hydrolyze starch into the sugars

2.1.5. Type 2 diabetes treatments

According to the extremely high number of type 2 diabetes patients, the disease need to be managed and controlled urgently to reduce the risk of developing others diabetes complications. The first recommendation are diet control and exercise, but if the patients cannot cope with this recommendation, the pharmacological

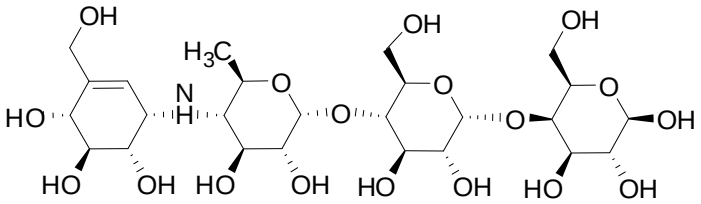
intervention should be applied. The first aim of therapy is to regulate insulin level. Up to now, there are six different classes of hypoglycemic agents are available including sulfonylureas (gliclazide), meglitinides (repaglinide), biguanides (metformin), thiazolidinediones (pioglitazone), alpha-glucosdase inhibitors (acarbose) and DPP-4 inhibitors (sitagliptin) and being used depending on the mechanism of action, site of action therapeutic application to control blood glucose level (Chakrabarti and Rajagopalan, 2002; Inzucchi, 2002; Kimmel and Inzucchi, 2005; TODA, 2008) (**Table 2.1)**.

Among six classes of drugs for treatment of type 2 diabetes, four classes: sulfonylureas, biguanides, thiazolidinediones, and α-glucosidase inhibitors have been extensively used to treat DM so far. The mechanisms of these drugs have different effects on insulin action (Derosa et al., 2011). For instance, sulfonylureas help increasing insulin level by effectively activating the pancreatic cell to promote secretion of insulin (Guney et al., 2002; Josse et al., 2003). The biguanides are related to insulin receptors to stimulate the adsorption of sugar into cells, therefore the glucose in the blood vessels is reduced (Defronzo et al., 1991). They are suitable to used in elderly patients. Thiazolidinediones are drugs that activate insulin to stimulate carbohydrate metabolism. α-Glucosidase inhibitors are drugs that involving in reduce metabolism of carbohydrate digestion. They can be used in insulin resistance patients, moreover, they can protect β cells of pancreas (Chiasson et al., 2002). Eventhough, their mechanisms are different but they play the same important role as to reduce the blood glucose levels by delay or prevent the complication of diabetes. Nevertheless, using these medicines, side effects such as heart failure, liver disease, renal problem and gastrointestinal effect may present. Therefore, nowadays alternative therapeutic agents to treat diabetes are popular instead of these medicines.

2.1.5.1. α-Glucosidase inhibitor

α-Glucosidase inhibitors are a group of drugs to inhibit the enzymes that break down the carbohydrate foods such as starch for treatment of diabetes mellitus type 2. Carbohydrates are normally converted into simple sugars (monosaccharides) which can be absorbed through the intestine. Hence α-glucosidase inhibitors reduce the impact of carbohydrates on blood sugar. Acarbose **[Figure 2.3 (1)]** (Precose®) is one

of the *α*-glucosidase inhibitor available on the market nowadays and has been available for clinical use for about 20 years (Standl and Schnell, 2012). Other *α*-glucosidase inhibitors are miglitol **(2)** (Glyset®) and voglibose **(3)** (**Figure 2.4**). Their roles are to delaying the absorbance of carbohydrates (complex form of sugar) (Van de Laar et al., 2009) in the gut. Acarbose, (*O*-4,6-dideoxy-4-[[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-(hydroxymethyl) -2-cyclohexen-1-yl] amino]-*α*-D-glucopyranoslyl-(1-> 4)-*α*-*D*-glucopyranosyl-(1-> 4)-D-glucose (Ibrahim et al., 2007) is an oligosaccharide to be used as an oral *α*-glucosidase inhibitor to decrease the breakdown of carbohydrate and delay the absorption of the glucose in the small intestine (Shibao et al., 2007). Acarbose is not only working act as inhibitor of *α*-glucosidase but it is also blocking pancreatic *α*-amylase enzyme in the lumen of the small intestine (Borges de Melo et al., 2006; Mogale et al., 2013). It is one of the powerful drug for the treatment of hyperglycemia in diabetic patients. Since many of diabetes drugs like acarboses have side effects, nowadays researchers have put many efforts to find safe inhibitors of *α*-glucosidase and *α*-amylase from natural products for treatment of diabetes to replace diabetes drugs (Bhandari et al., 2008).



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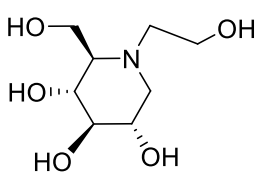

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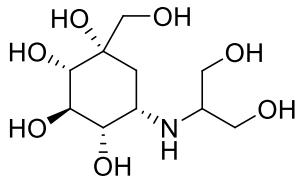


(3)

Figure 2.4: *α*-glucosidase inhibitors (1)=Acarbose, (2)=Miglitol, (3)=Voglibose

Table 2.1: Drugs for treatment of type 2 diabetes

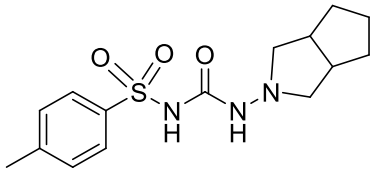
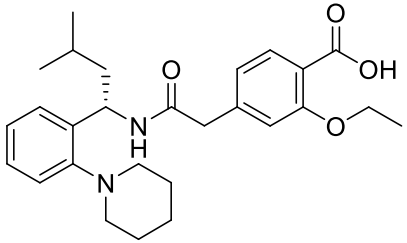
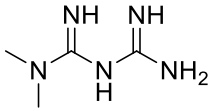
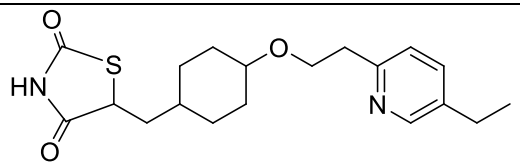
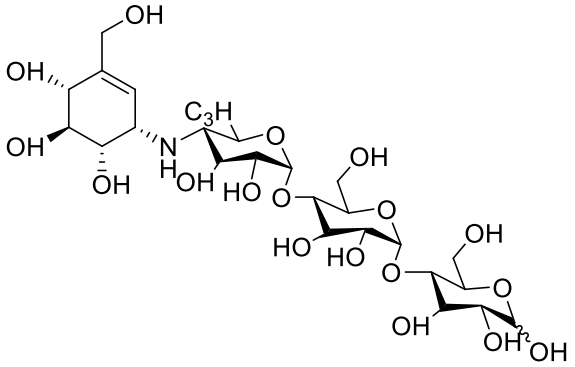
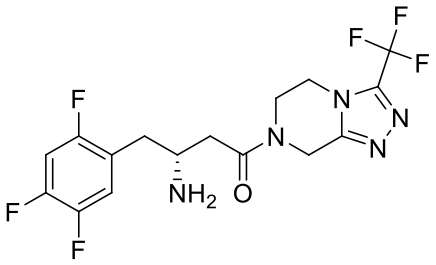
Drug class	Drug name	Chemical Structure	Mechanism of action	Side effect
Sulfonylurea	Gliclazide		-function on beta cells for endogenous insulin production.	-hypoglycemia -weight gain
Meglitinides	Repaglinide		-stimulate the release of insulin from the pancreatic beta cells	- weight gain - hypoglycemia
Biguanides	Metformin		-improving insulin sensitivity in hepatic	-weight loss -renal insufficiency -cardiogenic -congestive heart failure -liver disease -pulmonary insufficiency

Table 2.1: Drugs for treatment of type 2 diabetes (Continued)

Drug class	Drug name	Chemical Structure	Mechanism of action	Side effect
Thiazolidinediones	Pioglitazone		-decrease insulin resistance	-liver toxicity -weight gain -impaired renal function -hepatic dysfunction
α-Glucosdase inhibitors	Acarbose		-inhibit enzyme alpha-glucosidase	-abdominal discomfort -bloating -flatulence -diarrhea
DPP-4 inhibitors	Sitagliptin		-inhibit enzyme DPP-4 for increasing the levels of active incretin hormones	-weight gain -respiratory infection -runny nose -headache

2.2. *Etlingera elatior*

2.2.1. Botanical description of *Etlingera elatior*

Zingiberaceae is the largest monocotyledon family, with 52 genera and 1400 species (Kasarkar and Kulkarni, 2011) including *E. elatior* **(Figure 2.5)**. The typical habitat of *E. elatior* is widely distributed in tropical forests. This plant is native to Indonesia, Vietnam, Thailand and Malaysia and widely cultivated in South East Asia (Lachumy et al., 2010). The common names of this plant include torch ginger and the Philippine wax flower. In Malaysia, the flower of the *E. elatior* is commonly known as *bunga kantan*, in Indonesia as *bunga kecombrang* or *honje*, and in Thailand as *dalah* (ดาหลา). The species is a large tall ginger plant, growing in clumps to heights of up to 1.5 meter and having anatomic parts known as rhizome, flower, bud, stem, and leaf. Each rhizome can produce leafy shoots. Its inflorescences are terminal, borne either on leafy shoots or on erect shoots near the base of the plant, and they have waxy, red to pink, white-edged bracts and are pinecone-shaped with a skirt of larger bracts borne on stalks protruding from the ground or found at the soil level. The leaf blades are green, hairless, lanceolate in shape, and up to 81 cm long with young leaves sometimes flushed pink. The inflorescences and foliage are colorful and attractive. The pseudostems (formed by the leaf sheaths) emerge from underground rhizomes and are tall and arching.

2.2.2. Utilization of *E. elatior*

E. elatior has various traditional and commercial uses. It is vastly cultivated as spice for food flavouring including ingredients for curries and leaves are used as ornamentals. In Thailand, young leaves are utilized in salad or as vegetables and used to make cosmetic powder. In addition, leaves of *E. elatior* mixed with other aromatic herbs, are used by post-partum women for bathing to remove body odour. Leaves are also used for cleaning wounds, and sometimes eaten with betel nut to reduce abdominal pain (Chan et al., 2008). In Malaysia, the hearts of young shoots, inflorescences and fruits are consumed by indigenous communities as condiment, eaten raw or cooked as vegetable (Chan et al., 2011). Fruits are used to treat earache,

while the rhizomes are used by women during ailment, illness and confinement. Rhizomes are eaten raw or cooked as vegetables and used for food flavouring. Besides being consumed as food or spices *E. elatior* rhizome also shown potent antioxidant activity (Chan et al., 2011).

2.2.3. Phytochemical studies of *Etlingera elatior*

Phytochemical studies of *E. elatior* have been previously reported by a number of researchers (Chan et al., 2011). Several parts of *E. elatior* were subjected to the study such as leaves, rhizomes, flowers and whole plants.

The phytochemical evaluation has shown that diarylheptanoids, labdane, diterpeniods, steroids, phenolic, flavonoid, anthocyanin, and tannin were detected from this plant and are summarized in **Table 2.2**.

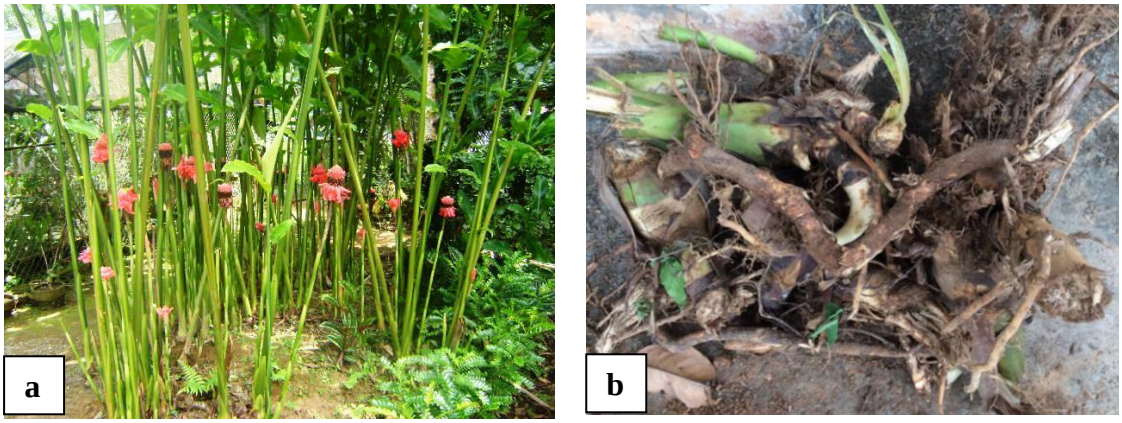


Figure 2.5: (a) *Etlingera elatior* (whole plant) (From: www.rv-orchidworks.com, accessed date December, 2014 and (b) rhizomes

Table 2.2: Chemical constituents from different parts of *Etlingera elatior*

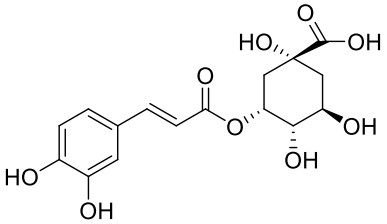
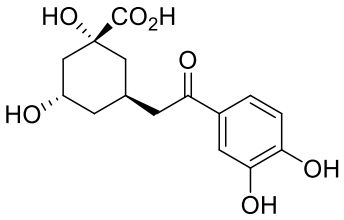
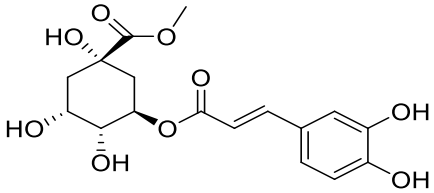
Chemical substance	Phytochemical classification	Part of Plant	References
1) 5-*O*-caffeoylquinic acid 	Phenolic	Leave	Abdelwahab et al., (2010); Chan et al., (2009)
2) 5-*O*-caffeoylquinic acid (chlorogenic acid) 	Phenolic	Leave	Abdelwahab e al., (2010); Chan et al., (2009)
3) 5-*O*-caffeoylquinic acid methylester 	Phenolic	Leave	Abdelwahab e al., (2010); Chan et al., (2009)

Table 2.2: Chemical constituents from different parts of *Etlingera elatior* (Cont).

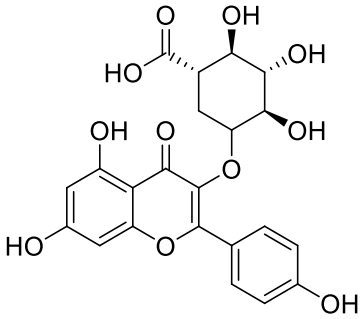
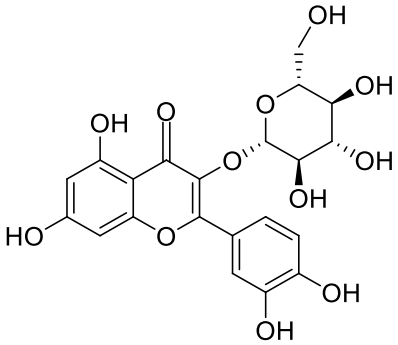
Chemical substance	Phytochemical classification	Part of Plant	References
4) kaempferol 3-glucuronide 	Flavonoid	Leave	Abdelwahab e al., (2010); Chan et al., (2009)
5) quercetin 3-glucoside 	Flavonoid	Leave	Abdelwahab e al., (2010); Chan et al., (2009)

Table 2.2: Chemical constituents from different parts of *Etlingera elatior* (Cont).

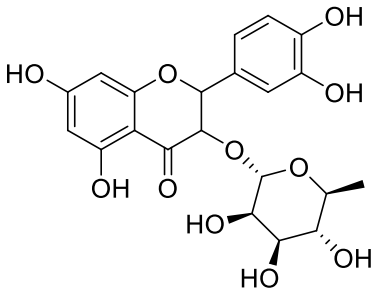
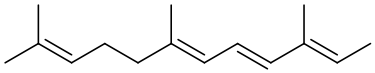
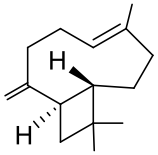
Chemical substance	Phytochemical classification	Part of Plant	References
6) quercetin 3-rhamnoside 	Flavonoid	Leave	Abdelwahab e al., (2010); Chan et al., (2009)
7) *(E)*-farnesene 	Terpenes	Leaves	Abdelwahab e al., (2010); Chan et al., (2011)
8) *(E)*-caryophyllene 	Terpenes	Leaves	Abdelwahab e al., (2010); Chan et al., (2011)

Table 2.2: Chemical constituents from different parts of *Etlingera elatior* (Cont).

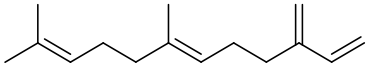
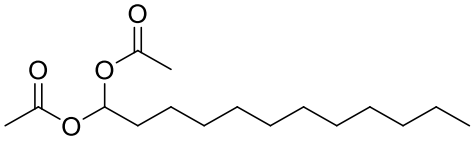
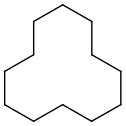
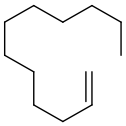
Chemical substance	Phytochemical classification	Part of Plant	References
9) (*E*)-*β*-farnesene 	Terpenes	Leave	Abdelwahab e al., (2010); Chan et al., (2011)
10) 1,1-dodecanediol diacetate 	Terpenes	Flower	Abdelwahab e al., (2010); Chan et al., (2011)
11) Cyclododecane 	Terpenes	Flower	Abdelwahab e al., (2010); Chan et al., (2011)
12) 1-dodecene 	Terpenes	Flower	Abdelwahab e al., (2010); Chan et al., (2011)

Table 2.2: Chemical constituents from different parts of *Etlingera elatior* (Cont).

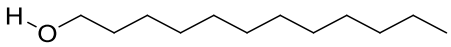
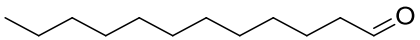
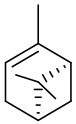
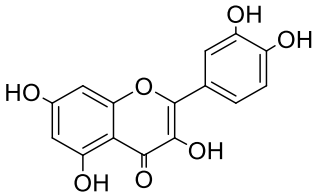
Chemical substance	Phytochemical classification	Part of Plant	References
13) dodecanol 	Terpenes	Inflorescence	Abdelwahab e al., (2010); Chan et al., (2011)
14) dodecanal 	Terpenes	Inflorescence	Abdelwahab e al., (2010); Chan et al., (2011)
15) α-pinene 	Terpenes	Inflorescence	Abdelwahab e al., (2010); Chan et al., (2011)
16) Quercetin 	Flavonoid	Inflorescence	(Jackie et al., 2011)

Table 2.2: Chemical constituents from different parts of *Etlingera elatior* (Cont).

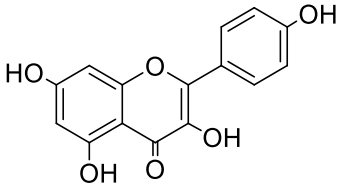
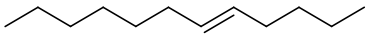
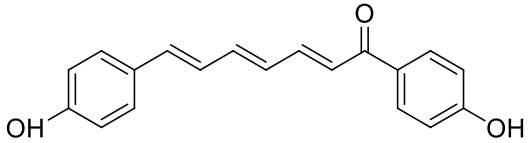
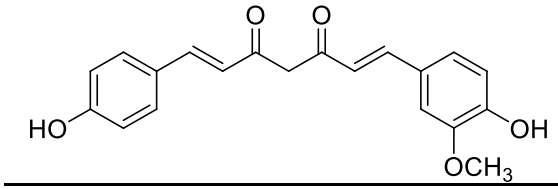
Chemical substance	Phytochemical classification	Part of Plant	References
17) kaempferol 	Flavonoid	Inflorescence	(Jackie et al., 2011)
18) (*E*)-5−dodecane 	Flavonoid	Stem	Chan et al., (2011) Habsah et al., (2005)
19) 1,7-bis(4-hydroxyphenyl)-2,4,6-heptatrienone 	Phenolic	Rhizome	Chan et al., (2013); Habsab et al., (2005); Mohamad et al., (2005); Tachai et al., (2014)
20) Demethoxycurcumin 	Phenolic	Rhizome	Chan et al., (2013); Habsab et al., (2005); Mohamad et al., (2005); Tachai et al., (2014)

Table 2.2: Chemical constituents from different parts of *Etlingera elatior* (Cont).

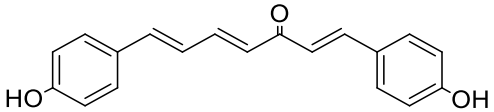
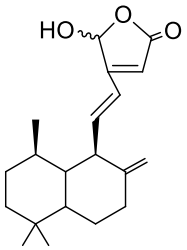
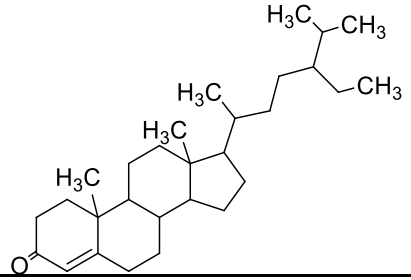
Chemical substance	Phytochemical classification	Part of Plant	References
21) 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one 	Phenolic	Rhizome	Chan et al., (2013); Habsab et al., (2005); Mohamad et al., (2005); Tachai et al., (2014)
22) 16-hydroxylabda-8(17),11,13-trien-15,16-olide 	Terpenes	Rhizome	Chan et al., (2013); Habsab et al., (2005); Mohamad et al., (2005); Tachai et al., (2014)
23) stigmast-4-en-3-one 	Steroid	Rhizome	Chan et al., (2013); Habsab et al., (2005); Mohamad et al., (2005); Tachai et al., (2014)

Table 2.2: Chemical constituents from different parts of *Etlingera elatior* (Cont).

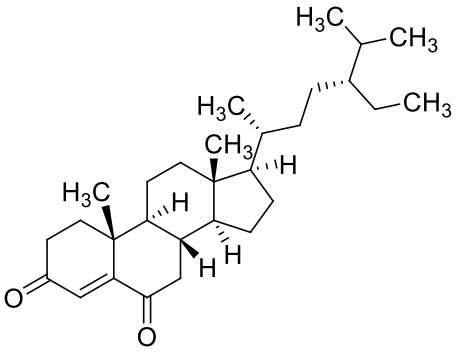
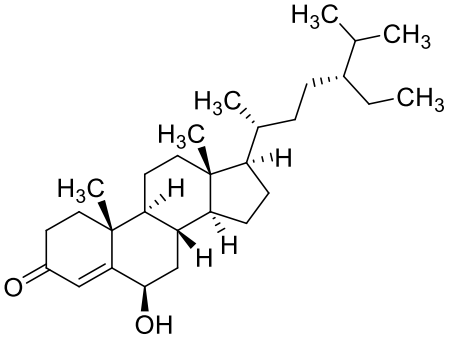
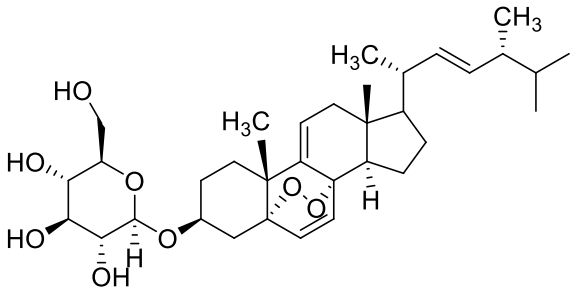
Chemical substance	Phytochemical classification	Part of Plant	References
24) stigmast-4-ene-3,6-dione 	Steroid	Rhizome	Chan et al., (2013); Habsab et al., (2005); Mohamad et al., (2005); Tachai et al., (2014)
25) stigmast-4-en6β-ol-3-one 	Steroid	Rhizome	Chan et al., (2013); Habsab et al., (2005); Mohamad et al., (2005); Tachai et al., (2014)

Table 2.2: Chemical constituents from different parts of *Etlingera elatior* (Cont).

Chemical substance	Phytochemical classification	Part of Plant	References
26) 5α,8α-epidioxyergosta 	Steroid	Rhizome	Chan et al., (2013); Habsab et al., (2005); Mohamad et al., (2005); Tachai et al., (2014)

2.2.4. Pharmacological activities of *Etlingera elatior*

E. elatior contains many phytochemicals with biologically activities including antimicrobial, antifungal, antioxidant, cytotoxic, tyrosinase inhibition and hepatoprotective activities as following.

2.2.4.1. Antioxidant activity

The highest antioxidant activities of *E. elatior* were found in rhizome and leaves (Habsah et al., 2005). The study of *E. elatior* by Williams and Harborne, (1977) showed that the flavonoids, kaempferol-3-glucuronide, quercetin-3-glucuronide, quercetin-3-glucoside and quercetin-3-rhamnoside were capable of scavenging reactive oxygen species (Haleagrahara et al., 2010). These compounds could increase the synthesis and release antioxidant enzyme to protect our cells and keep oxygen under control.

2.2.4.2. Antimicrobial activity

Inflorescences and leaves have shown to have antimicrobial activities. The ethanolic extract from inflorescences was found to be effective against *Pseudomonas aeruginosa, Bacillus megaterium,* and *Escherichia coli* with minimum inhibitory concentration (MIC) of 200 µg/mL, 400 µg/mL, and 800 µg/mL, respectively. Methanol extracts of inflorescences were reported to be active against *Staphylococcus aureus, Bacillus thuringiensis, Bacillus subtilis, E. coli* and *Proteus mirabilis* with MIC ranging from 1.563-50.000 mg/mL (Lachumy et al., 2010). Eventhough leaves showed no activity against gram-negative bacteria such as *E. coli, P. aeruginosa, and Salmonella choleraesuis,* it inhibited gram-positive bacteria such as *Bacillus cereus, Micrococcus luteus,* and *S. aureus*. The essential oils from leaves also inhibited gram-positive bacteria including *B. cereus, M. luteus, and S. aureus* with MIC values of 25, 6.3, and 50 mg/mL, respectively.

2.2.4.3. Antifungal activity

E. elatior was found to have pronounced inhibitory activities against a wide variety of human pathogenic fungi, including strains resistant to the common antifungals amphotericin B and ketoconazole (Ficker et al., 2003). Hexane extracts from young inflorescences demonstrated high inhibitory activity on mycelial growth of *Colletotrichum gloeosporioides* with EC_{50} value of 804 µg/mL.

2.2.4.4. Cytotoxicity

Leaves of *E. elatior* were found to be non-cytotoxic to normal liver and kidney cells. Ethyl acetate extract of *E. elatior* showed strong cytotoxic activity against CEM-SS and (MCF-7) cell lines (Habsah et al., 2005). Among chemical compounds isolated from rhizome 1,7-bis(4-hydroxyphenyl)-2,4,6-heptatrienone, demethoxycurcumin, 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one, 16-hydroxylabda-8(17),11,13-trien-16,15-olide, stigmast-4-en-3-one, stigmast-4-ene-3,6-dione, stigmast-4-en-6*β*-ol-3-one, 5*α*,8*α*-epidioxyergosta-6,22-dien-3β-ol were the compounds that showed cytotoxic activity (Habsah et al., 2005). Two of these compounds, stigmast-4-en-3-one and stigmast-4-en-6*β*-ol-3-one, displayed high antitumour activity with IC_{50} against CEM-SS and MCF-7 cell lines= 4.0 and 6.3 mg/mL (Chan et al., 2011). In addition, ethanolic extracts from *E. elatior* leaves and inflorescences are cytotoxic to HeLa cells (Ali et al., 1996).

2.2.4.5. Tyrosinase inhibitory activity

Tyrosinase inhibitors are chemical agents capable of reducing the activity of the enzyme responsible for the development of melanin in cells. Anti-tyrosinase activity of methanolic leaf extract is typically expressed as a percent of kojic acid equivalent values and quercetin equivalent values (QE). The tyrosinase inhibitory activity of *E. elatior* (leaves) typically falls in the range of 49-55% (Chan et al., 2008; Lim et al., 2009), therefore displaying activity.

2.2.4.6. Hepatoprotective activity

Many studies have reported that lead toxicity is associated with impaired functioning of brain, liver, kidney, tastes, and the hematopoietic system. *E. elatior* (inflorescence) has ability to protect lead-induced hepatotoxicity in rat (Haleagrahara et al., 2010). The treatment with *E. elatior* (inflorescence) significantly decreased hepatic lipid hydroperoxides (LPO), protein carbonyl content (PCC), total antioxidants (TA), superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione S-Transferase (GST) levels and caused histopathological changes in liver to accustomed liver, normal architecture of hepatocytes (normal nucleus, clear sinusoids in many parts, and no fatty).

2.3. Enzyme inhibitory activities of phytochemical compounds

There are many reports about poly-phenols were found on carbohydrates hydrolyzing enzyme especially, they a play role to inhibit of α-glucosidase and sucrose activities (Bello et al., 2011; Mohamed, 2014). A large number of flavonoid compounds have been tested for their inhibitory activity against α-glucosidase and α-amylase. The result showed that luteolin, myricetin, and quercetin were potent inhibitors against porcine pancreatic α-amylase. Their potency are related to the number of hydroxyl groups on the B ring of the flavonoid scaffold (Matsui et al., 2002; Tadera et al., 2006; Lo Piparo et al., 2008). Moreover, steroids and terpenes compounds extract from *Hyptis suaveolens* have been reported anti-diabetic activity (Danmalam et al., 2009).

2.4. Enzyme kinetics study

Enzyme kinetics studies the rates of enzyme-catalyzed reaction and how the rates are affected by changing the conditions. The importance of enzyme-catalyzed reactions is saturation by increasing the concentrations of substrates until the reaction rate is no longer dependent on concentration. Substrates of enzymes are targeted bind into an active site of enzymes. There are two kinds of mechanisms (single-substrate and multiple-substrate). In particular, the substrates position looks like the keys and enzyme position looks like the locks, so the keys and the locks should be affinity to

unlock. For kinetic study, the enzyme will only bind to one substrate **(Figure 2.6)**. The useful of enzyme kinetics study is: knowing how to control its activities, how to use drugs or inhibitor against the enzyme activities and transformed into products through the steps of enzymatic mechanism (https://en.wikipedia.org/wiki/Enzyme_ kinetics).

According to Lineweaver-Burk and Michaelis-Menten plots of kinetic model were widely determined by measuring the rate of reaction [V] over a range of increasing substrate concentrations [S]. When the concentration of the substrate is higher, the enzyme becomes saturated and the rate reaches V_{max}, that is the maximum rate of enzyme to give the different types of enzyme inhibition.

By Lineweaver-Burk and Michaelis-Menten kinetic plot, the enzyme kinetic is divided into 3 types namely competitive inhibitors, noncompetitive inhibitors and uncompetitive inhibitors. **Competitive inhibitors** usually the inhibitor blocks the binding of the substrates and enzymes at the active site by binding itself with enzymes. The competitive inhibitors graph is shown in **Figure 2.7**. **Noncompetitive inhibitors** are different from competitive inhibitors, normally the inhibitors of noncompetitive and substrates are binding together with enzyme because substrates have been bound with the enzyme already so, it will be bound with other sites and not completely bound at the active site. The noncompetitive inhibitors graph is shown in **Figure 2.8**. **Uncompetitive inhibitors** are called anti-competitive inhibitor because they only bind with the complex formed between the enzyme and the substrate. The uncompetitive inhibitors graph is shown in **Figure 2.9**.

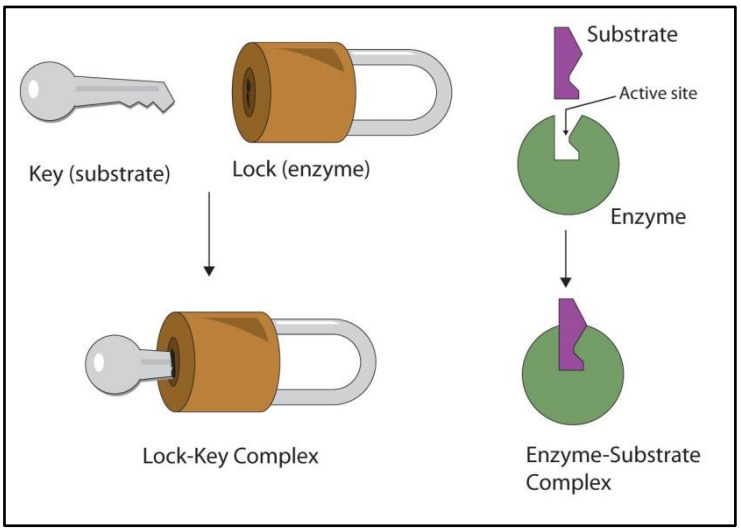


Figure 2.6: Lock and key model of substrate-enzyme reaction

(From: www.2012books.lardbucket.org, accessed date: January, 2015)

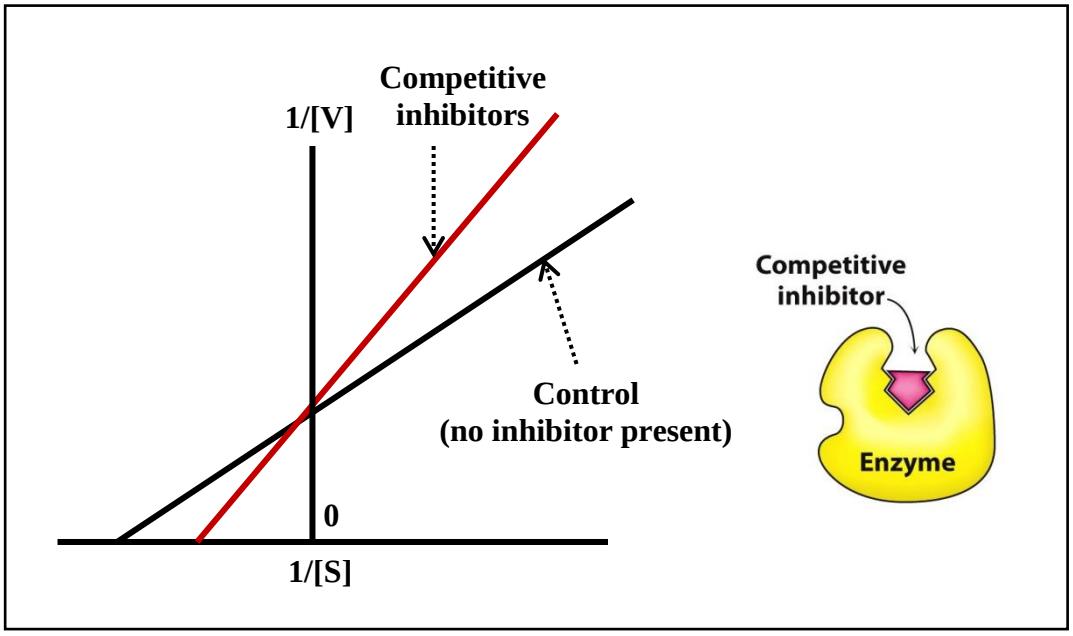


Figure 2.7: The plot of competitive inhibitors (the inhibitors are bound with enzyme) and the graph of the control and uncompetitive inhibitors are cut on Y axis

(From:www.oregonstate.edu, accessed date: January, 2015)

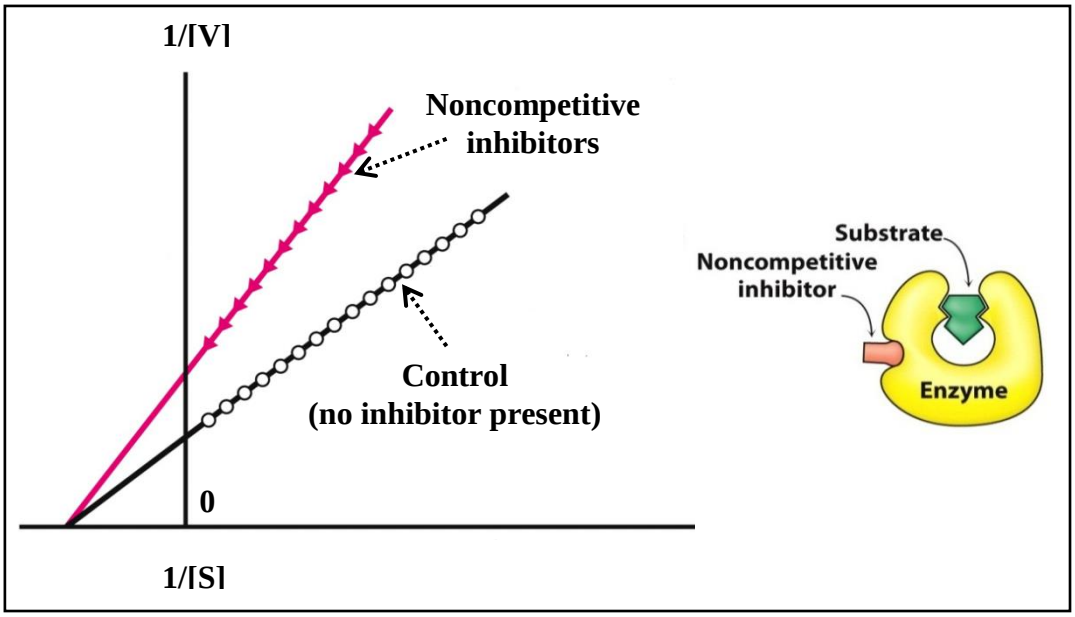


Figure 2.8: The plot of noncompetitive inhibitors (the inhibitors are opposite from competitive enzyme) and the graph of the control and uncompetitive inhibitors are met on X axis
(From:www.oregonstate.edu, accessed date: January, 2015

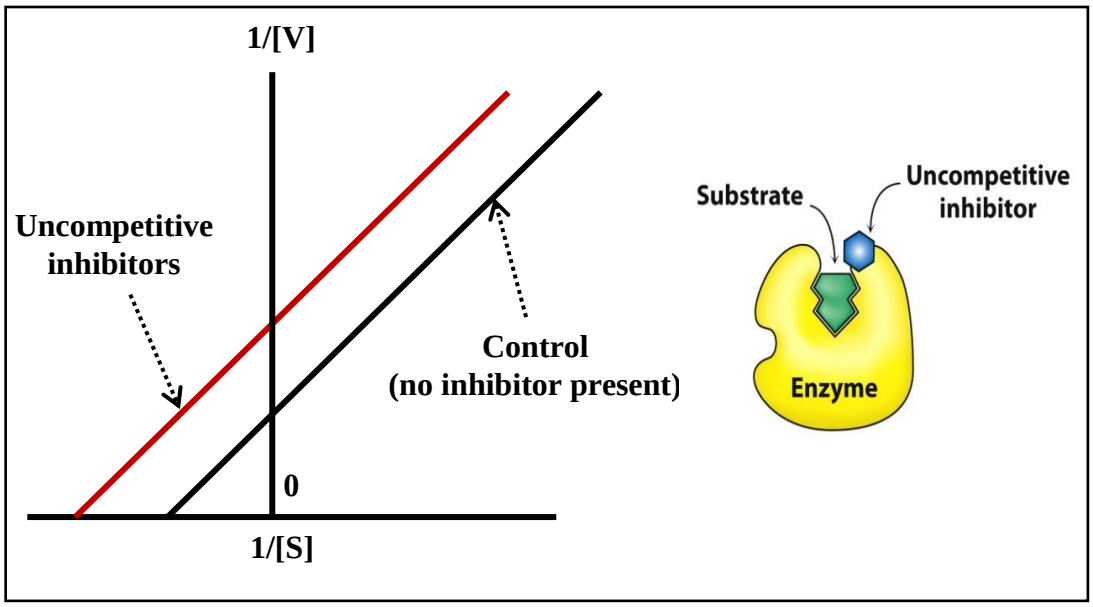


Figure 2.9: The plot of uncompetitive inhibitors (the inhibitors are bound with other site of enzyme) and the graphs of the control and uncompetitive inhibitors are parallel
(From:www.oregonstate.edu, accessed date: January, 2015)

2.5. Thin layer chromatography

Thin layer chromatography or TLC, can be used to qualitatively determine the number of components in a mixture, identifying and purifying compounds. During TLC when mobile phase moves through the stationary phase it will carry various components of the mixture through the stationary phase at different rates. The crude extract is normally chromatographed on a pre-coated aluminium plate (silica gel G 60 F254, 0.2 mm thick) by placing the plate in a glass tank with a mobile phase system solvent and waiting for the solvent front to rise to a marked height (solvent front). The plates were then removed and dried, and spots were visualized under UV light (254 and 365 nm) or sprayed with anisaldehyde in sulfuric acid and heating at 100-110 ºC. The mobile phases used for TLC are solvents of varying polarity. The R_f values of chemical compounds were determined using different mobile phases to show which solvent would be suitable for further use in separation of compounds by column chromatography.

R_f = distance of spot traveled from origin line/distance of solvent front

2.6. Column Chromatography

Column chromatography is the separation process using a glass column with various diameters for separation of compounds using the same principles as TLC, but can be applied to separate larger quantities and more convenient than TLC.

2.6.1. Methods of column wet packing

Below is the process for column packing which is normally used in preparation of column chromatography for sample isolation.

1. Used long rod to add a plug of cotton to the bottom of the glass column.
2. Clamp the column to a ring stand and fill the column about 1/4 full with the initial eluent.
3. Pouring stationary phase (SP) into the beaker and stir until the slurry is obtained.
4. Add the same solvent (about 20 mL) used for making slurry into the column.

5. Quickly pour the slurry into the column by using funnel and remove the pinch clamp at the end of the column to allow the solvent to drip into a clean flask. Tap on the side of the column with a rubber stopper or neoprene tube to help the resin settle uniformly.
6. Use a Pasteur pipette to rinse any SP that sticks to the sides of the column. Allow the SP to settle while eluent continues to drip into the flask.
7. Add some sand on top of the settle surface.

2.6.2. Load and elute sample on to the wet column

Below is the typical loading and eluting sample on to the column chromatography for separation.

1. Remove solvent from the column until no solvent remains above the surface of the silica gel.
2. Use a long Pasteur pipette to carefully apply the sample solution to the column.
3. Remove eluent from the column until no sample remains above the surface of the silica gel.
4. Use about 1 mL of eluent to rinse the container and pipette this solvent into the column. Drain eluent from the column until no liquid remains above the surface of the sand.
5. Repeat step 4 two or three times to completely transfer the sample onto the stationary phase.
6. Sample fractions are collected from the column by additional eluent to the top of the column.

2.6.3. Analyzing the fractions

Analyzing chemical compositions in each fraction is usually performed by using thin-layer chromatography to determine the separation power the components. If the fractions contain more than one component and have the same TLC patterns as other fractions, then these fractions can be combined without affecting the purity of these fractions.

2.6.4. Normal phase column chromatography

Silica gel is a granular, vitreous form of silicon dioxide (SiO_2) synthetically made from sodium silicate (Na_2SiO_3). Any types of mixtures can be separated and can be used with wide choices of mobile phase (solvents). In this study, silica gel was purchased from Vertical Chromatography Co., Ltd. VertiFlask^{TM} silica 60A, 60-200 μm. One gram of sample is normally applied on used 40 g of silica gel for column chromatographic separation. The main concept to consider in separation using silica gel column chromatography is polarity. Polar compounds will adsorb onto the stationary phase to a greater extent than non-polar compounds, therefore, different travelling distance will be observed, so the separation occur.

2.6.6. Ion exchange column chromatography

Diaion HP-20 was purchased from Sigma, Sigma-Aldrich, Germany and stored at room temperature. These ion-exchange resins are widely used in different separation, purification, and decontamination process. They are suitable for adsorbing large molecules because of their relatively large pore sizes and have superior adsorption/desorption. Most commercial resins are made of polystyrene sulfonate. One gram of sample can normally be applied on about 40 grams of Diaion HP-20.

2.6.7. Size exclusion column chromatography

Sephadex column chromatography or gel filtration chromatography is a trade mark for cross-linked dextran gel. The sample were separated according to their sizes and some cases molecular weight, this can be also called size-exclusion chromatography. Sephadex LH-20 (20-100 μm, Sigma-Aldrich Co.) was eluted with organic solvents such as dichloromethane, chloroform, ethyl acetate, *n*-butanol and methanol. Solvents such as methanol, ethanol, or isopropanol can cause these resins to swell. These resins should be stored in aqueous acetone or alcohol either in refrigerator (Hagerman, 2002).

2.7. Standardization of herbal medicine

The traditional herbal medicines have been used long time ago in many countries such as China, Korea, Japan, Thailand etc. Recently, the use of herbal products are still increasing and these products are available in food shops and pharmacies for health care in both developed and developing countries. WHO has estimated that 80% of people worldwide will be rely on herbal medicines as a part of their primary health care in a near future (Fabricant and Farnsworth, 2013). The herbal market is thought to be around hundred and $60 billion globally each year (Mentreddy, 2007). Eventhough herbal medicines are popular today, they still have many problems related to the lack of written verification from manufacturers, the lack of relevant regulation, the presence of undeclared agents and the lack of standardization of formulations as well as the right procedure or dosage for particular treatment. More researches are therefore necessary to build confidence for the users in the utilization of herbal medicines as potentially reliable alternatives to conventional modern medicines. Although, herbal medicines have been popular and used extensively during the last decade, some of herbal medicines have not been officially recognized in most countries.

According to the WHO guidelines, an herbal product needs to be standardized with respect to safety before going into the market. Unstandardized herbal products can cause health problems such as direct toxic effects, allergic reactions, effects from contaminants, some are not effective and some may interact with other drugs. So, standardization of herbal medicine is an important step for the establishment of biological activity and quality assurance (Shankar and Amritpal, 2011).

Standardization is a system to find amount of quantity, quality and therapeutic effect of ingredients in each dose. The development of these traditional herbal medicines need to be safety, efficacy and quality in the healthcare (Choudhary and Sekhon, 2011). There are some researchers that have been done on standardization of herbal products such as demethoxycurcumin, bisdemethoxycurcumin and curcumin are the major curuminoids in the *Curcuma longa* species was used as commercial for functional food and for clinical trials (Sandur et al., 2007; Goel et al., 2008). Chlorogenic acid (CGA) or 5-caffeoylquinic

acid contain 40% in the leaves of *E. elatior* was used as a biomarker in standardization of the leave's extract (Clifford, 2000; Chan et al., 2011).

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals

3.1.1. Solvents

-Hexane (*n*-C_6H_{14}), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), 95% ethanol (EtOH), and methanol (MeOH) were purchased from LabScan Asia Co, Thailand. All of these solvents were used for extraction and isolation techniques.

-Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich (Sigma Aldrich, USA) and used for dissolving the samples.

-Deuterated chloroform ($CDCl_3$) from Isotope Laboratories, Inc., Cambridge was used as a solvent for NMR determinations.

3.1.2. Reagents

-*p*-Nitrophenyl-α-D-glucopyranoside (p-NPG) from Sisco Research Laboratories Pvt. Ltd., India was used as a substrate for biological activities testing of α-glucosidase.

- Starch azure from Sigma-Aldrich, Germany was used as a substrate for biological activities testing of α-amylase.

-Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) from Merck®, Germany was used for preparation of phosphate buffer of α-glucosidase and α-amylase testing.

-Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$) from Merck®, Germany was used for preparation of phosphate buffer of α-glucosidase testing.

-Sodium Carbonate (Na_2CO_3) from Sigma-Aldrich, Germany was used as reagent for stopping reaction of α-glucosidase activity testing.

-Tris (hydroxymethyl) from Sigma-Aldrich, Germany was used for preparation of Tris-HCl (phosphate buffer). This buffer is used for dissolving starch azure of α-amylase testing.

-Sodium hydroxide (NaOH) from LabScan, Thailand was used to adjust pH of the phosphate buffer for α-amylase testing.

-Sodium chloride (NaCl) from Sigma-Aldrich, Germany was used in phosphate buffer preparation for α-amylase testing.

-Calcium chloride ($CaCl_2$) from Liuxlang, China was used for Tris-HCl (phosphate buffer) preparation for dissolving starch azure of α-amylase testing.

-Acetic acid was purchased from LabScan, Thailand and used for stopping reaction of α-amylase activity testing.

-2,2-Diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide, trichloroacetic acid, ferric chloride, sodium hydroxide, concentrated sulphuric acid and Dragendroff reagent were purchased from Sigma, Sigma-Aldrich, Germany and were used for 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity assay (DPPH assay).

-Anisaldehyde (6% in 100 mL Methanol and H_2SO_4 (LabScan, Thailand) were used as a spraying reagent.

3.1.3. Enzymes

-*α*-Glucosidase enzyme was purchased from Sisco Research Laboratories Pvt. Ltd., India and used for α-glucosidase activity.

-Porcine pancreatic *α*-amylase enzyme was purchased from Sigma-Aldrich, Germany and used for α-amylase activity.

3.1.4. Standard

-Acarbose was purchased from Sigma-Aldrich, Germany and used as a standard drug for α-glucosidase and α-amylase inhibitory studies.

-Ascorbic acid was purchased from Sigma-Aldrich, Germany and used as a standard of antioxidant testing.

-*p*-Nitrophenol from Merck®, Darmstadt was used for construction of calibration curve for the product from p-NPG clearage.

-Quercetin was purchased from Sigma-Aldrich, Germany and was utilized as positive standard for antioxidant testing.

3.1.5. Chromatography

In the separation procedures, Silica gel (SiO_2) was purchased from Silicycle® Inc., Canada and sephadex LH-20, Diaion HP-20 were purchased from Sigma-Aldrich, Germany. Semipreparative column chromatography (Luna® 10 µM, C_{18} 100 ^{o}A, 250x10 mm, Phenomenex, USA) was used in the separation procedure with HPLC system. Thin layer chromatography, Silica gel (SiO_2, 60, F_{254}) adsorbent pre-coated on Aluminum sheets was used for separation power determination and purity testing.

3.1.6. Instruments

-Ultraviolet light (UV Lamp UVGL-58 Handheld, Cambridge, UK) was used for fluorescence detection.

-The Ultraviolet Absorption (UV) were measured with a UV-visible spectrophotometer (Hewlett Packard 8452A, Diode Array Spectrometer, U.S.A).

-Microplate reader (DTX 880, Multimode Detector, Beckman Coulter, Inc., Austria) was used for testing bioassay activities of α-glucosidase and α-amylase inhibitory activity.

-Microplate reader (Bio-Tec instruments, Inc., U.S.A) was used for testing 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and determination of ferric reducing antioxidant power (FRAP).

- Cary 100 Series UV-Vis Spectrophotometer was used for kinetic study (Agilent Technologies, Malaysia).

-HPLC was operated on Water® with Binary HPLC pump (model), autosample (Water® 2707), and photodiode array detector (Water® 2998) using semi-preparative reversed-phase column (Luna 10µM, C_{18} 100^{o}A, 250x10 mm, Phenomenex®, USA).

-IR spectra were measured with KBr disc on FT-IR spectrophotometer (Spectrum One, Perkin Elmer Ltd., UK).

-^{1}H and ^{13}C NMR spectra were recorded with Varian Unity Inova 500 FT-NMR spectrometer.

- Rotary evaporator (Buchi, Switzerland) was used for removing solvents.

-96-well microplates were purchased from Corning Incorporate, U.S.A.

3.2. Plant materials

The rhizome of *E. elatior* was collected from Songkhla Province, Thailand in September 2013. The voucher specimen no. SKP 201050501 has been deposited at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Science, Prince of Songkla University. The rhizome of *E. elatior* was cleaned with tap water and cut into small pieces (3-5 mm) **(Figure 3.1)**, then dried in the air at room temperature for 48 hours **(Figure 3.2)** followed by drying in the oven at 45 ºC (3 days). The dried rhizome was further ground to a fine powder by the grinder machine **(Figure 3.3)**. The dried powder was kept at 4 ºC in a refrigerator until uses.

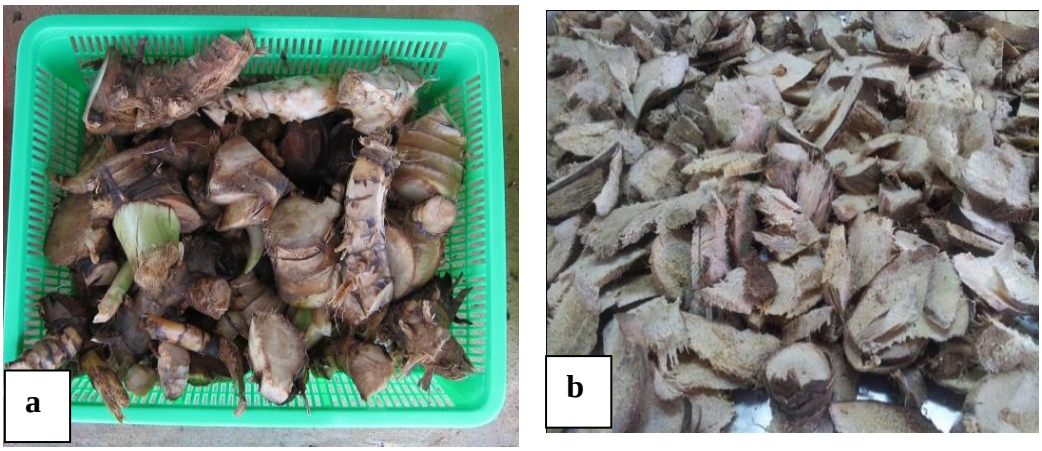


Figure 3.1: The processing of cleaning (a) and slicing the *E. elatior* rhizome (b)

Figure 3.2: Drying *E. elatior* rhizome in the air (48 hours) at room Temperature

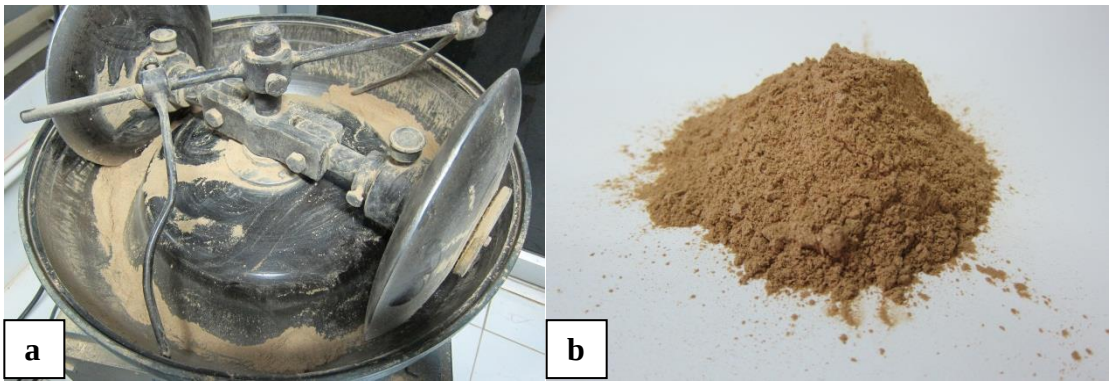


Figure 3.3: The grinder machine (a) and *E. elatior* rhizome in powder form (b)

3.3. Extraction process

Processes of extraction of *E. elatior* rhizome are summarized in **Scheme 3.1** and the detail of extraction processes are as following.

Ground rhizomes (2600 g) were macerated with EtOH in triplicate (3x3.5 L) for 48 hours in a glass jar by scattered around with aluminum foil to prevent the light **(Figure 3.4)**. The EtOH extract was obtained by simple filtration through a filter paper (Whatman® No. 1) **(Figure 3.5)** and subjected to removal of EtOH by using rotary evaporator at 45 ºC under vacuum (Buchi, Switzerland) **(Figure 3.6)**. The crude EtOH extract **(Figure 3.7)** was fractionated by using solvent partition method

(Figure 3.8) with four organic solvents, namely hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) and water **(Scheme 3.2)**. This was done by first dissolving 127.33 g of the extract in 1000 mL of 10% methanol in water and transferred to a 2 L separatory funnel. Hexane (750 mL) was added to the solution and the funnel was shaken vigorously. The upper layer of the hexane fraction was then separated. The partition was performed in triplicate (3x750 mL). After that CH_2Cl_2 (750 mL) was then added to the 10% MeOH fraction and the flask was shaken. The upper layer of CH_2Cl_2 fraction was then separated and the partition repeated for another two times (2x750 mL). After complete extraction of CH_2Cl_2 fraction, then EtOAc (750 mL) was further added to the 10% MeOH fraction and the funnel was well shaken. The EtOAc fraction was then separated and the partition continued for two more times with EtOAc (2x750 mL). After separation of EtOAc fraction, then, *n*-BuOH was added to the 10% MeOH fraction and the funnel was well shaken. The *n*-BuOH fraction was separated and 10% MeOH fraction was continued partition with 2x750 mL *n*-BuOH. The combined partitioned fractions of each organic solvent were subjected to removal of solvent using rotary evaporator under vacuum. The 10% MeOH fraction was first evaporated to remove methanol and the remaining water was then removed by using freezed drying method. Finally, 5 extracts were obtained: hexane extract, CH_2Cl_2 extract, EtOAc extract, *n*-BeOH extract and water extract. Each extract was subjected to α-glucosidase and α-amylase inhibitory activities testing. The extract that gave high inhibitory activity will be further utilized for chromatographic isolation process.

Figure 3.4: The *E. elatior* rhizome powder was macerated with 95% ethanol in triplicate (3x3.5 L) for 48 hours in a glass jar

Figure 3.5: The EtOH extract was filter through a filter paper (Whatman® No. 1)

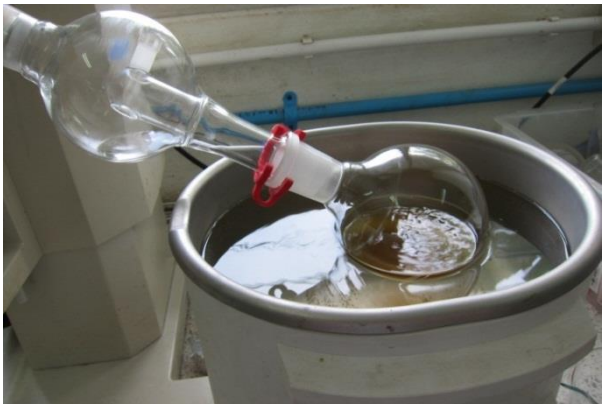
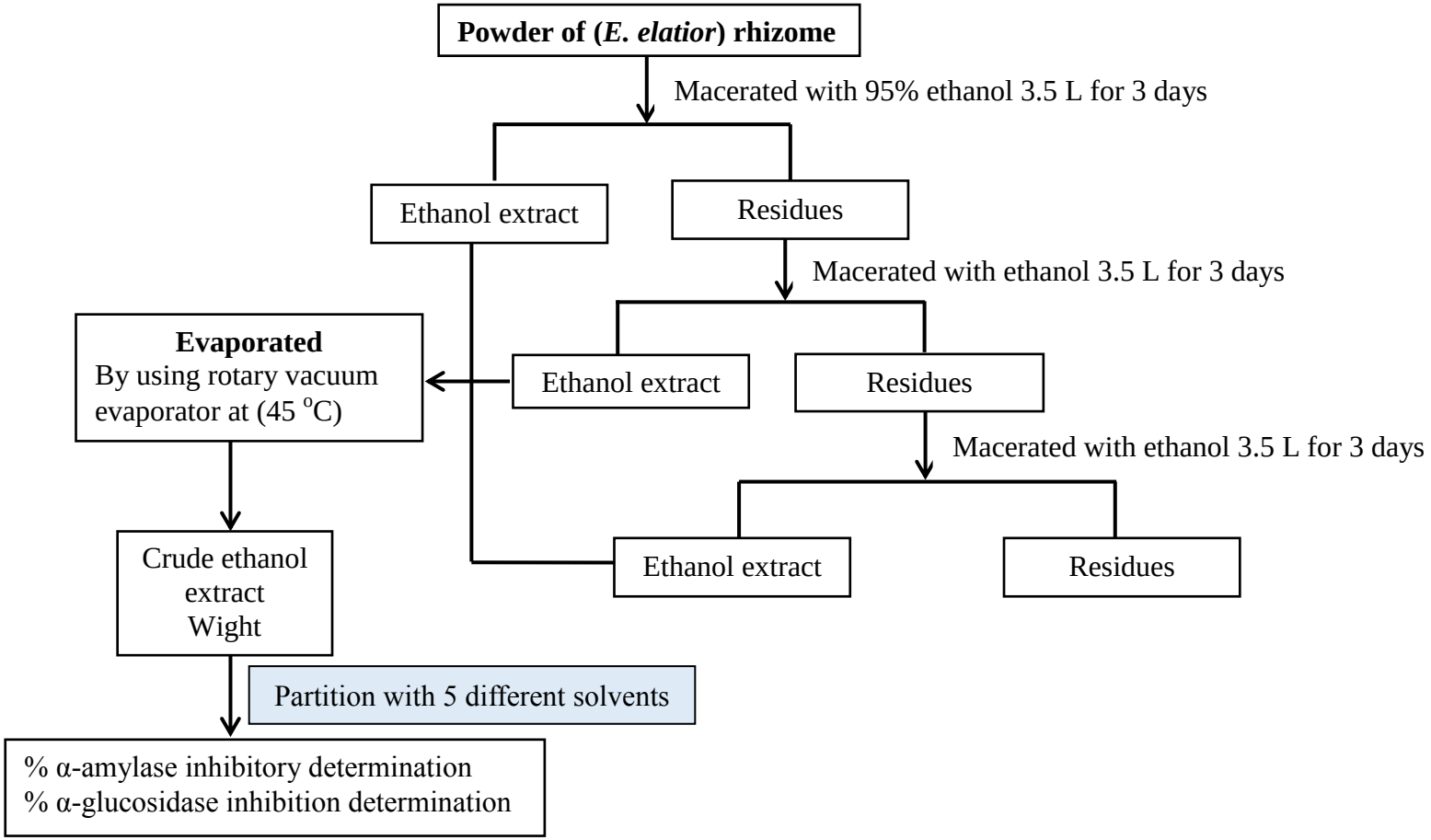


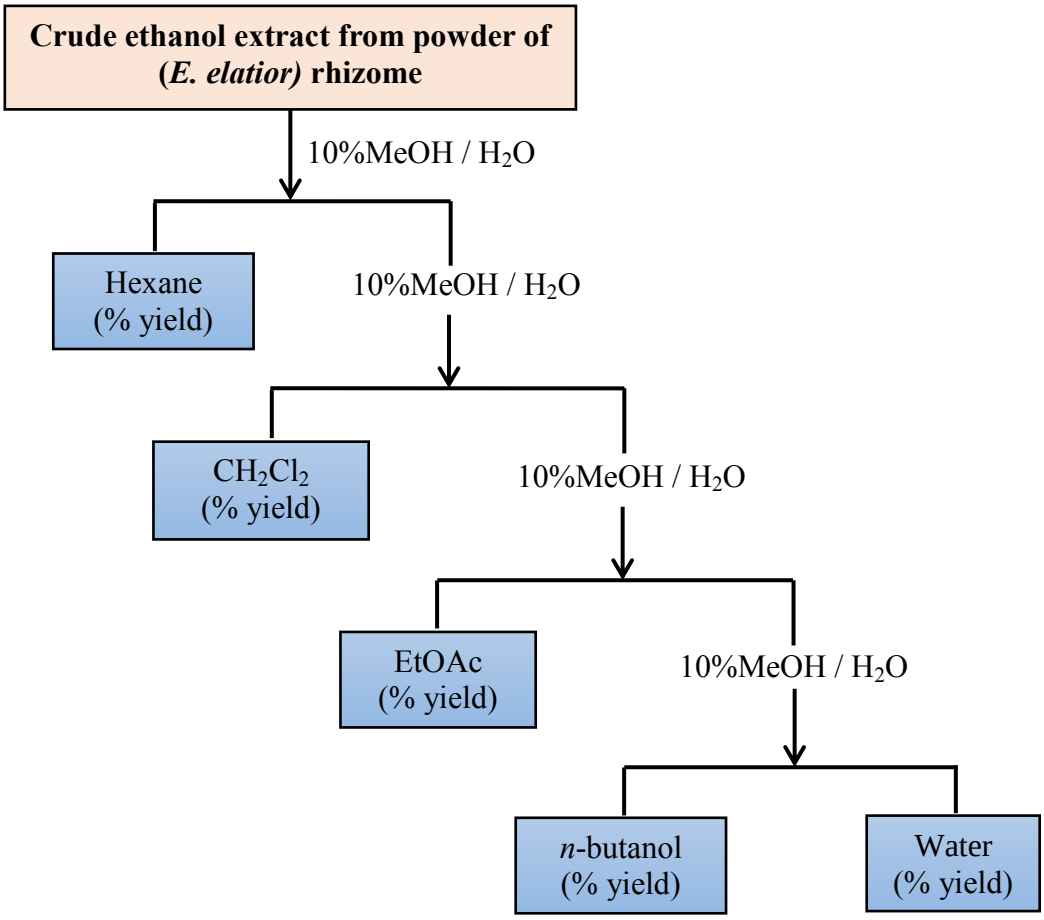
Figure 3.6: EtOH was removed from the EtOH extract using rotary evaporator at 45ºC under vacuum

Figure 3.7: The EtOH crude extract (dark brownish color)

Figure 3.8: The process of liquid-liquid extraction to obtain 5 fractions (hexane, CH_2Cl_2, EtOAc, *n*-BuOH and water)



Scheme 3.1: Summary of extraction process



Scheme 3.2: Summary of partition extraction process

3.4. Chromatographic isolation processes

The separation was performed by using four types of chromatographic processes.

3.4.1. Normal phase column chromatography

Silica gel was utilized by using wet packing into a glass column. Sample elution process was performed by using different solvents including hexane, CH_2Cl_2, $CHCl_3$, EtOAc and MeOH. Each fraction was collected and tested by using silica gel TLC (SiO_2 60 F_{254}) before combining the fractions which have the same chromatographic patterns.

3.4.2. Size exclusion chromatography

Gel filtration chromatography was performed to separate compounds by means of their sizes. Sephadex LH-20 was used in the separation process in a glass column chromatography. Sample elution process was done by using a number of solvents including CH_2Cl_2, $CHCl_3$, EtOAc and MeOH. Each fraction was collected and tested by using silica gel TLC (SiO_2 60 F_{254}) before combining the fractions which have the same chromatographic patterns.

3.4.3. Diaion HP-20 exchange chromatography

Diaion HP-20 exchange chromatography was performed in order to isolate compounds in the extracts by mean of their ionic affinity interaction with the stationary phase. The separation was carried out by using a glass column. The elution process was done by using solvents including EtOAc, MeOH, EtOH and water. Each fraction was collected and tested by using silica gel TLC (SiO_2 60 F_{254}) before combining the fractions which have the same chromatographic patterns.

3.4.4. Reversed phase-HPLC (RP-HPLC)

The samples which were partially purified from the above methods were further purified by using RP-HPLC method.

The chromatographic conditions were:

Column	:	Semi-preparative reversed-phase column (Luna 10 µM, C_{18} 100 A, 250x10 mm, Phenomenex®, USA).
Mobile phase	:	MeOH and water in gradient elution system
Detector	:	Photodiode array detector (λ 210 nm)
Flow rate	:	4 mL/min
Pump	:	Binary HPLC
Inject volume	:	100 µL

Each fraction was collected and tested by using silica gel TLC (SiO_2 60 F_{254}) from Merck®, Germany before combining the fractions.

3.4.5. Detection Method

To determine the identity of compounds and thy purity of a sample, the primary detection was performed by ultraviolet light detection and chemical determination using spraying reagents.

3.4.5.1. Ultraviolet light detection

Ultraviolet (UV) light detection was performed by using the light with 254 nm wavelength so the compounds that have strong UV absorption ability in the range 220-280 nm can be detected. On the TLC silica gel plates pre-coat with silica, an inorganic fluorescent agent (<0.5%) was presence and impregnated into the adsorbent layer and act as the indicator. This indicator in the TLC plate will absorbed light at this wavelength and if no compound on the TLC plate, it will give fluorescence emission in bright green color. If the sample on TLC is the compound that can absorb UV light, it will quench the fluorescent light, therefore, the spot will become blue, dark blue up to black spot. This method is helpful to locate the position of sample on TLC.

3.4.5.2. Detection by using Spraying reagents

Since compounds on TLC cannot be detected by using observation under UV-Vis light, due to the compounds do not contain chromophore. Therefore, chemical detection procedures need to be used. The method was performed by using different types of spraying reagents. In this study the spraying reagent including *p*-anisaldehyde-sulfuric acid, 10% sulfuric acid and1% aluminum chloride solution were utilized. *p*-Anisaldehyde-sulfuric acid solution [prepared by mixing 0.5 mL of *p*-anisaldehyde with 10 mL of conc. acetic acid, 5 mL of conc. sulfuric acid and 85 mL of methanol] was used for detection of a group of compounds including phenols, sugars, steroids and terpenes. The presence of these compounds will give violet, blue, red, grey, or green spots after spraying and heating on the hot plate at about 105 ^{o}C. 10% Sulfuric solution was used for detection of in volatile organic compounds and that will give dark brown. Flavonoids can be detected by spraying 1% w/v aluminum chloride solution in ethanol and the presence of flavonoids will give yellow fluorescence spot under long wavelength (360 nm).

3.5. Phytochemicals screening processes

Each extract was tested for its phytochemical compositions by using chemical testing procedures. The test for tannins, phenolic, flavonoids and alkaloids according to the reported procedures were utilized in the screening process (Ayoola et al., 2008).

3.5.1. Screening for tannins

Twenty five milligrams of each extract were boiled with 5 mL of distilled water and then filtered through a filter paper (Whatman® No. 1) while hot. The filtrate was left standing until cool to room temperature before testing. A few drops of 0.1% w/v ferric chloride solution was added to the filtrate. The presence of tannins was demonstrated by the brownish or dark blue coloration.

3.5.2. Screening for phenolic compounds

Each extract (25 mg) was dissolved in 2 mL of 95% ethanol. After that a few drops of 5% w/v ferric chloride was added to the solution. The presence of phenolic compounds was observed by the appearance of a bluish black color.

3.5.3. Screening for flavonoids

Each extract (25 mg) was dissolved in 2 mL of 95% ethanol and mixed with a few drops of 10% w/v NaOH solution to give a yellow color solution followed by addition of 1 mL of concentrated sulfuric acid to the solution. The presence of flavonoids was observed by disappearance of the yellow solution after addition of concentrate sulfuric acid.

3.5.4. Screening for terpenoids

Each extract (25 mg) was dissolved in 2 mL chloroform. A few drops of concentrate sulfuric acid were added to the solution to form double layers. The presence of terpenoids was indicated by the formation of a reddish brown color at the interface between the double-layer.

3.5.5. Screening for alkaloids

Each extract (25 mg) was mixed with 10% HCl solution and boiled for 15 mins. The solution was filtered through a filter paper (Whatman® No. 1) while hot to obtaining a filtrate. Dilute ammonia solution (pH~8) 2 mL was added to the filtrate. The filtrate was then extracted with 5 mL chloroform. The remaining aqueous layer was mixed with 10 mL glacial acetic acid. Then, a few drops of Dragendorff's reagent [prepared by dissolving 8 g of bismuth subnitrate in 20 mL of concentrate nitric acid and pour slowly into a solution prepared by dissolving 22.7 g of potassium iodide in about 25 mL of water and allow to stand until the potassium nitrate has precipitated. Filter, and dilute the filtrate with water to 100 mL] was added to the solution. If the extract contains alkaloid components, a reddish brown precipitates was observed.

3.6. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The DPPH radical scavenging activity of the plant extracts was determined using a literature method (Brand-Williams et al., 1995) with slightly modification. The activity was determined using DPPH solution prepared by dissolving 24 mg of DPPH in 100 mL methanol to form a stock solution. DPPH working solution was prepared by diluting 10 mL of the stock solution (24% w/v) with methanol (10 mL) whose absorbance value is in a range of 1.1±0.02, measuring at 515 nm by UV-visible spectrophotometer **(Figure 3.9)**. DPPH working solution (170 µL) was mixed well with plant extract solution (30 µl) and kept in the dark at room temperature for 30 min. After that the mixture was measured for an absorbance value at 515 nm using microplate reader **(Figure 3.10)** by using 200 µL methanol as a blank comparison. Ascorbic acid was used as a standard positive control. 50% Inhibition concentration (IC_{50}) was achieved by plotting a graph between % inhibition of samples with different concentrations ranges.

% Inhibition was calculated according to the following equation. The test was performed in triplicate and the results are expressed as the mean ± SD.

$$\textbf{\% inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

-$A_{control}$: Absorbance value at 515 nm of 170 µL DPPH working solution mixed with 30 µL Methanol.

-A_{sample}: Absorbance value at 515 nm of the test sample solution (30 µL) mixed with 170 µL DPPH working solution.

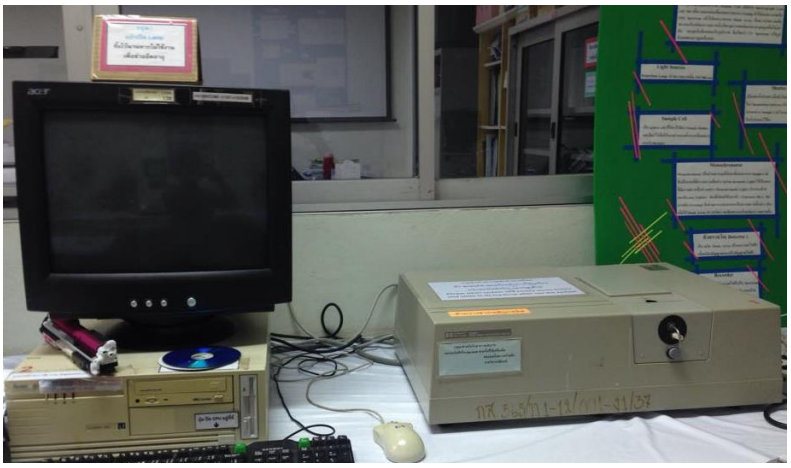


Figure 3.9: UV-visible spectrophotometer (Hewlett Packard 8452A, Diode Array Spectrophotometer, U.S.A) used for measure the absorbance at 515 nm for preparing the DPPH solution in a range of 1.1±0.02

Figure 3.10: Microplate reader (Bio-Tec instruments, Inc., U.S.A) using absorbance value at 515 nm

3.7. Determination of Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing antioxidant power of the plant extract was determined by using a literature method (Yildirim et al., 2001) with slightly modifications. Briefly, 200 µL of extract solution (250 µg/mL) was mixed with 500 µL of phosphate buffer (0.2 M, pH 6.6) and 500 µL of potassium ferricyanide solution

(1% w/v) and incubated for 30 min at 50 ^{o}C. Then 500 µL of trichloracetic acid solution (10% w/v) was added before being centrifuged at 3000 rpm for 30 min. The upper part of the mixtures (600 µL) was taken and mixed with 600 µL of distilled water and 120 µL of 0.1% w/v ferric chloride solution. The mixture was measured for an absorbance value at 700 nm using microplate reader (Bio-Tec instruments, Inc., U.S.A). Methanol was used as a blank. Quercetin was used as a positive standard and performed the testing in the same manner. Ferric reducing antioxidant power of the extract was expressed as mg quercetin equivalents (quercetin mg/g sample).

3.8. Enzyme inhibitory activity testing

3.8.1. α-Amylase inhibitory activity determination

Porcine pancreatic α-amylase inhibitory activity of a sample was determined using a literature method (Hansawasdi et al., 2000; Gao and Kawabata, 2005) with some modification. The enzyme substrate, starch azure (2 mg, Sigma-Aldrich), was suspended in 0.2 ml of 0.05 M Tris-HCl buffer (pH 6.9) containing 0.01 M $CaCl_2$ and then boiled for 10 min at 100 ºC and incubated for another 5 min at 50 ºC, before being used in the assay. The starch azure solution was pre-incubated at 37 ºC for 5 min before mixing with sample solution. The extracted sample (2 mg) to be tested was first dissolved in dimethyl sulfoxide (1 mL). α-Amylase (1 mg) activity about 1.6 Unit/mL was used to prepared solution by adding 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride (Ali et al., 2006). The reaction was started by mixing the substrate (200 µL), enzyme solution (100 µL) and with the extract solution (200 µL) and incubated at 37 ºC for 10 min. After the required time the reaction was stopped by adding 0.5 ml of 50% acetic acid. The reaction mixture was then centrifuged at 3000 rpm for 5 min at 4 ºC. The absorbance of the resulting supernatant at 595 nm was measured by using microplate reader (Beckman Coulter, Inc., Austria) and 200 µL of 20 mM phosphate buffer (pH 6.9) was used as a blank. The test was performed in triplicate. The effect of the extract sample on enzyme activity was determined by comparison with a negative control in which the enzyme (100 µL) was mixed with 200 µL of 20 mM phosphate buffer (pH 6.9) and 200 µL of starch azure

solution without the extract sample. The results were expressed in mean % inhibition ± SD compared with acarbose as a positive control and used at the same concentration of the sample.

% α-Amylase inhibition was calculated according to the following equation

% α-Amylase inhibition = $[(A_{c+}-A_c)-(As-Ab)]/ (A_{c+}-A_{c-}) \times 100$

- A_{c+}= Absorbance value at 595 nm of enzyme solution (100 µL), starch azure substrate solution (200 µL) and 200 µL of 20 mM phosphate buffer (pH 6.9). It is the absorbance of 100% enzyme activity (only the solvent with the enzyme).
- A_c= Absorbance value at 595 nm of only (200 µL) of 50 mM phosphate buffer (pH 6.9) without the enzyme.
- *As*= Absorbance value at 595 nm of enzyme solution (100 µL), starch azure substrate solution (200 µL) and sample or acarbose solution (200 µL) in 20 mM phosphate buffer (pH 6.9). It is the absorbance of test sample with the enzyme.
- *Ab*= The blank of sample without the enzyme in the absorbance value at 595 nm solution of 300 µL [20 mM phosphate buffer (pH 6.9)] and sample solution 200 µL.

3.8.2. α-Glucosidase inhibitory activity determination

The α-glucosidase inhibitory activity was performed by using similar procedure in the reported method with a slightly modification (Kumar et al., 2013). The α-glucosidase inhibitory assay was performed in a 96 well plate. Various concentrations of the extract sample solution (50 µL) in 50 mM phosphate buffer (pH 6.9) was mixed with 50 µL of enzyme α-glucosidase solution (0.57 unit/mL) in 50 mM phosphate buffer (pH 6.9) and the mixture was pre-incubated at 37 ± 1 ºC for 10

min. The assay was started by adding 50 µL of a substrate (5 mM) *p*-nitrophenyl α-D-glucopyranoside (p-NPG) in 50 mM phosphate buffer (pH 6.9) and incubated at 37 ± 1 ºC for 20 min. The reaction was terminated by adding 50 µL of (1M) sodium carbonate solution. The absorbance of the solution produced was measured at 405 nm by using microplate reader (Beckman Coulter, Inc., Austria), using 50 µL of 50 mM phosphate buffer (pH 6.9) as a blank. The test was performed in triplicate. The effect of the extract sample was determined by comparison with a negative control in which the enzyme solution (50 µL) was pre-incubated with 50 µL of 50 mM phosphate buffer (pH 6.9) without the extract sample. The results were reported in mean % inhibition ± SD compared with acarbose as a positive control and used at the same concentration of the sample.

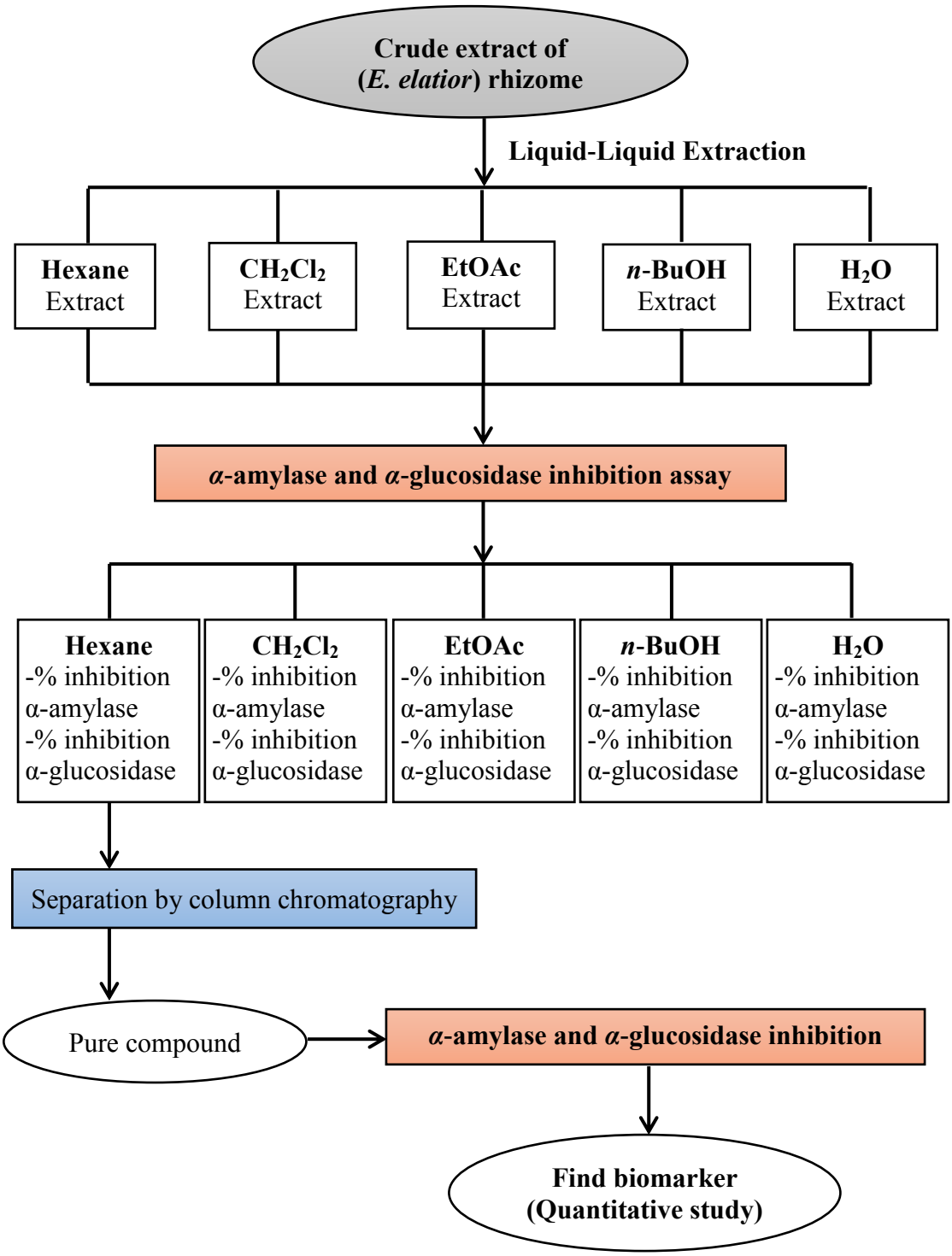
The % inhibition of α-glucosidase was calculated according to the following equation.

% α-Glucosidase inhibition = $[(A_{c+}\text{-}A_c)\text{-}(As\text{-}Ab)]/ (A_{c+}\text{-}A_{c-}) \times 100$

- A_{c+}= Absorbance value at 405 nm of enzyme solution (50 µL), p-NPG substrate solution (50 µL) and 50 µL of 50 mM phosphate buffer (pH 6.9). It is the absorbance of 100% enzyme activity (only the solvent with the enzyme).
- A_c= Absorbance value at 405 nm of only (200 µL) of 50 mM phosphate buffer (pH 6.9) without the enzyme.
- *As*: Absorbance value at 405 nm of enzyme solution (50 µL), p-NPG substrate solution (50 µL) and sample or acarbose solution (50 µL) in 50 mM phosphate buffer (pH 6.9). It is the absorbance of test sample with the enzyme.

-*Ab*= The blank of sample without the enzyme in the absorbance value at 405 nm solution of 300 µL [50 mM phosphate buffer (pH 6.9)] and sample solution 200 µL.

In order to find biomarkers for the quality control of the extract of *E.elatior* rhizome, the separation of pure compound that give the highest inhibitory activity was performed and the whole separation process is summarized in **scheme 3.3**. This process started from maceration of *E. elatior* rhizome powder, followed by partitioned with different solvents. Each fractions was tested for α-glucosidase and α-amylase inhibitory activity to find % inhibition. From the result of inhibitory activity, % inhibition at the same concentration of hexane and *n*-butanol fractions gave the best activity. However, in this study hexane fraction was selected to be further used in the separation step. Several separation processes by using column chromatographic techniques including normal phase chromatography using silica gel, ion-exchange chromatography using Diaion-HP20, size exclusion chromatography using Sephadex LH-20 and reverse-phase HPLC were utilized in order to get pure compounds. The obtained compounds were then tested for α-glucosidase and α-amylase inhibitory activity to find their IC_{50}. The compound that give the highest activity will be used as biomarkers.



Scheme 3.3: Summary of a whole separation process

3.9. Mode of α-glucosidase and α-amlyase inhibition

Slightly modified method according to Kim et al., (2005) was used in order to described the mode of inhibition of *α*-glucosidase using ethanol crude extract as a test sample. The experiment was carried out in triplicate compared with the control. In the beginning p-NPG was prepared in advance and used as a substrate. It was accurately weighed (15 mg) of p-NPG and dissolved in 10 mL of 50 mM of phosphate buffer (pH6.9) to give 5 mM of p-NPG solution. Then 250 µL this solution was transferred to a 10 mL volumetric flask, phosphate buffer 50 mM (pH 6.9) was added to volume to give 0.125 mM of p-NPG solution. Another concentration of p-NPG solution (0.25 mM) was prepared in the same manner but using 500 µL of 5 mM p-NPG solution stock. Further serial dilutions were carried out in similar manner to give 0.5 mM, 0.75 mM, 1 mM and 1.5 mM of p-NPG solutions. The test was performed by first weighing, 1 mg of crude extract and dissolved in 1 mL DMSO and further diluted with 50 mM phosphate buffer (pH 6.9) to get the concentration of 3 µg/mL. 100 µL of the extract solution was mixed with 100 µL of enzyme solution (0.57 unit/mL) in 50 mM phosphate buffer (pH 6.9) in a cuvette and incubated at 37 ± 1 ºC for 10 min. 100 µL of p-NPG solution at in different concentrations from 0.125, 0.25, 0.5, 0.75, 1.0 and 1.5 mM was added. The absorbance was measured at 405 nm by using Cary 100 Series UV-Vis Spectrophotometer **(Figure 3.11)**. The absorbance was monitored at selected time interval (2, 4, 6, 10, 15, 20 min) during incubation at 37 ± 1 ºC. The standard curve as of *p*-nitrophenol (1, 10, 20, 30, 40 and 50 µg/mL) was obtained by measuring the absorbance at 405 nm. The concentration of the products produced (reducing sugars as maltose and *p*-nitrophenol) at each time point was determined from corresponding standard curves by converted absorbance of test sample to the concentration of *p*-nitrophenol by using the standard curve. The concentration of *p*-nitrophenol at each time point was plotted against the time. The reaction rate was obtained by using the equation below.

$$\text{Reaction rate (V)} = \frac{\text{Amount of product produced at selected time } (\mu g.mL^{-1})}{\text{Time (min)}}$$

Mode of inhibition was achieved by plotting lineweaver burk plot between 1/[V] and 1/[S] according to the Michaelis-menten kinetic equation.

$$\textbf{Michealis Menten Equation } (\upsilon) = \frac{Vmax \times [S]}{Km + [S]}$$

[V] : is initial velocity of enzymatic reaction

[S] : is substrate concentration

Vmax : maximum velocity of enzymatic reaction

Km : Substrate concentration when [V] is one-half of Vmax

Figure 3.11: Cary 100 Series UV-Vis Spectrophotometer (Agilent Technologies, Malaysia) used for mode of inhibition study

3.10. Structure elucidation

Spectroscopic methods including infrared spectroscopy (IR) and nuclear magnetic resonance (NMR) spectroscopy were used to characterize the chemical structures of the isolated compounds. To measure a precise molecular formula of sample molecules nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C) (500 MHz and 125 MHz, Varian Unity Inova 500 FT-NMR spectrometer, UK) are used to give structural information of the pure isolated compounds. Major functional groups of the

compounds were identified by using FT-IR spectrometer (Spectrum One, Perkin Elmer, UK). The well characterized compounds which are isolated from *E. elatior* rhizome extract are then used in α-glucosidase and α-amylase inhibitory activity to find their IC_{50} values. Any compounds that exhibited high activities in α-glucosidase and α-amylase inhibition will be considered to be used as biomarker for quality control of the extract.

3.12. Statistical analysis

The final results are expressed as the mean ± SD. % Inhibition of the biological activities and IC_{50} values were calculated by using the Microsoft Excel program. The group means was evaluated for their significant was analyzed by using one-way analysis of variance (ANOVA) SPSS 16.0, followed by LSD. Values were determined to be significant when p was less than 0.05 ($p<0.05$).

CHAPTER 4

RESULTS AND DISCUSSION

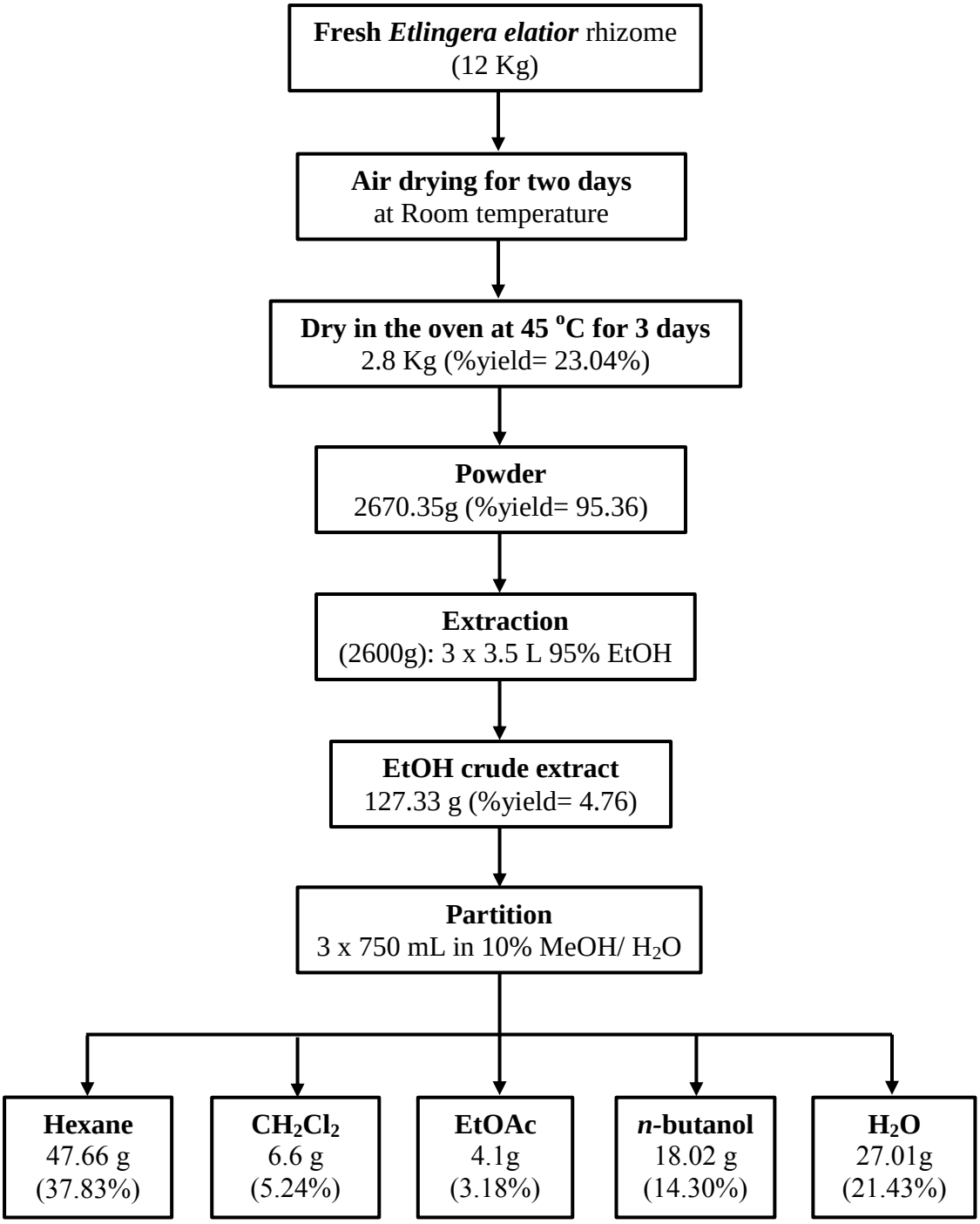
Different parts of *Etlingera elatior* are widely used such as edible both raw and cooking and also to make their extract into a lot of products like soap, shampoo and perfume. Moreover, *E. elatior* was used as traditional medicine to treat earache, cleaning wounds, remove body odour and used by women during ailment. Pharmacological activities as medicinal plant included antimicrobial agents against various microorganisms, anti-bacteria, antifungal, antioxidant, tyrosinase inhibitory, anti-inflammation, antitumor, anti-diabetes, and cytotoxic activities (Karim and Munir, 2011). Eventhough, *E. elatior* has been known as traditional medicine and medicinal plant, there are no reports about mechanism on carbohydrate digestive with α-amylase and α-glucosidase activities in type 2 diabetes. This chapter is divided into three parts. The first part is focused on bioassay of anti-alpha glucosidase and anti-alpha amylase enzyme. The second is the isolation process to find the active compounds and the third part is to investigate for the mode of enzyme inhibition.

4.1. Extraction of *Etlingera elatior* rhizome

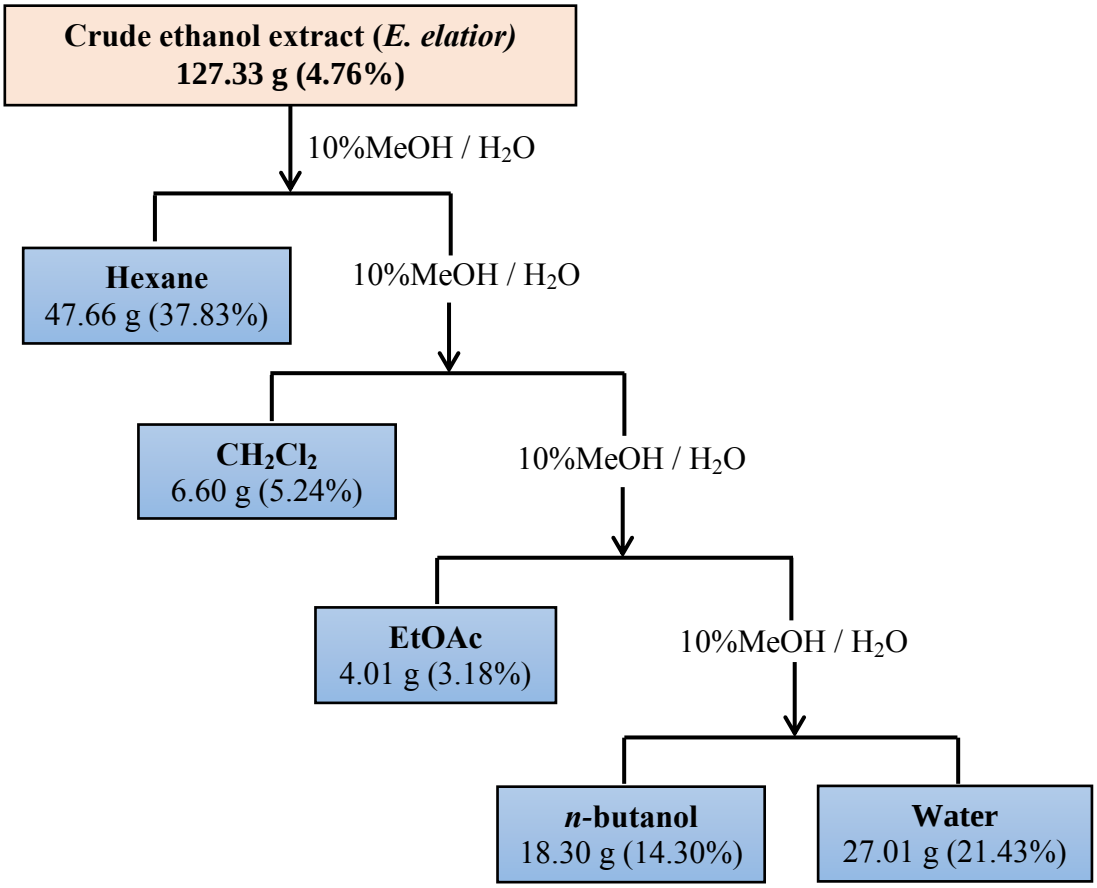
The fresh *E. elatior* rhizome 12 Kg was dried in the air for two days after chopping to small pieces 3-5 mm followed by drying in the oven at 45 ^{o}C for 3 days to give 2.8 Kg (% loss of water = 76.67%). Comparing the fresh and the dried rhizome, weight was reduced from 12 Kg to 2.8 Kg, it meaned that the rhizome contained a lot of water. Then the dried rhizome was ground into powder using the mortar machine to obtain 2.67 Kg. The rhizome powder (2.6 Kg) was macerated in a small jar with 3.5 liters of 95% ethanol for 3 times. The maceration was kept at room temperature protected from light for 48 hours each time. Filtration the extract solution through a filter paper (Whatman® No.1) and after solvent evaporate using rotary evaporator at 45 ^{o}C, this will provide EtOH crude extract in 127.33 g (4.76 % yield, **Table 4.1, entry 1)**. The appearance of EtOH crude extract was dark brown viscous liquid **(Figure 4.1, a)**. The EtOH crude extract was further fractionated by using

partition method with four solvents, namely hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and water (H_2O). This was done by first dissolving 127.33 g of the ethanol extract in 1000 mL of 10% methanol in water. For partition method 750 mL of each solvent was used for 3 times. After extraction processes using variety of solvents from non-polar to polar were completed, five extracts were obtained. The process of extraction method was shown in **Scheme 4.1**. The result of the % isolated yield from each extraction solvents are summarized in **Scheme 4.2** and **Table 4.1**. After solvent evaporation by using rotary evaporator n-hexane extract was obtained in 47.66 g (37.88 % of crude ethanol extract, **Table 4.1, entry 2**) as a dark brown color and viscous liquid **(Figure 4.1b)**. Dichloromethane extract was obtained in 6.60 g (5.24% of crude ethanol extract, **Table 4.1, entry 3**), as a yellow orange solid **(Figure 4.1c)**. Ethyl acetate extract was obtained in 4.01 g (3.18% of crude ethanol extract, **Table 4.1, entry 4**) as a deep red solid **(Figure 4.1d)**. *n*-Butanol extract was obtained in 18.3 g (14.30% of crude ethanol extract, **Table 4.1, entry 5**) as a dark brown viscous liquid **(Figure 4.1e)**. The aqueous extract obtained from the water part was appeared in a brown viscous liquid **(Figure 4.1f)** in 27.01 g (21.43% yield of crude ethanol extract, **Table 4.1, entry 6)**.

Each extract was utilized for further study in phytochemical screening.



Scheme 4.1: The diagram displays the extraction processes



Scheme 4.2: The diagram displayed the product yields from each partition methods

Table 4.1: The appearances of crude extracts

Entry	Sample	Color	Physical appeareance	Weight (g)	%Yeild	Note
1	Ethanol crude extract (a)	Dark brown	Viscous liquid	127.33	4.76%	Compare with dried rhizome
2	Hexane extract (b)	Dark brown	Viscous liquid	47.67	37.83%	Compare with crude ethanol extract
3	Dichloromethane extract (c)	Yellow-orange	Solid	6.60	5.24%	
4	Ethyl acetate extract (d)	Deep red	Solid	4.01	3.18%	
5	n-butanol extract (e)	Dark brown	Viscous liquid	18.03	14.30%	
6	Water extract (f)	Brown	Viscous liquid	27.01	21.43%	

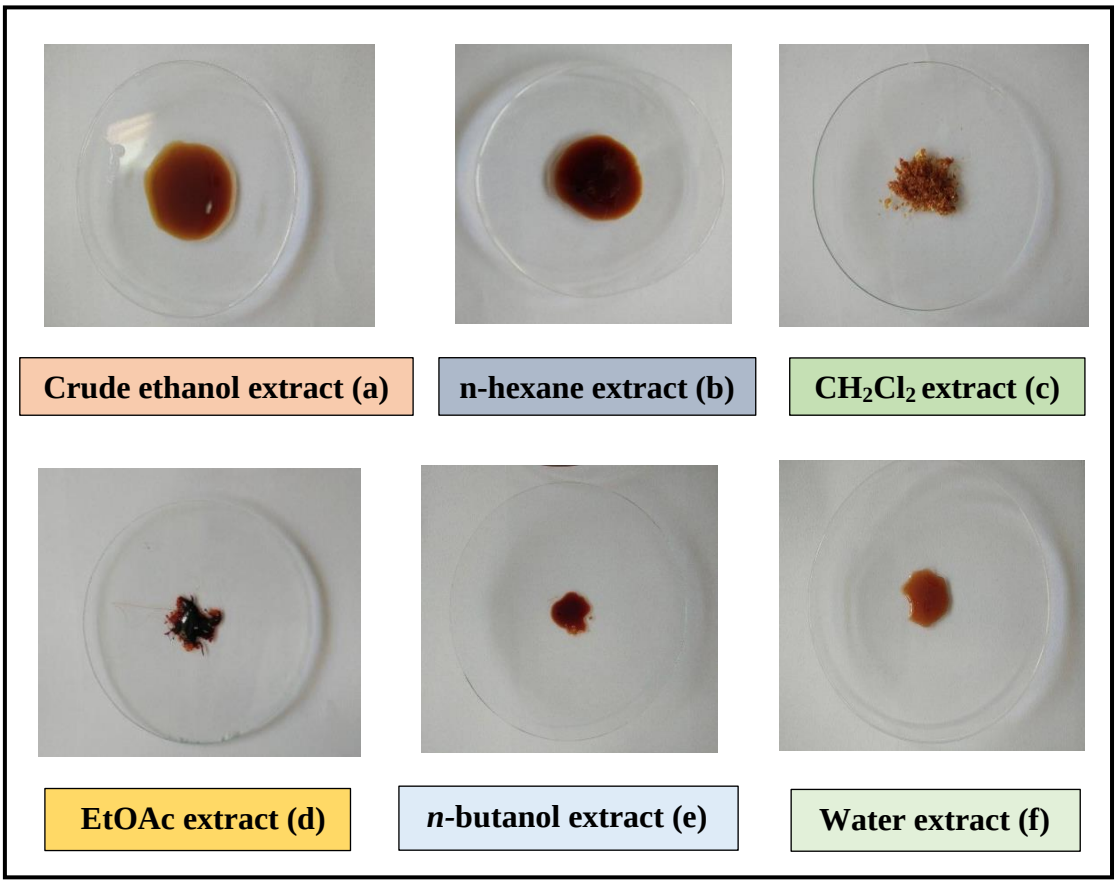


Figure 4.1: The appearance of crude extracts obtained from different solvents

4.2. Phytochemical study of *E. elatior* rhizome

According to many reports on phytochemical study, some chemical compounds such as alkaloids, flavonoids, and polysaccharides have potential to inhibit α-amylase and α-glucosidase enzyme (Ueda et al., 1998; Sou et al., 2000; Gao et al., 2007). Moreover, some chemical compounds such as phenolic compounds and tannin can precipitate enzyme (Benoit and Starkey, 1968; Martin and Martin, 1982). Therefore, phytochemical study is very important for enzyme activities testing like α-amylase and α-glucosidase. *E.elatior* rhizome extracts obtained from previous part were subjected to phytochemical composition screening to find the presence of chemical component namely tannins, phenolics, flavonoids, terpenoids and alkaloids. The results **(Table 4.2)** showed that phytochemicals components of *E. elatior* rhizome were similar to that containing in the flower of *E. elatior* reported by (Lachumy et al.,

2010). Phenolics and flavonoids were presence in the crude EtOH and all partition extracts. Tannin was not obtained in only n-hexane extract, whereas alkaloids were not found in all extracts. Terpenoids were not detected in ethyl acetate and water extracts **(Table 4.2)**.

Table 4.2: Results of phytochemical screening of crude ethanol extracts and partition extracts

Extract	Tannin	Phenolic	Flavonoid	Terpenoid	Alkaloid
Crude ethanol	+	+	+	+	-
Hexane	-	+	+	+	-
Dichloromethane	+	+	+	+	-
Ethyl acetate	+	+	+	-	-
n-Butanol	+	+	+	+	-
Water	+	+	+	-	-

(+)= Detected

(-)= Not detected

4.3. The inhibitory activities of α-glucosidase and α-amylase

Human can break down food into small molecule easily for support the body living by enzyme activities. α-Amylase is an enzyme in the mouth secreted from saliva gland and the pancreas for hydrolyzing the starch at α-1,4 glycosidic bond into oligosaccharides and maltose (Nater and Rohleder, 2009; Feng et al., 2011; Mogale et al., 2013). α-Glucosidase is an enzyme that continues to break down oligosaccharides, maltose, sucrose and lactose into glucose by hydrolyzing α-1,4 glycosidic bond in carbohydrate digestion process (Gao and Kawabata, 2005). So, if both enzymes are inhibited by inhibitors, the level of glucose in blood stream will be reduced and therefore the diabetes mellitus will be managed or controlled. The ethanol extract and all extracts from partition of *E. elatior* rhizome were tested for α-glucosidase and α-amylase inhibitory activities and the result are as the following.

4.3.1. *α*-glucosidase inhibitory testing of *E.elatior* rhizome

p-NPG was used as a substrate for *α*-glucosidase inhibitory activity (Chapdelaine et al., 1978). The mechanism of *α*-glucosidase enzyme on p-NPG (No color) will give *p*-nitrophenol (yellow) and glucose as products **(Figure 4.2)**. However, when the enzyme is inhibited by inhibitor, the color will be reduced from yellow to pale yellow or no color **(Figure 4.3 and 4.4)**. The existing of *p*-nitrophenol can be monitored by determine the absorbance at 405 nm by microplate reader **(Figure 4.5)**. The color of *p*-nitrophenol was changed to pale yellow or colorless when the inhibitors are in active form **(Figure 4.4)**.

EtOH crude extract and five extracts from partition process were tested for *α*-glucosidase inhibitory activities and were compared with that of acarbose used as standard drug (positive control). There are many kinds of drugs used to control type 2 diabetes such as acarbose (Chiasson et al., 2002; Josse et al., 2003), miglitol, voglibose and etc., but the most popular standard drug for *α*-glucosidase inhibitory activity is acarbose because it is specific on glycemic control to reduce fasting blood glucose and post-load insulin levels compared to other drugs (Van de Laar et al., 2005).

All extracts and acarbose were tested for *α*-glucosidase inhibitory activity at concentration of 25 μg/mL and the results are summarized in **Figure 4.6**. The inhibition of EtOH crude extract was 95.34% and showed much higher activity when compared with acarbose (11.12%). This may be due to its extract contained a lot of components including tannin, phenolic, flavonoid and terpenoid **(Table 4.2)**. All extracts from partition process showed good *α*-glucosidase inhibitory activity but in different extents. They were ranged from the highest to the lowest as following n-butanol (99.79%), hexane (94.36%), water (92.10%) and EtAOc (86.36%). CH_2Cl_2 extract showed poor inhibitory activities (28.36%) compared to the other extracts but still higher than that of acarbose (11.12%). The %inhibition of EtOH crude extract, hexane, CH_2Cl_2, EtOAc, n-butanol and water were not significantly different.

Eventhough, acarbose is an *α*-glucosidase inhibitor and available in a market, this drug is not a potent inhibitor of this enzyme. As in our study using a concentration of acarbose at 25 μg/mL gave only 11.12% inhibition. Similar study

found that using 120 µg/mL of acarbose gave only 38.25% inhibition of glucosidase enzyme (Ye et al., 2010).

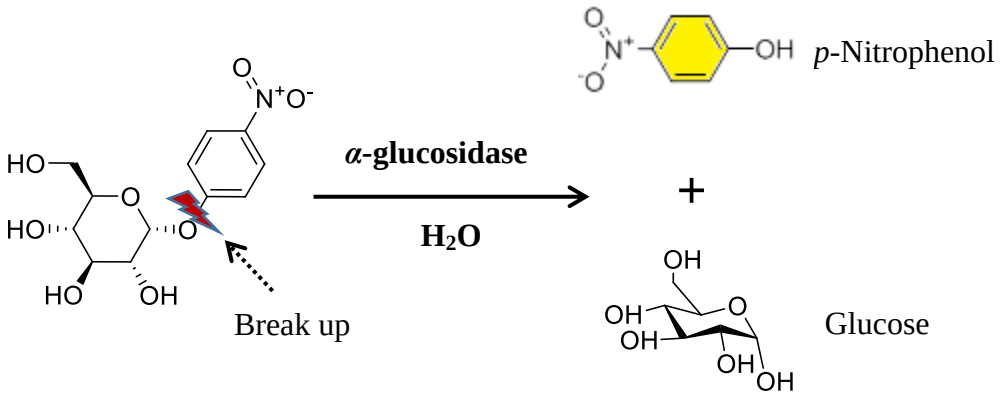


Figure 4.2: The mechanism of *α*-glucosidase enzyme without inhibitors in enzyme activity testing

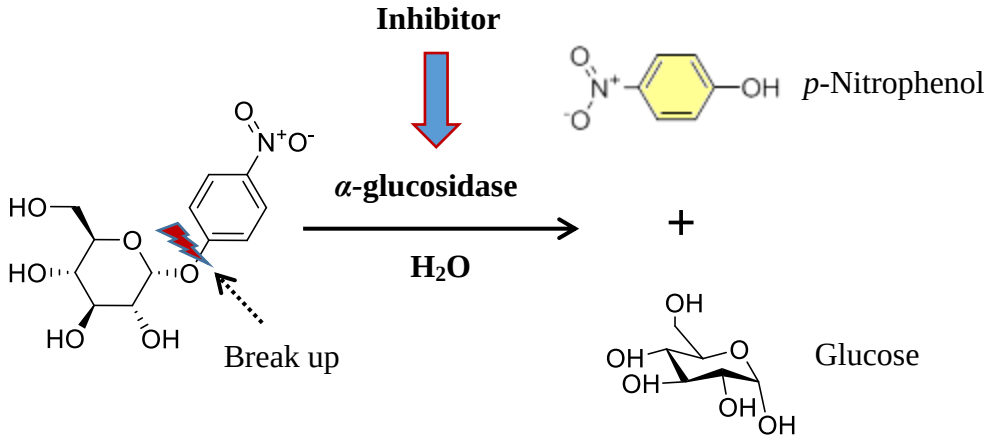


Figure 4.3: The mechanism of *α*-glucosidase enzyme with inhibitors in enzyme activity testing

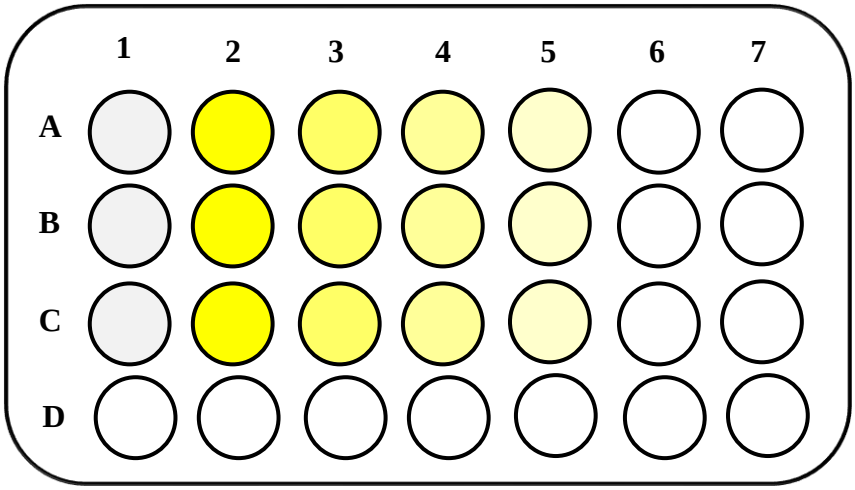


Figure 4.4: Displayed example of 96-well microplate on α-glucosidase inhibitory activity testing

The presence of yellow color demonstrateds the active enzyme. Less yellow color or no-color wells demonstrate less enzyme activity or no activity.

Figure 4.5: Microplate reader (Beckman Coulter, DTX 800, Austria) used for measuring the absorbance of enzyme activity testing

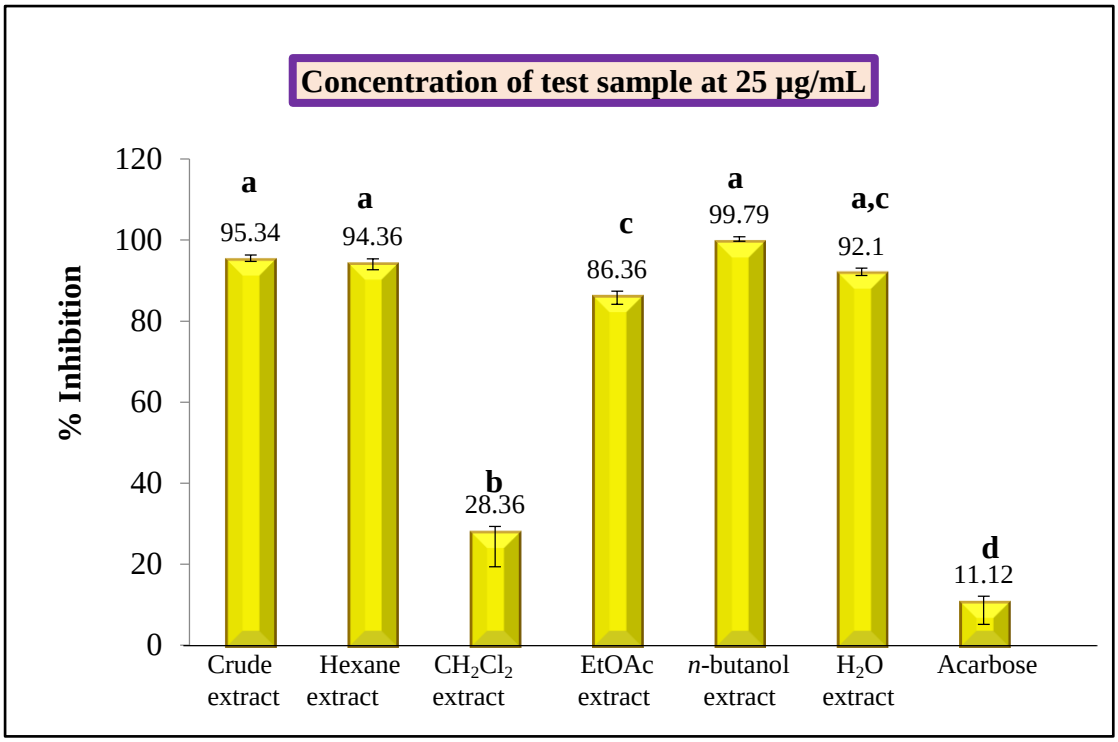


Figure 4.6: Graph displayed the % inhibition of α-glucosidase activities of EtOH crude extract and five extract fractions (hexane, CH_2Cl_2, EtOAc, *n*-butanol and water) at 25 µg/mL

The different letters showed the statistically significant.

4.3.2. α-Amylase inhibitory testing of *E.elatior* rhizome

Starch azure was used as a substrate for α-amylse inhibitory activity (Hansawasdi et al., 2000). Starch azure is obtained from combination of potato starch covalently linked with remazol brilliant blue R which is helpful in the activity determination processes.

When mixing α-amylase with starch azure, the enzyme will cleave the starch azure to give brilliant blue R and maltose as the products **(Figure 4.7)**. Therefore, when the enzyme is still active the final solution after incubation will give dark blue color solution which can be determined the absorbance at 595 nm **(Figure 4.7)**. However, when the inhibitor was also incubated with the enzyme, the enzyme can be less active or not active, hence, less blue color no color would be observed **(Figure**

4.8 and 4.9). Therefore, if any extract samples have α-amylase inhibitory activity, they will give less blue or no color after testing.

The preliminary screening of α-amylase inhibitory activity of all extracts were performed by using sample solutions having concentration at 25 µg/mL, and compared their activity against acarbose (positive drug) at the same concentration. The results demonstrated that all extracts have shown to have α-amylase inhibitory activity in a range of 35.91-58.13% inhibition **(Figure 4.10)**. Crude ethanol extract (45.39%), dichloromethane extract (50.77%), *n*-butanol (55.31%) extract and H_2O extract (35.91%) gave higher inhibitory activity than acarbose (43.39%), whereas hexane extract (35.91%) showed lower inhibitory activity than acarbose. EtOAc extract (43.56%) provided similar % inhibition to that of acarbose.

From this result, it can be seen that using medium polar or high polar solvents in the extraction process could obtain the compounds that have better α-amylase inhibitory activity than using non polar solvent such as hexane. Since, hexane did not contain tannin but contain phenolic, flavonoid and terpenoid compounds, therefore, the α-amylase inhibitory activity of hexane should be according to the phenolic, flavonoid or terpenoid contents. Good inhibition of crude ethanol extract, dichloromethane, EtOAc, *n*-butanol and water extracts could be due to the tannin contents in these extracts, since it is known that tannin can precipitate protein. However, other compounds such as phenolic compound, flavonoid and terpenoid could also are the compounds that play important role in α-amylase inhibitory activity.

Ethyl acetate and *n*-butanol eventhough, not detected terpenoid but they are still gave % inhibition of α-amylase activity higher than hexane, therefore, terpenoid not may play important role in α-amylase inhibitory activity.

To compare % inhibition between α-glucosidase and α-amylase inhibitory activities at the same concentration (25 µg/mL) found that all extracts displayed α-glucosidase inhibitory activity better than α-amylase inhibitory activity. The purpose of this research was to find the most active compound for treatment of diabetes type 2 to be used as a biomarker for *E. elatior* rhizome qualitative determination. So, the fractions which showed the highest activity were selected for further isolation of pure

compounds which would be further subjected to the testing of inhibitory activity against both enzymes (*α*-glucosidase and *α*-amylase). According to the results **(Table 4.3)** eventhough four fractions showed high *α*-glucosidase inhibitory activity including hexane (94.36%), ethyl acetate (86.36%), *n*-butanol (99.79%) and water (92.10%). Only hexane fraction was selected for further isolation of pure compounds since from the phytochemical screening demonstrated that hexane fraction did not contain tannin but contain phenolic, flavonoid and terpenoid. These compounds are normally known to have some interesting biological activities.

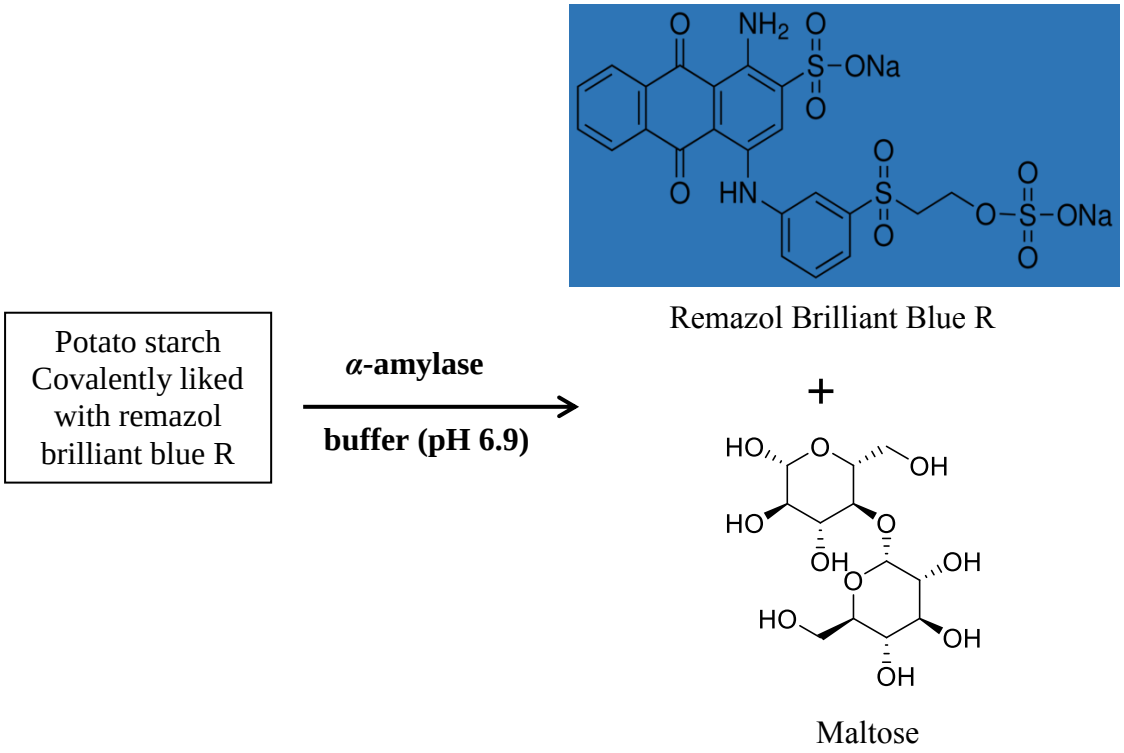


Figure 4.7: The mechanism of *α*-amylase enzyme without inhibitor

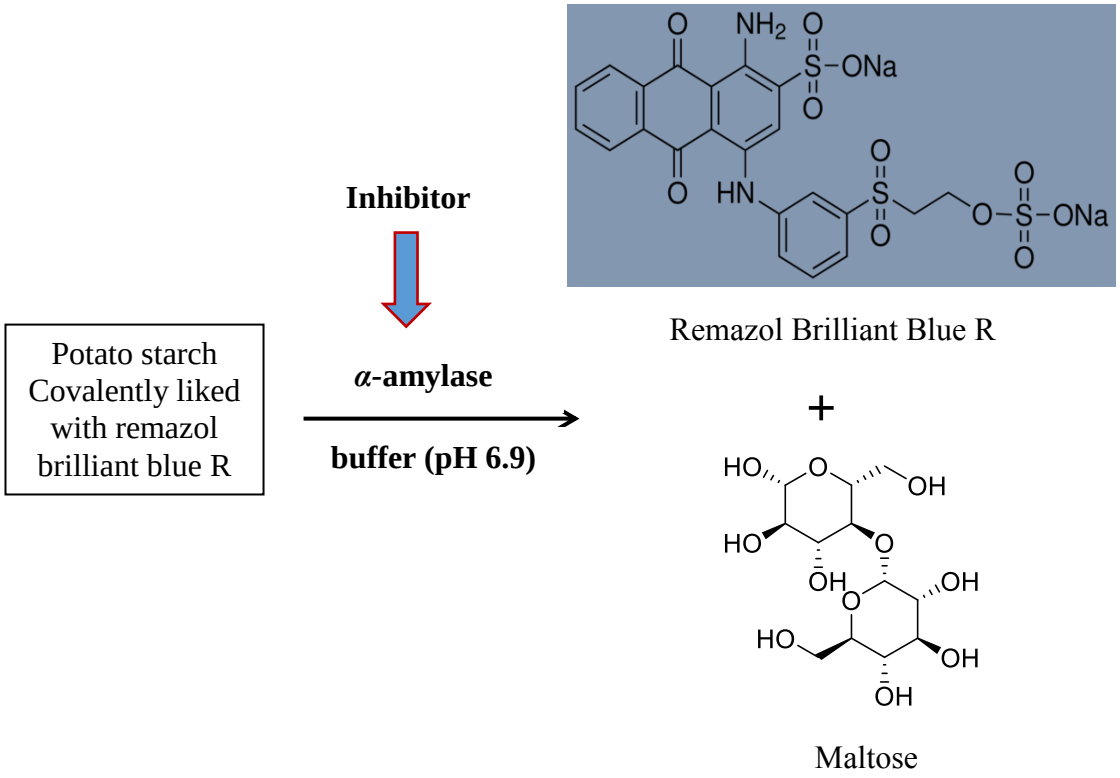


Figure 4.8: The mechanism of α-amylase enzyme with inhibitor

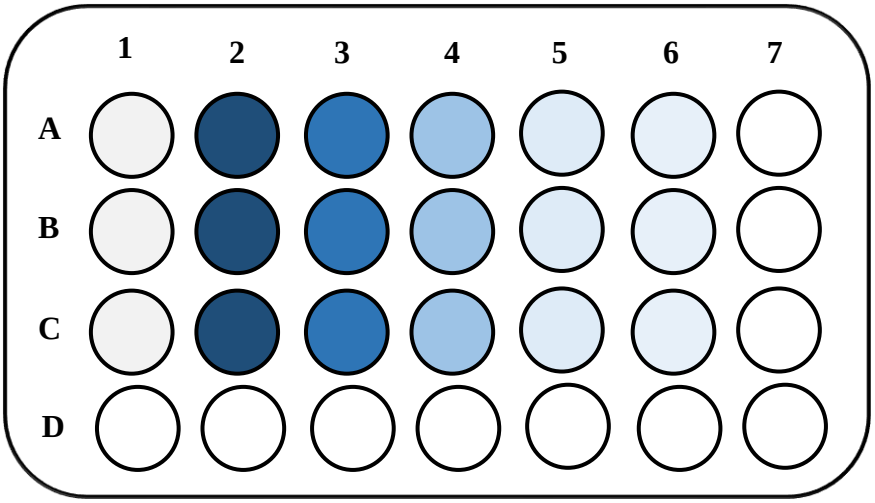


Figure 4.9: The picture of 96-well microplate for determination of α-amylase inhibitory activity

The presence of dark blue color demonstrate the active enzyme. Less blue or no color wells demonstrate less enzyme activity or no activity.

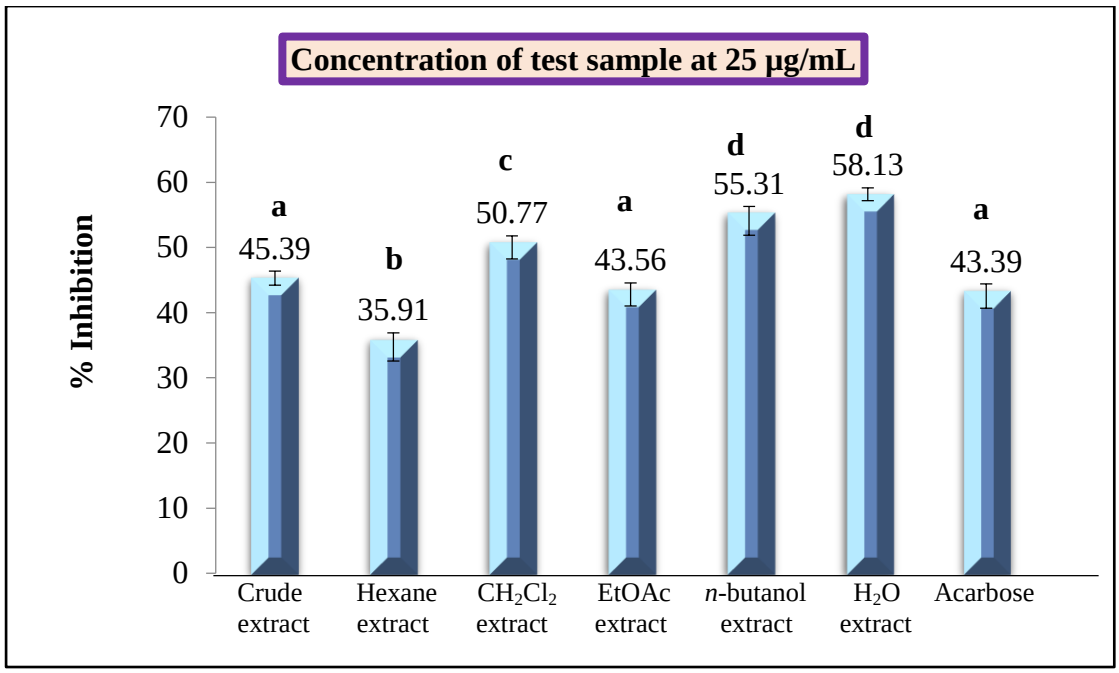


Figure 4.10: Graph displayed the % inhibition of α-amylase activities of EtOH crude extract and five extract fractions (hexane, CH_2Cl_2, EtOAc, *n*-butanol and water) at 25 µg/mL

The different letters showed the statistically significant.

Table 4.3: % Inhibition of crude ethanol extract and partition extracts against α-glucosidase and α-amylase at 25 µg/mL

Sample	**% Inhibition**	
	α-glucosidase	**α-amylase**
EtOH crude extract	95.34 ± 0.57^{a}	$45.39\pm3.34^{a'}$
Hexane extract	94.36 ± 1.65^{a}	$35.91\pm2.52^{b'}$
Dichloromethane extract	28.36 ± 8.95^{b}	$50.77\pm2.52^{c'}$
Ethyl acetate extract	86.36 ± 2.23^{c}	$43.56\pm3.46^{a'}$
n-Butanol extract	99.79 ± 0.05^{a}	$55.31\pm0.96^{d'}$
Water extract	$92.10\pm0.80a^{c}$	$58.13\pm2.70^{d'}$
Acarbose	11.12 ± 5.93^{d}	$43.39\pm1.19^{a'}$

The different letters showed the statistically significant.

4.4. Extraction and isolation of compounds from the hexane fraction of *E. elatior* rhizome

From previous part, hexane fraction was selected to further purification due to it gave high % inhibition of α-glucosidase, eventhough it did not have high % inhibition of α-amylase. But hexane extract did not show to have tannin, therefore, the compounds in this fraction could be the compound that play important role in the enzyme inhibition activity.

In order to isolate compound from this fraction, column chromatographic techniques were utilized in the purification process including silica gel (normal phases) chromatography, ion-exchange chromatography (Diaion HP-20), size-exclusion chromagography (Sephadex LH-20) and reverse-phase HPLC. In each separation process, eluted fractions were collected and tested for their purity by using TLC technique. Several types of solvents were utilized in the separation processes, including hexane, CH_2Cl_2, $CHCl_3$, diethyl ether, EtOAc, MeOH and water. Appropriate solvent mixtures were optimized before using as an eluent in each separation step.

The process of isolation of the desired compounds were conducted as following **(Scheme 4.3)**. The hexane extract (5 g) from partition process was first purified by column chromatography using sephadex LH-20 as adsorbent in a glass column (2 cm diameter, 100 cm length) and eluted with 100% of MeOH **(Figure 4.13 and Table 4.4)** to give 11 fractions: Fraction EH1 (2 mg), EH2 (1560 mg), EH3 (1640 mg), EH4 (811 mg), EH5 (1000 mg), EH6 (340 mg), EH7 (34 mg), EH8 (6 mg), EH9 (9 mg), EH10 (2 mg), EH11 (1 mg). The obtained fractions were subjected to analyze for their α-glucosidase inhibitory activity in order to choose the best active fraction for further isolation. However, only seven fractions were used in the activity testing since they have enough amount for testing, EH1, 8, 10, and 11 gave insufficient amount for further testing. Fractions EH2-7 and 9 were used in the activity testing by preparing the test sample having concentration of 25 μg/mL. The % inhibitory activity of α-glucosidase of each fraction was summarized in **Table 4.4**. The % inhibitions were obtained in a range of 97.15-66.71. The % inhibition ranging from the highest to the lowest were EH6 (97.15±0.13%), EH7 (95.61±0.23%), EH2

(94.22±0.26%), EH3 (93.09±0.13%), EH9 (90.37±0.48%) and EH7 (66.71±0.67%), respectively. The result from the activity testing demonstrated that fractions EH2 and EH3 which provided high isolated residues and high % α-glucosidase inhibitory activities. These two fractions were selected for further purification processes.

Fraction EH2 (1560 mg) gave % α-glucosidase inhibitory activity at 25 μg/mL = 93.09±0.13% was further separated by ion exchange chromatography (Diaion HP-20) using MeOH and water in a gradient mode from 25% to 100% MeOH and finally cleaning with 100% EtOAc. The separation by the ion exchange chromatography provided five combined fractions (EH2.1-EH2.5). Next step, fraction EH2.3 (725 mg) was selected for further separation with size exclusion chromatography (Sephadex LH-20) by using 50% CH_2Cl_2 in MeOH as an isocratic eluent. Five combined fractions (EH2.3.1-2.3.5) were obtained. From these five fractions, fraction EH2.3.3 (467 mg) was chosen to further purifying by using silica gel column chromatography. A gradient system start from 50% hexane in ethyl acetate to 100% EtOAc following by increasing polarity by additional of MeOH from 10% to 50% MeOH in EtOAc was utilized as eluents. After, silica gel column chromatography, 10 combined fractions were obtained (EH2.3.3.1-EH2.3.3.10). These fractions were determined for their purity by using silica gel TLC and the results showed that fraction EH2.3.3.8 contained almost pure compounds, therefore, this fraction was selected to further purify by reverse phase HPLC using 100% MeOH as a mobile phase **(Figure 4.14)**.

Five combined fraction were collected from the HPLC system, however, the most interesting fraction and seem to give pure compound was fraction EH2.3.3.8.3 (7 mg). The result of silica gel TLC (SP=silica gel, MP= 50% hexane in EtOAc) demonstrated that only one spot on the TLC was observed by anisaldehyde-sulfuric acid spraying reagent **(Figure 4.11)**. The separation process of fraction EH2 was displayed in **Scheme 4.4**.

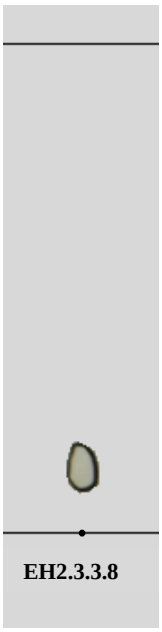


Figure 4.11: TLC analysis of Fraction EH2.3.3.8.3 (SP= silica gel, MP= Hexane:EtOAc, 5:5) (detected by anisaldehyde-sulfuric acid)

The residue after solvent evaporation of fraction of fraction EH2.3.3.8 was subjected to structural determination by using ^{1}H-NMR, ^{13}C-NMR and FT-IR.

Another interesting fraction which gave high product yield was fraction EH3 (1640 mg). This fraction gave % α-glucosidase inhibitory activity at 25 µg/mL= 94.22±0.26%, therefore, this fraction was also selected to further purification. This fraction was first separated by silica gel column chromatography using a gradient elution system. The elution started from 90% hexane in EtOAc and the increment of EtOAc from 10% to 100% EtOAc was utilized. Once 100% EtOAc elution was achieved, additional of MeOH to the EtOAc from 10% to 50% MeOH was performed. The silica gel column chromatography gave eight fractions (EH3.1-EH3.8). Fraction EH3.4 (117 mg) was selected to further separate by size exclusion chromatography (Sephadex LH-20) using an isocratic system of 20% CH_2Cl_2 in MeOH as eluent to afford seven fractions (EH3.4.1-EH3.4.7).

Finally, fraction EH3.4.4 (74 mg) was chosen to purify with silica gel column chromatography and eluted with 10% hexane in $CHCl_3$ to give six fractions.

Fraction EH3.4.4.3 (37.9 mg) was subjected to analyzed for the purify by using silica gel TLC developed with a mixture of 10% hexane and 90% Chloroform and gave TLC pattern as shown in **Figure 4.12**. The separation process of fraction EH3 was displayed in **Scheme 4.5**.

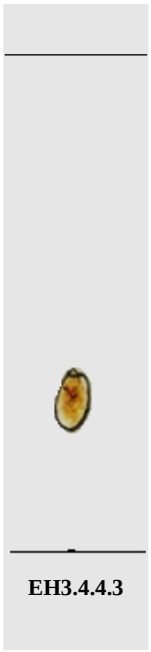


Figure 4.12: TLC analysis of Fraction EH3.4.4.3 (SP= silica gel, MP= Hexane:$CHCl_3$, 1:9) (detected by anisaldehyde-sulfuric acid)

From the TLC chromatogram, indicated that this fraction seems to be quite pure, therefore the residue after solvent removal was subjected to further determine for the structure of the compound containing in this fraction by using ^{1}H-NMR, ^{13}C-NMR and FT-IR.

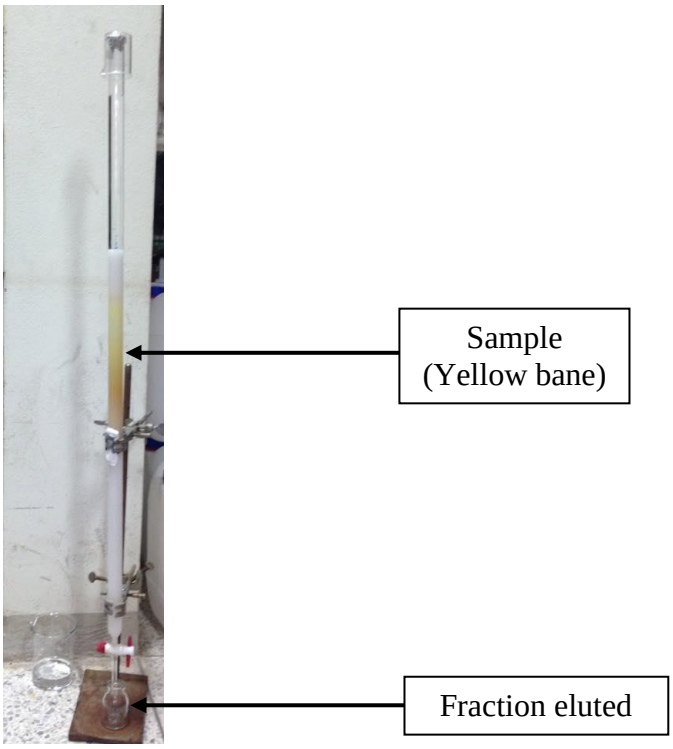


Figure 4.13: Picture of sephadex LH-20 column chromatography (size 2x100 cm)

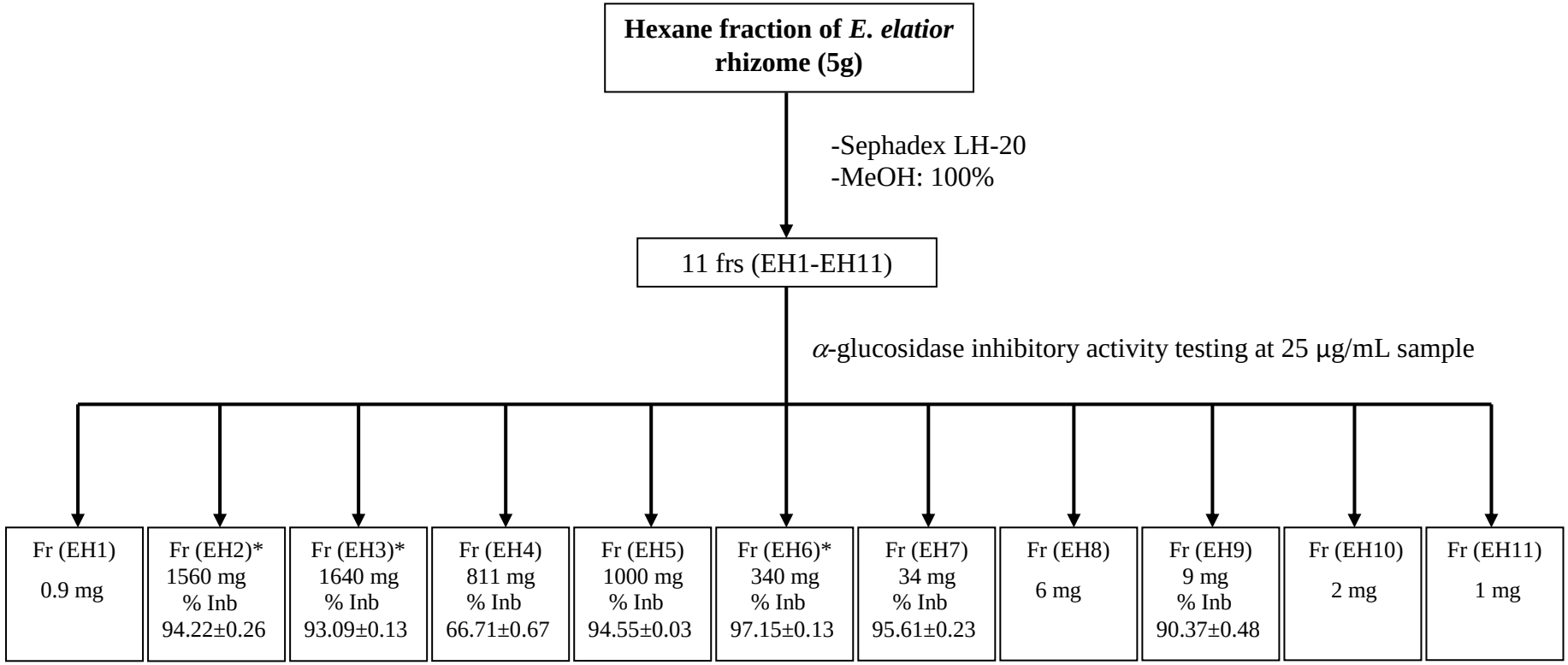
Figure 4.14: Reversed phase HPLC column chromatography used in purification processes

Table 4.4: Weight of residue from separation by sephadex LH-20 and % inhibitory α-glucosidase activities of fraction (EH1-EH11)

Fractions	Weight (mg)	% Yield	% Inhibition of α-glucosidase Activities*
Acarbose			8.62±7.51
EH1	0.0009	N/A	N/A
EH2	1560	31.2	94.22±0.26
EH3	1640	32.8	93.09±0.13
EH4	811	16.22	66.71±0.67
EH5	1000	20.00	94.55±0.03
EH6	340	6.80	97.15±0.13
EH7	34	0.68	95.61±0.23
EH8	6	0.12	N/A
EH9	9	0.18	90.37±0.48
EH10	2	0.04	N/A
EH11	1	0.02	N/A

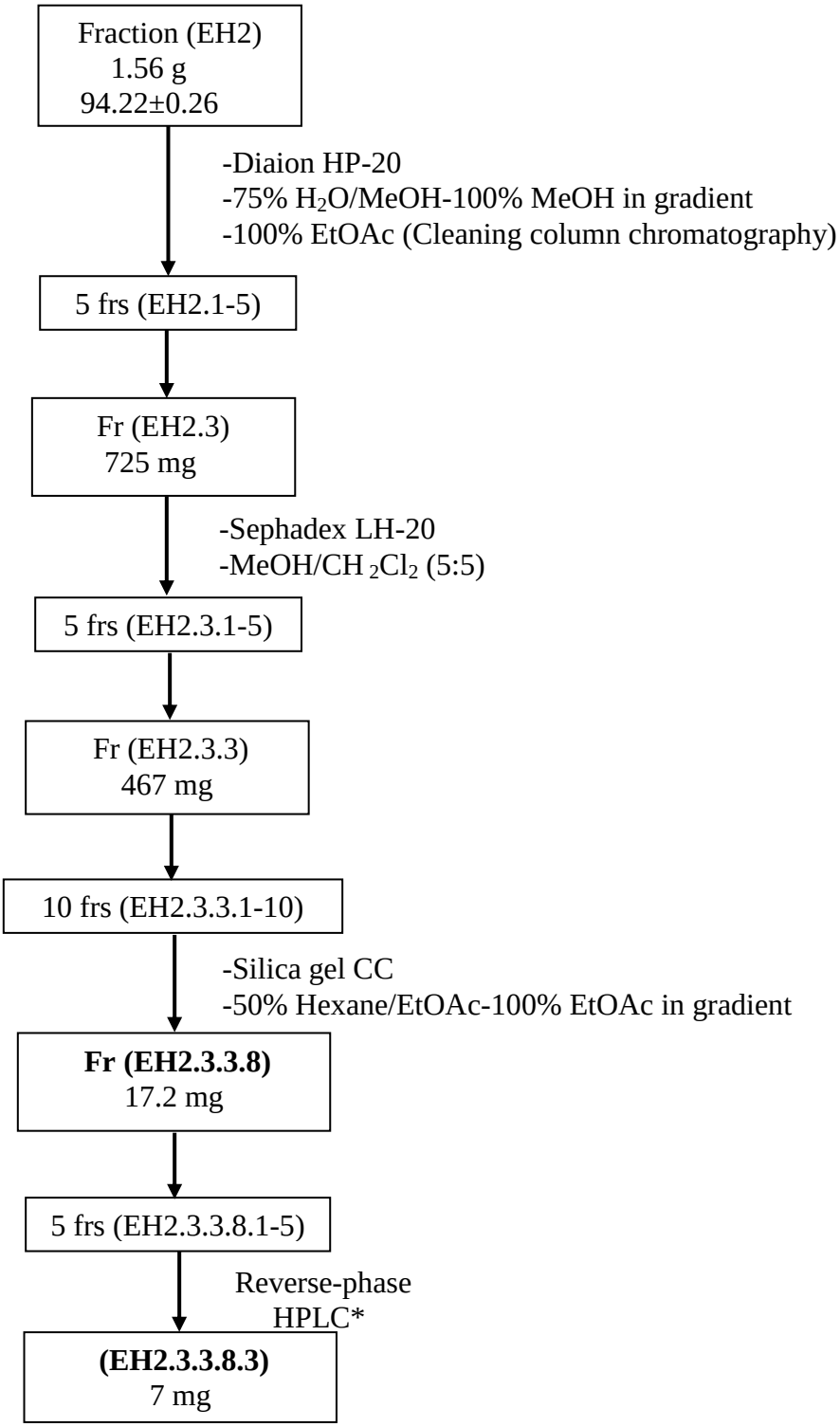
N/A= Cannot determine due to not enough sample

* All samples were tested at the concentration of 25 μg/mL



***=** Indicated the fractions that were chosen for further separation.

Scheme 4.3: Diagram displays the separation process of hexane fraction of the *E. elatior* rhizome



Scheme 4.4: Diagram displays the separation process of fraction EH2

***Note:** HPLC chromatographic system column was performed by using a semi-preparative reversed-phase column.

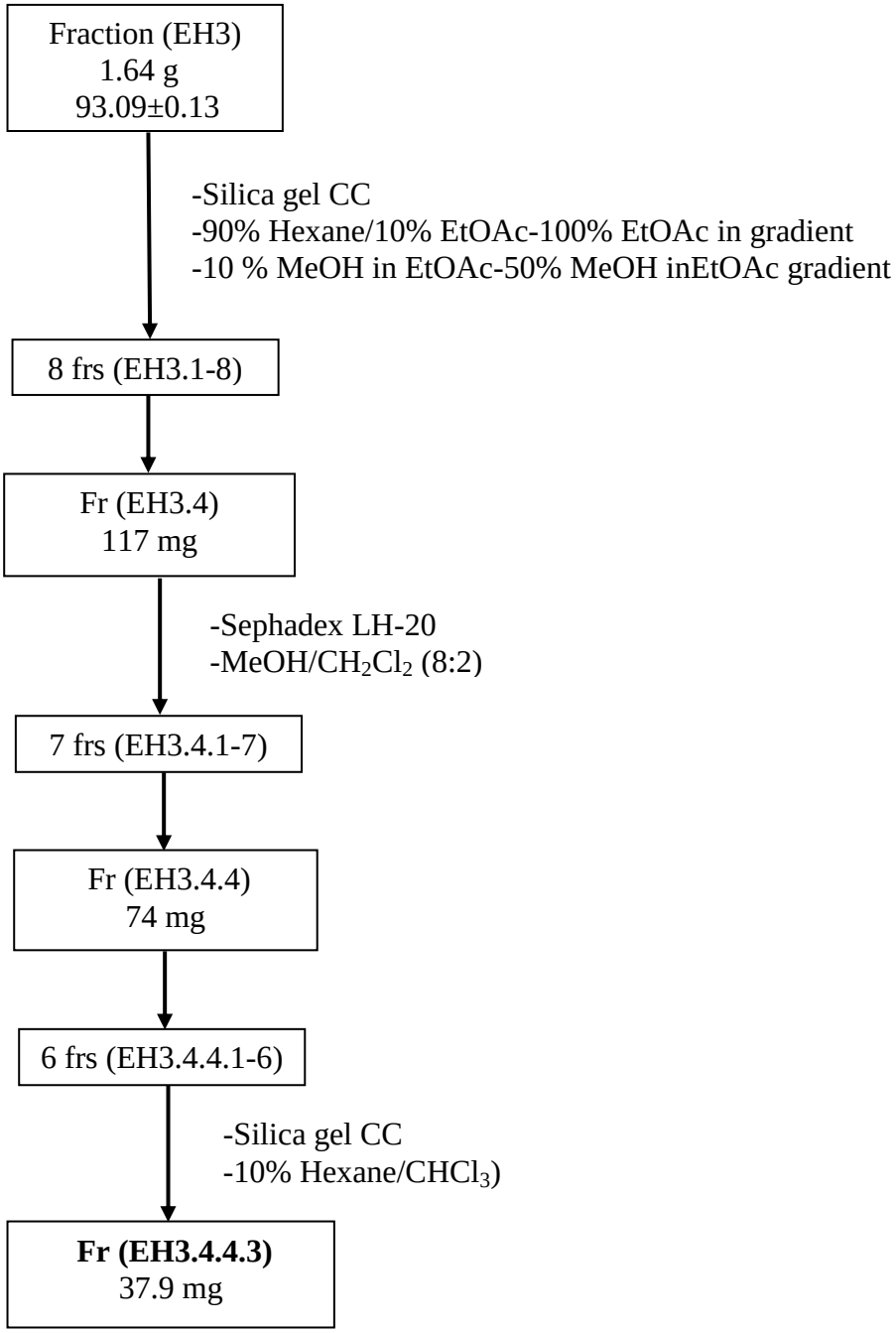
-Pump : Binary HPLC pump (Water® 2707, USA)

-Detector : Photodiode array detector, λ 210 (Water® 2998, USA)

-Flow rate : 4 mL/min

-Mobile phase : 100% MeOH

-Inject volume : 100 μL



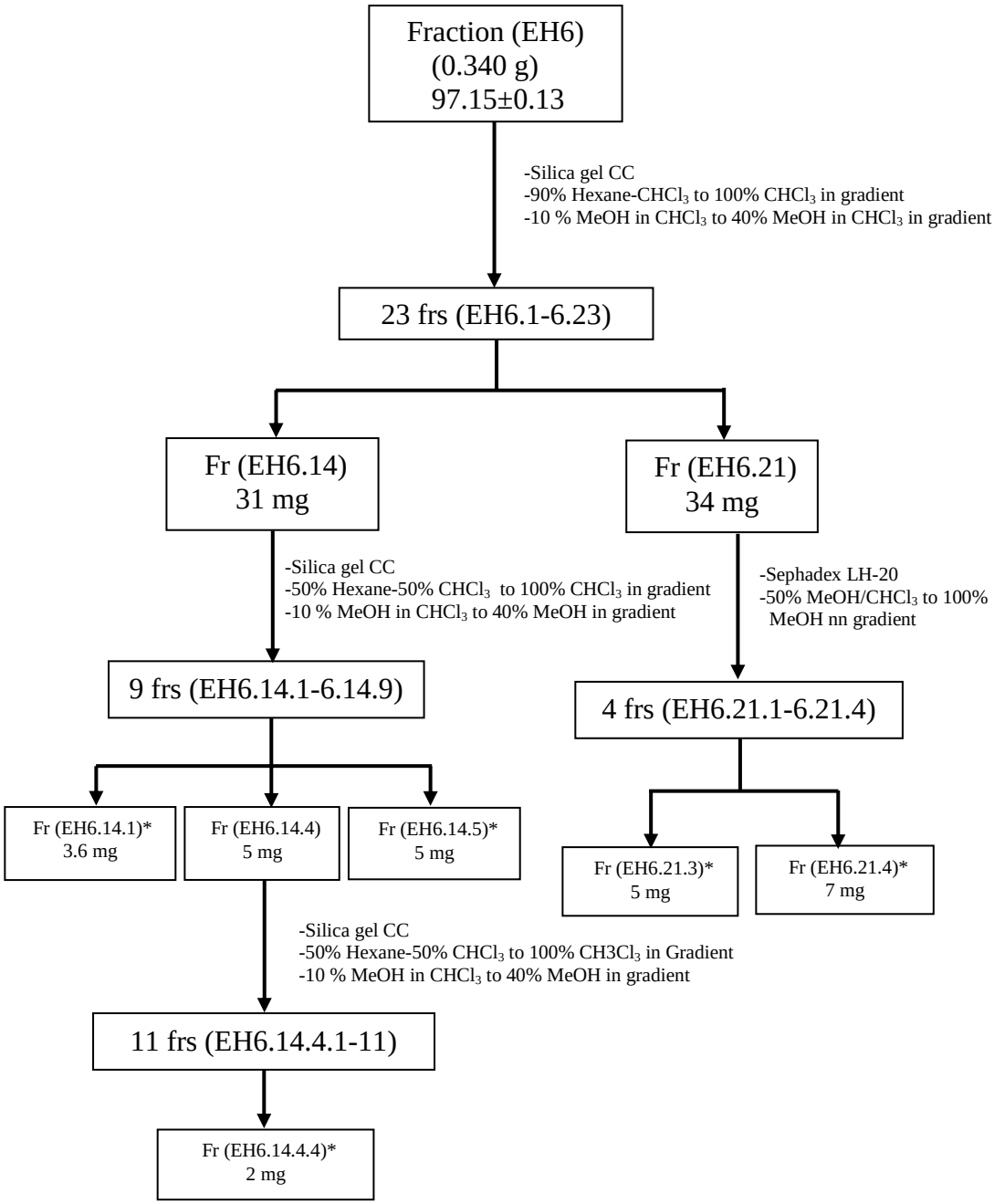
Scheme 4.5: Diagram shows the separation process of fraction EH3

Fraction EH6 was a fraction that gave the highest % α-glucosidae inhibition (97.15%), therefore, this fraction was also selected for further separation to find biomarkers. The separation started by using silica gel column chromatography using a gradient system of 90% hexane in $CHCl_3$ and chloroform content was increased until 100% $CHCl_3$. The elution was then continued by additional of MeOH to the $CHCl_3$ started from 10% to 40% MeOH in $CHCl_3$ in a gradient system. From this separation, 23 fractions were obtained (EH6.1-6.23). Two fractions that gave the most high product yields were EH6.14 (31 mg) and EH6.21 (34 mg) were chosen for further purification process.

EH6.14 was subjected to the separation by using silica gel column chromatography. The gradient elution was utilized in the separation start from 50% hexane in $CHCl_3$ and keep increasing the volume of $CHCl_3$ to 100% following by adding MeOH form 10% to 40% MeOH in $CHCl_3$. Nine fractions (EH6.14.1-6.14.9) two fractions (EH6.14.1 and EH6.14.5) showed one spot on TLC plate (determined by anisaldehyde-sulfuric acid spray reagent). Therefore, these fractions may contained pure compound. Fraction EH6.14.4 was also selected for a further separation by using silica gel column chromatography using the same gradient system as for separation of EH6.14 to provide 9 fractions. Among these fractions, fraction EH6.4.14.4 (2 mg) showed on spot on TLC determined by anisaldehyde-sulfuric acid.

Fraction EH6.21 (34 mg) was also interesting since it provides high quantity. It was then further isolated by sephadex-LH-20 eluted by 50% MeOH in $CHCl_3$ and keep increasing amount of MeOH to 100% MeOH in gradient elution system. Four fractions were obtained (EH6.21-EH6.21.4) from this separation process. From these fractions, fraction EH6.21.3 (5 mg) and EH6.21.4 (7 mg) displayed only one spot on TLC system (detected by anisaldehyde-sulfuric acid). The separation process of fraction EH6 was displayed in **Scheme 4.6**.

From these separation processes, five fractions, (EH6.14.1, EH6.14.4.4, EH6.14.5, EH6.21.3 and EH6.21.4) which were seem to contain pure compound by TLC determination. These fractions were under investigation for their structures by spectroscopic methods.



Note: *= One spot on silica gel TLC

Scheme 4.6: Separation fraction EH6 from hexane extract

4.5. Structure elucidation of compounds

4.5.1. Structure elucidation of compounds containing in fraction EH2.3.3.8.3

The residue obtained from fraction EH2.3.3.8.3 gave 7 mg in a viscous oil characteristic. The result of TLC chromatogram showed a purple spot with anisaldehyde-sulfuric acid spraying reagent **(Figure 4.11)**. Anisaldehyde-sulfuric acid was used to detect of phenols, sugars, steroids, and terpenes by turn violet, blue, red, grey or green. The result showed that the spot on TLC turn purple color **(Figure 4.11)** therefore this compound can be belong to phenol, sugar, steroids or terpene type. Further study was performed by using ^{1}H-NMR, ^{13}C-NMR and FT-IR to investigate for their structures.

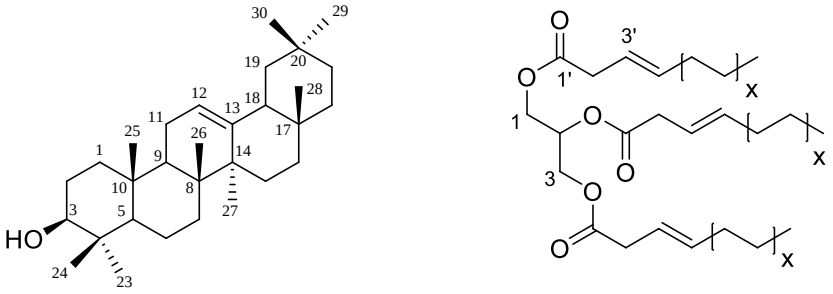
The ^{1}H-NMR (500 MHz, $CDCl_3$) showed characteristic of ursane type triterpenoids having eight methyl singlets at δ_H 1.23 (*s*), 0.98 (*s*), 0.85 (*s*), 0.82 (*s*), 0.80 (*s*), 0.79 (*s*), 0.78 (*s*), and 0.65 (*s*). 3.43 (*ddd, J* = 2.5, 4.5, 10 Hz), 0.68 (*d, J* = 11 Hz), 5.34 (*t, J* = 3), 1.99 (*td, J* = 4, 14 Hz), 193 (*td,* 4, 13.5), 1.93 (*dd,* 3.5, 13) and 1.80 (*m*) **(Figure A.2-A.7)**.

It's ^{13}C-NMR (125 MHz, $CDCl_3$) spectrum displayed identified signals of δ 38.7 (C-1'), 27.1 (C-2'), 79.5 (C-3'), 37.2 (C-4'), 56.0 (C-5'), 18.7 (C-6'), 31.8 (C-7'), 39.1 (C-8'), 50.1 (C-9'), 36.1(C-10'), 22.6 (C-11'), 122.1 (C-12'), 140.2 (C-13'), 42.2 (C-14'), 26.0 (C-15'), 24.9 (C-16'), 31.9 (C-17'), 45.78 (C-18'), 42 (C-19), 29.7(C-20'), 34.2 (C-21'), 36.7 (C-22'), 28.2 (C-23'), 11.9 (C-24'), 11.8 (C-25'), 14.1 (C-26'), 24.2 (C-27'), 29.3 (C-28'), 33.8 (C-29'), 23.0 (C-30') **(FigureA.8-A.11)**.

FT-IR of this sample **(Figure A.1)** showed distinguished broad peaks at absorption band of hydroxyl group (O-H) at 3400 cm^{-1}, the absorption band of C-H stretching sharp peak at 2924 and 2851 cm^{-1} and showed the absorption band of carbonyl stretching peak at 1723 cm^{-1} **(Figure A.1)**.

By comparison these data with the previously reported **(Table 4.5)** suggested that residue in fraction EH2.3.3.8.3 contains a mixture of *β*-amyrin **(Compound 1a)** and fixed oil **(Compound 1b)**. The ^{1}H-NMR spectrum demonstrated the typical characteristic of glycerol part of fixed oil at at δ_H 5.32 (*m*, H-2), 4.43 (*d, J* = 5.5, 12.5

Hz, H_2-1 or H_2-3) and 4.25 (*d*, *J* = 2.5, 12.0 Hz, H_2-3 or H_2-1), respectively. In addition, the ^{1}H NMR spectrum (**Figure A.2**) of mixture of compounds **1a** and **1b** also showed typical of fatty acid part, which was fused to glycerol unit at δ_H 2.32 (*m*, -CH_2-2′) and 3.60-3.28 (an alkene parts; CH-3′ and CH-4′) (Boonnak, 2011; Liu et al., 2012). The ^{13}C NMR spectrum signals (**Figure A.9**) at δ_c 174.8 ppm relative to C-1′ position of the ester unit of the fixed oil.



1a **1b**

Figure 4.15: Structures of *β*-amyrin **(1a)** and possible fixed oil **(1b)**

Table 4.5: Comparison NMR spectral data of *β*-amyrin as compared with reference Vázquez et al., (2012)

Position	Chemical shift of *β*-amyrin			
	Compound (1a)		*β*-amyrin Vázquez et al., (2012)	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1		38.8		38.7
2		27.1		27.2
3	3.43 (*ddd*, 2.5, 4.5, 10)	79.5	3.15 (*dd*, 4.4, 10.8)	79.3
4		37.2		38.5
5	0.68 (*d*, 11)	56.0	0.68 (*d*, 11)	55.1
6		18.7		18.6
7		31.8		32.4
8		39.1		39.8
9		50.1		47.6
10		36.1		36.9
11		22.6		23.6
12	5.34 (*t*, 3)	122.1	5.12 (*t*, 3.2)	121.7
13		140.2		145.2
14		42.2		41.7
15	1.99 (*td*, 4, 14)	26.0	1.89 (*td*, 4.0, 14.0)	26.2
16	1.83 (*td*, 4, 13.5)	24.9	1.70 (*td*, 4.3, 13.5)	26.1
17		31.9		32.6
18		45.78		47.2
19	1.93 (*dd*, 3.5, 13)	42	1.93 (*dd*, 4.0, 13.7)	46.8
20		29.7		31.0
21		34.2		34.7
22	1.80 (*m*)	36.7	1.80 (*m*)	37.1
23	0.78 (*s*)	28.2	0.77 (*s*)	28.0
24	0.82 (*s*)	11.9	0.90 (*s*)	15.4

Table 4.5: Comparison NMR spectral data of *β*-amyrin as compared with reference Vázquez et al., (2012) (Cont.)

Position	Chemical shift of *β*-amyrin			
	Compound (1a)		*β*-amyrin Vázquez et al., (2012)	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
25	0.65 (*s*)	11.8	0.73 (*s*)	15.4
26	0.85 (*s*)	14.1	0.93 (*s*)	16.8
27	1.23 (*s*)	24.2	1.19 (*s*)	25.9
28	0.98 (*s*)	29.3	1.07 (*s*)	28.4
29	0.80 (*s*)	33.8	0.87 (*s*)	33.8
30	0.79 (*s*)	23.0	0.80 (*s*)	23.7

4.5.2. Structure elucidation of compounds containing in fraction EH3.4.4.3

Residue from fraction EH3.4.4.3 was obtained in 37.9 mg as a while needles. The result from silica gel TLC using a mixture of hexane and chloroform in (1:9) as an eluent showed only one spot. The spot gave yellow spot by anisaldehyde-sulfuric acid **(Figure 4.12)** spraying reagent, suggested that this compound can be a member of phenolic, sugar, steroid or terpene group.

The ^{1}H-NMR (500 MHz, $CDCl_3$) **(Figure A.13-A16)** showed peaks at showed an oxymethine proton at δ 3.57-3.47 (*m*), three olefinic protons at δ 5.36-5.34 (*dd, J* = 8.4, 15.1 Hz) and 5.01 (*dd, J* = 8.4, 15.1 Hz). The singlets at δ_H 0.65 (*s*) and 0.99 (*s*) and at 0.90 (*d, J* = 6.6), 0.83 (*t, J* = 7.3), 0.82 (*d, J* = 6.8) and 0.79 (*d, J* = 6.8).

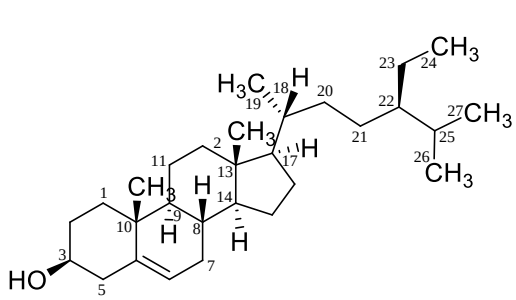
The ^{13}C (125 MHz, $CDCl_3$) showed peaks at δ 37.2 (C-1'), 39.5 (C-2'), 71.7 (C-3'), 42.2 (C-4'), 140.7 (C-5'), 121.6 (C-6'), 31.8 (C-7'), 31.8 (C-8'), 50.0 (C-9'), 36.4 (C-10'), 21.0 (C-11'), 39.7 (C-12'), 42.2 (C-13'), 56.7 (C-14'), 25.9 (C-15'), 28.2 (C-16'), 55.9 (C-17'), 36.1 (C-19'), 18.9(C-20'), 33.8 (C-21'), 24.2 (C-22'), 45.7

(C-23’), 23.0 (C-24’), 11.9 (C-25’), 29.0 (C-26’), 19.8 (C-27’), 19.3 (C-28’), 18.7 (C-29’), 11.8 (C-30’) **(Figure A17-A.19)**.

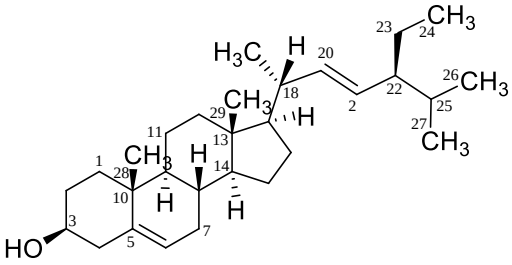
FT-IR spectrum displayed several important peaks at absorption band of hydroxyl group (O-H) at 3422 cm^{-1}, the absorption band of C-H stretching sharp peak at 2937 and 2862 cm^{-1} and carbon carbon double peak showed the absorption band of at 1462 cm^{-1} **(FigureA.12)**.

By comparison these data with the previously reported data **(Table 4.6)** demonstrated that residue in fraction EH3.4.4.3 was a mixture of *β*-sitosterol **(2a)** and stigmasterol **(2b)** (Thongdeeying, 2005).

In order to purify *β*-amyrin out from fixed oil and to separate *β*-sitosterol and stigmasterol out of stigmasterol further chromatographic reparations are needed. However, due to lack of time in this study, these mixture were continued studying for their inhibitory activities without further purification.



2a



2b

Figure 4.16: Structures of *β*-sitosterol **(2a)** and stigmasterol **(2b)**

Table 4.6: Comparison NMR spectral data of *β*-sitosterol and stigmasterol with previously reported Chaturvedula and Prakash, (2012)

Position	**Chemical shift of *β*-sitosterol**			
	Compound (2a)		***β*-sitosterol Chaturvedula and Prakash, (2012)**	
	δ_H (ppm)	**δ_C (ppm)**	**δ_H (ppm)**	**δ_C (ppm)**
1		37.2		37.5
2		39.5		31.9
3	3.50 (*tdd*, 4.8, 4.6, 4.4)	71.7	3.53 (*tdd*, 4.5, 4.2, 3.8)	72.0
4		42.2		42.5
5	5.33 (*dd*, 5.3, 2.2)	140.7	5.36 (*t*, 6.4)	140.9
6		121.6		121.9
7		31.8		32.1
8		31.8		32.1
9		50.0		50.3
10		36.4		36.7
11		21.0		21.3
12		39.7		39.9
13		42.2		42.6
14		56.7		56.9
15		25.9		26.3
16		28.2		28.5
17		55.9		56.3
18		36.1		36.3
19	0.90 (*d*, 6.6)	18.9	0.93 (*d*, 6.5)	19.2
20		33.8		34.2
21		24.2		26.3
22		45.7		46.1
23		23.0		23.3

Table 4.6: Comparison NMR spectral data of *β*-sitosterol and stigmasterol with previously reported data Chaturvedula and Prakash, (2012) (Cont.)

Position	Chemical shift of *β*-sitosterol			
	Compound (2a)		*β*-sitosterol Chaturvedula and Prakash, (2012)	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
24	0.83 (*t*, 7.3)	11.9	0.84 (*d*, 7.2)	12.2
25		29.0		29.4
26	0.82 (*d*, 6.8)	19.8	0.83 (*d*, 6.4)	20.1
27	0.79 (*d*, 6.8)	19.3	0.81 (*d*, 6.4)	19.6
28	0.65 (*s*)	18.7	0.68 (*s*)	19.0
29	0.99 (*s*)	11.8	1.01 (*s*)	12.0

Note: This spectrum indicated that the mixture contains about 3% of stigmasterol based on NMR spectrum according to the integral data of signals of at δ 5.12 *dd*, 5.13, 8.79, (H-12) and 4.99 *dd*, 15.13, 8.79 (H-20) whereas, carbon spectrum showed at 138.3 (C-20) and 129.2 (C-21), respectively.

4.6. Determination of IC_{50} of *α*-glucosidase and *α*-amylase of ethanol crude extract of *E. elatior* rhizome

IC_{50} is the half of maximal inhibitory concentration by determination of 50% inhibition of enzyme activity in a range of dose-response curve. IC_{50} was obtained by varying concentration of the sample (inhibitor) solutions from low to high and used for the inhibition against the test enzymes. The ethanol crude extract of *E. elatior* rhizome, exhibited IC_{50} of *α*-glucosidase 1.22 µg/mL which much better than that of acarbose, 139.85 µg/mL **(Figure 4.17)**. From the previous reports, (Elya et al., 2011; Mun'im et al., 2013) IC_{50} of *α*-glucosidase inhibition of acarbose was 117.20 µg/mL, hence IC_{50} of acarbose obtained from this study was not significantly different from the previous study. The result indicated that the ethanol crude extract of *E. elatior* rhizome have more potent in *α*-glucosidase inhibition than acarbose. Therefore, the

ethanol extract of *E. elatior* could be used to inhibit the glucose production from carbohydrate food to reduce the glucose level in diabetic patients. Moreover, α-amylase of crude ethanol extract showed the IC_{50} of 68.13 µg/mL compared that of acarbose, IC_{50} 5.67 µg/mL. This result indicated that the activity of crude ethanol extract on α-amylase inhibition was lower than that of acarbose. This result is also supported by the IC_{50} of previously reported of acarbose by Ye and workers in 2010, which indicated that IC_{50} of acarbose was 40 µg/mL. Therefore, the crude ethanol was not a potent α-amylase inhibitor, however, it still can be used to work together with α-glucosidase inhibition activity to reduce the blood glucose level in diabetic patients. The inhibitory activity of both enzymes by the crude ethanol extract of *E. elatior* rhizome could be due to it's phytochemical compositions including tannin, phenolic, flavonoid and terpenoid. However, the biomarker need to be a further investigated.

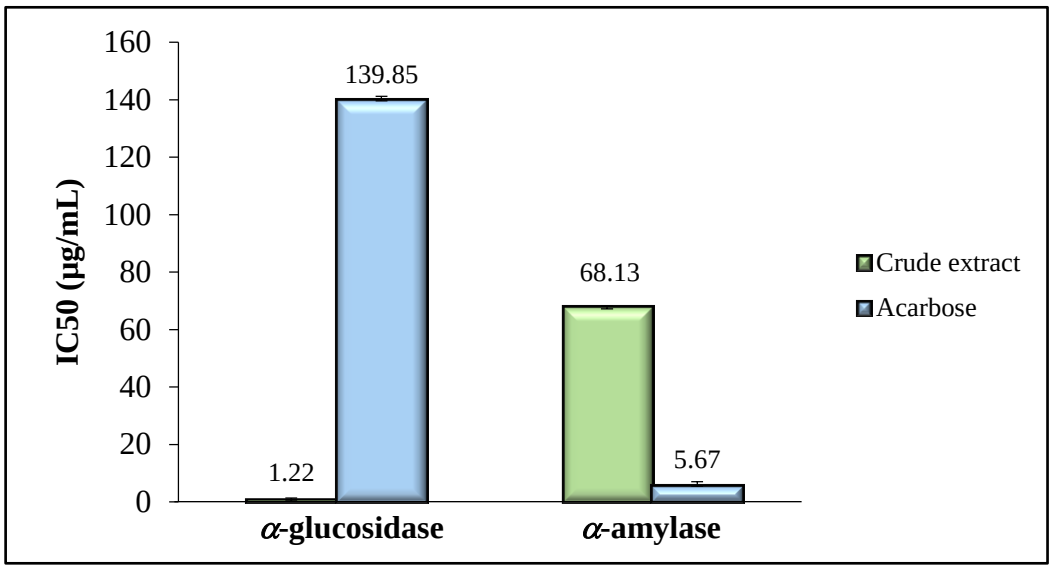


Figure 4.17: IC_{50} (µg/mL) of α-glucosidase and α-amylase of *E. elatior* crude ethanol extract and acarbose

4.7. Determination IC_{50} of α-glucosidase and α-amylase of residues from EH2.3.3.8.3

A mixture of β-amyrin (1a) and fixed oil (1b) in EH2.3.3.8.3 fraction and a mixture of β-sitosterol (2a) and sitgmasterol (2b) in EH3.4.4.3 fraction were subjected for their inhibitory activities against α-glucosidase and α-amylase enzymes by using similar method for crude extracts testing and compare the result with acarbose as a positive control. A mixture of β-amyrin and fixed oild showed better α-glucosidase inhibitory activities (IC_{50} 12.11 µg/mL) than the mixture of β-sitosterol and stigmasterol (IC_{50} 193.5 µg/mL) while that of acarbose had IC_{50} 139.85 µg/mL. The α-amylase inhibitory activity of the mixture of 1a and 1b was 43.29 µg/mL but no estimation for that of the mixture of 2a and 2b, whereas that of acarbose was, IC_{50} 5.67 µg/mL **(Table 4.7)**.

Table 4.7: IC_{50} of α-glucosidase and α-amylase of compounds compared with acarbose

Compounds	IC_{50} (µg/mL)	
	α-glucosidase	**α-amylase**
β-amyrin and fixed oil	12.11±5.00	43.29±6.14
β-sitosterol and sitgmasterol	193.5±2.56	N/A
Acarbose	139.85±1.33	5.67±0.26

N/A: Values was not estimated.

4.8. Mode of inhibition of α-glucosidase of *E. elatior* ethanol extract

Mode of enzyme inhibition can be determined by enzyme kinetic study. Enzyme kinetic is related to the rate of reaction mechanism between enzymes and substrates. Mode of enzyme inhibition of *E. elatior* rhizome crude ethanol extract against both α-glucosidase and α-amylase were analyzed and compared with that of acarbose. In order to find mode of inhibitor of crude ethanol extract against α-

glucosidase, different concentrations of α-glucosidase substrates (p-NPG) were prepared (0.125, 0.25, 0.5, 0.75, 1.0 and 1.5 mM) and used in the inhibition study. The sample of crude ethanol extract at concentration of 3 µg/mL (about 2-time of its IC_{50}) was used in the testing whereas acarbose at the concentration of 500 µg/mL (about 3-time of its IC_{50}) was utilized. The study was performed in comparison with the enzyme that have no inhibitor. Rate of the inhibition reaction was achieved by incubate of sample of inhibitor with enzyme in the presence of various concentration of substrate. The measurement was performed at 405 nm to determine absorbance values of the product produced by the cleavage of p-NPG by α-glucosidase at the selected time interval at 2, 4, 6, 10, 15 and 20 mn. The amount of *p*-nitrophenol that was produced from the reaction was then calculated from the standard curve of *p*-nitrophenol **(Figure 4.18)** and expressed in mM values.

The rates of the reaction were obtained by plotting the amount of *p*-nitrophenol which was produced at the selected time against time. The slope was the rate of raction [V] expressed in mM/min value **(Table 4.8)**. Mode of enzyme inhibition was obtained by plotting the values of 1/[V] and 1/[S] from data of control (no inhibitor) and sample (as inhibitor) **(Figure 4.19 and 4.20)**.

The results demonstrated that mode of α-glucosidase inhibition of crude ethanol extract of *E. elatior* rhizome was in competitive mode whereas of acarbose was in non-competitive mode **(Figure 4.19 and 4.21)**. In competitive mode of inhibition by crude ethanol extract of *E. elatior* rhizome indicted that the active phytochemical component would bind toe the active site of the enzyme and compete with the enzyme substrate, therefore, the enzyme will not be able to break down disaccharide to monosaccharide and hence reducing blood glucose level. In non-competitive mode of inhibition by acarbose revealed that acarbose will not compete with the substrate for binding to the active site.

These results are similar to that previously reported by McDougall et al., (2005) that the polyphenol of crude acetone extract of *Cassia abbreviata* also showed α-glucosidase inhibitory activity in a non-competitive manner and acarbose, miglitol, and voglibose showed competitive mode of inhibition of the same enzyme (Matsuda et al., 2002; Shai et al., 2010). Since, crude ethanol extract of *E. elatior* rhizome

contains also phenolic compounds, it is therefore, possible that the phenolic components may play important roles in this inhibitor activity.

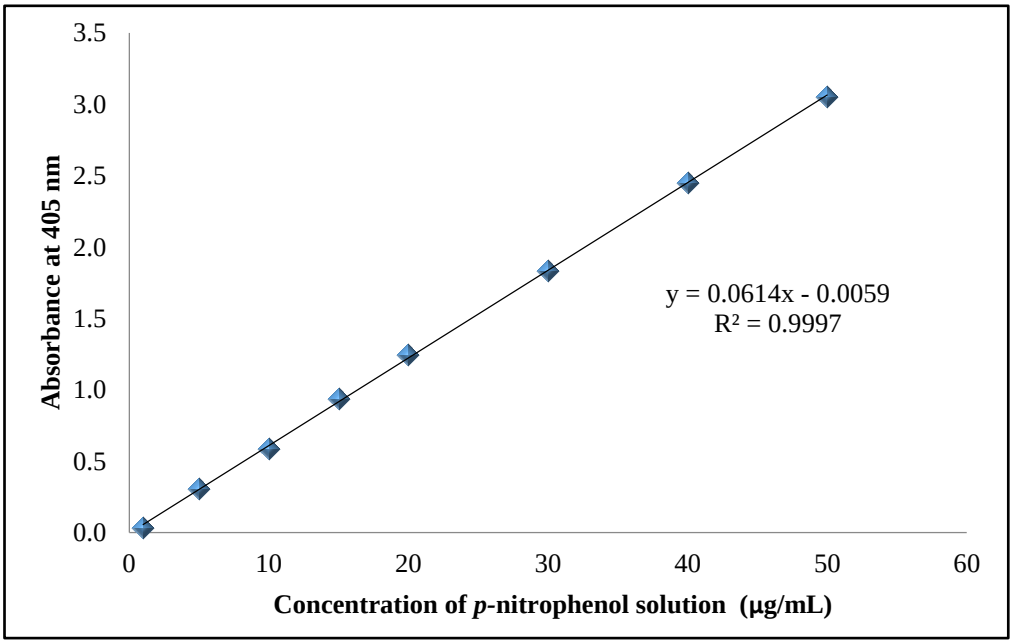


Figure 4.18: Calibration curve of *p*-nitrophenol solutions (concentration= 1-50 µg/mL) the product from the enzymatic cleavage p-NPG by gucosidase

Table 4.8: Slope values of the linearity part of the graph plotted between amount of *p*-Nitrophenol produced (mM) and time (min)

Concentration of p-NPG (mM) [S]	**Slope [V] of the linearity part of the graph plotted between amount of *p*-nitrophenol produced mM and time (min)**		
	No inhibitor	**Crude ethanol extract 3 at µg/mL**	**Acarbose at 500 µg/mL**
0.125	0.27±0.04	0.15±0.00	0.11±0.00
0.25	0.74±0.01	0.39±0.02	0.23±0.00
0.5	1.19±0.00	0.59±0.00	0.31±0.05
0.75	1.27±0.00	0.78±0.09	0.55±0.02
1	1.71±0.02	0.87±0.06	0.62±0.00

Table 4.9: The 1/[V] values of ethanol crude extract (3µg/mL) and acarbose (500 µg/mL with of various substrates concentrations [S]

1/[S] (mM)$^{-1}$	**1/[V] (mM/min)$^{-1}$**		
	No inhibitor	**Crude ethanol extract at 3 µg/mL**	**Acarbose at 500 µg/mL**
8	3.57	6.55	8.80
4	1.34	2.51	4.33
2	0.83	1.69	3.16
1.33	0.78	1.27	1.80
1	0.58	1.14	1.60

[V] : is initial velocity of enzymatic reaction

[S] : is substrate concentration

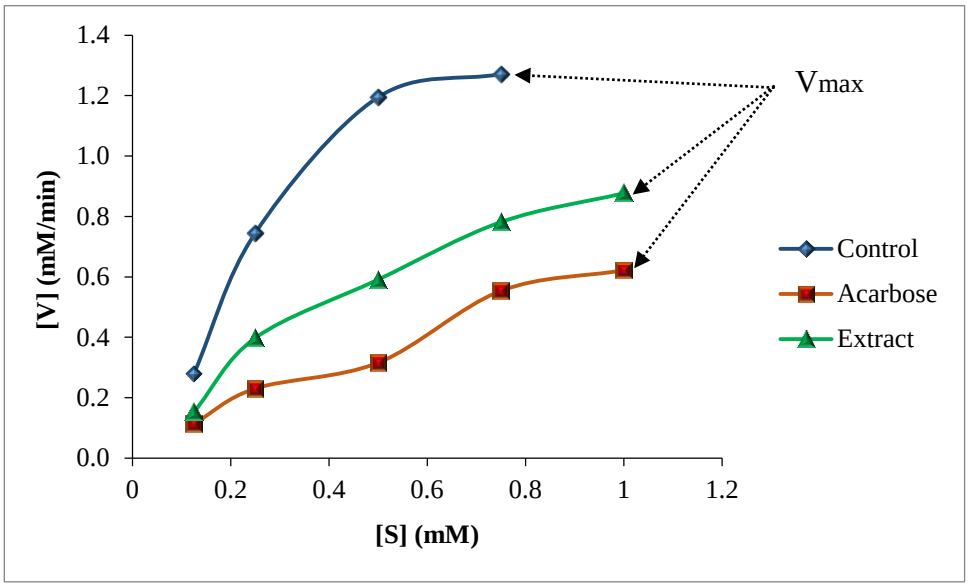


Figure 4.19: Michalis-Menten plot of α-glucosidase inhibitory activity of *E. elatior* rhizome extract (3 µg/mL) and control (no inhibitor) against concentration [S] of p-NPG

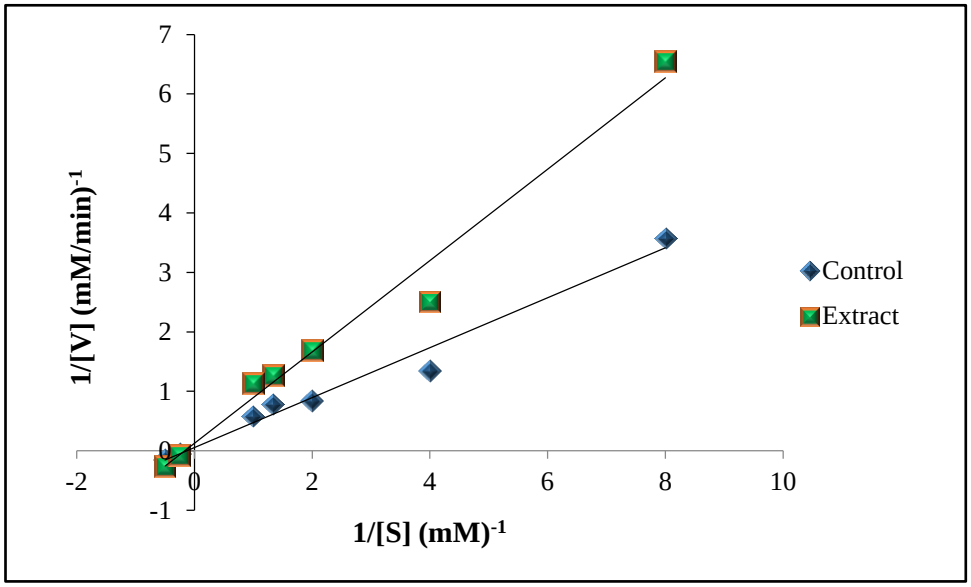


Figure 4.20: Lineweaver-Burk plot of α-glucosidase inhibition by ethanol extract of *E. elatior* rhizome plant extract at 3 µg/mL and without plant extract (control,

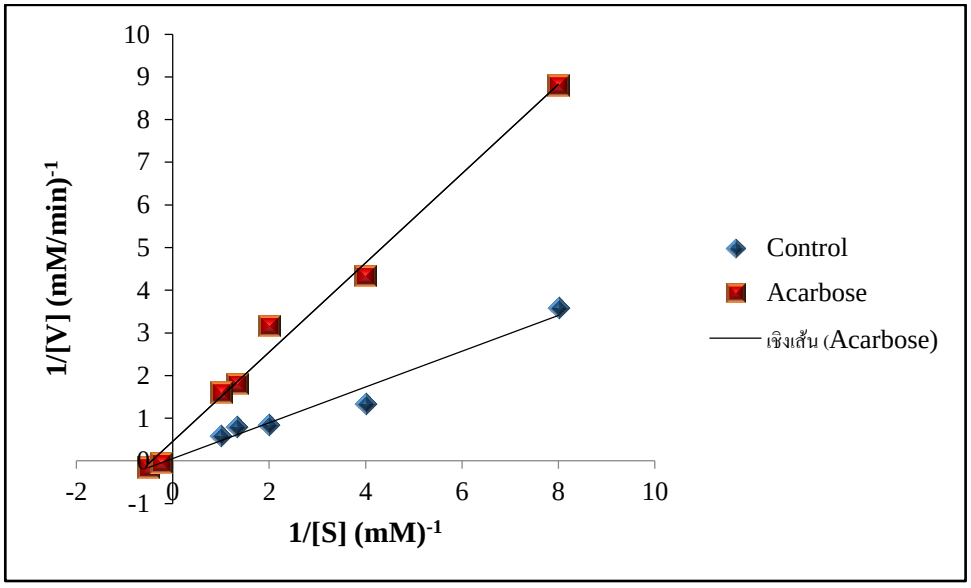


Figure 4.21: Lineweaver-Burk plot of α-glucosidase inhibition of acarbose at 500 µg/mL and without acarbose (control,

It is known that, long term diabetes is usually associated with many types of oxidative stress (Vincent et al., 2004). In diabetic patients, oxidative stress may causes insulin resistance, *β*-cell dysfunction, impaired glucose tolerance and also is related with macrovascular and microvascular complications (Banerjee and Vats, 2014). All of these problems will make diabetes more complicate. It is normally known that herbal extracts always present antioxidant activity, in order to investigate for the ability of *E. elatior* rhizome in antioxidant capacity, two types of antioxidant testing were performed including DPPH radical scavenging assay and ferric reducing antioxidant power (FRAP).

4.9. DPPH radical scavenging assay of the plant extracts of *E. elatior* rhizome

DPPH is a common method that has been used in general to test antioxidant capacity of test samples. In the study, DPPH radical was first generated in situ and provide purple solution. If the sample has antioxidant capacity, it will scavenge DPPH radical to the reduced form and the purple color will change to yellow solution **(Figure 4.23 and 4.24)**.

Sample including crude ethanol extract, hexane partition extract, dichloromethane partition extract, ethyl acetate partition extract, *n*-butanol partition extract and water partition extract were subjected to the DPPH radical scavenging assay and compared the result with positive control (ascorbic acid). The results **(Figure 4.22)** demonstrated that all samples have DPPH antioxidant activity but in the different capacities. Ascorbic acid is known to be a good antioxidant and can be seen from the lowest IC_{50} (4.22 µg/mL).

Ethanol crude extract showed moderate antioxidant activity with IC_{50}= 134.27 µg/mL. However, after the crude ethanol was partitioned by different polarity solvents, the partitioned extracts gave obviously different antioxidant activity. The best antioxidant was found in the EtOAc extract with IC_{50}= 12.6 µg/mL. This extract contains tannin, phenolic and flavonoid compounds which may play important role in its antioxidant activity. Dichloromethane and *n*-butanol extracts have similar IC_{50} values in 26.61 and 24.77 µg/mL, respectively. These fractions contain tannin, phenolic, flavonoid and terpenoids compounds which may be important in DPPH

scavenging activity. Water extract, eventhough contains tannin, phenolic, terpenoid and flavonoid component, it did not have good antioxidant activity. This could be due to its phytochemical components are not good proton donator by free radical reaction. It was interesting that hexane part which contains no tannin compound also did not show good antioxidant activity. Therefore, the compounds that are composition in this extract do not have good free radical scavenging activity.

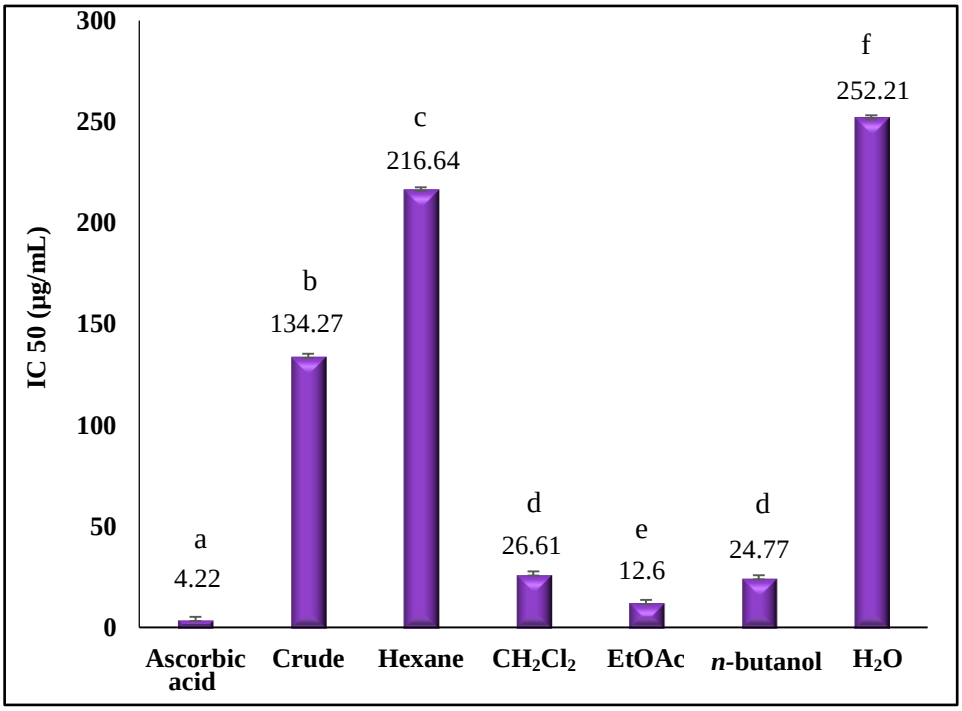


Figure 4.22: IC_{50} values from DPPH radical scavenging assay of crude EtOH extract and other extracts from partition process

The different letters were shown the statistically significant different data.

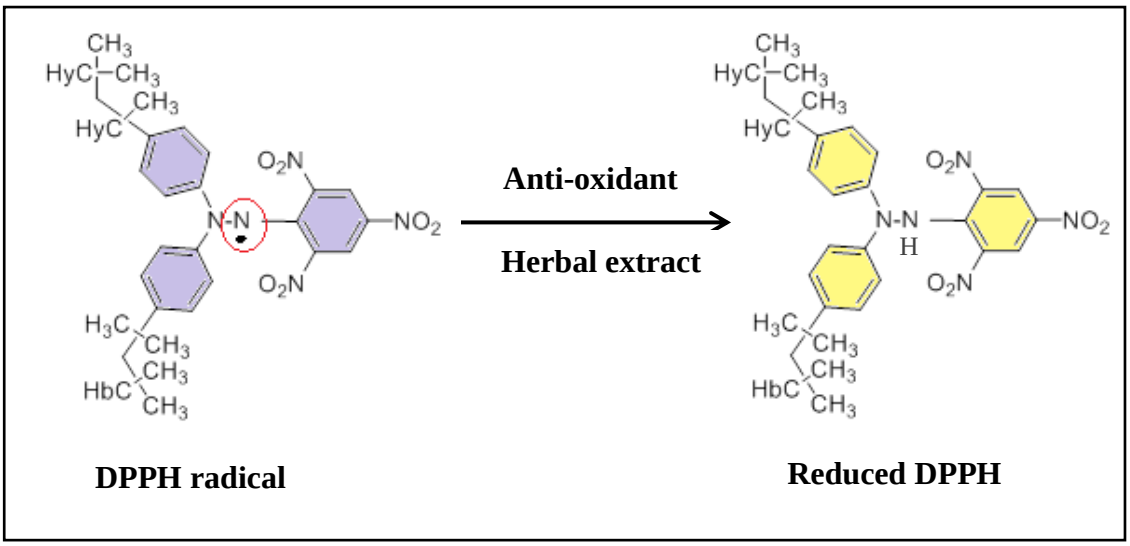


Figure 4.23: Displayed the change in colors of DPPH radical scavenging assay

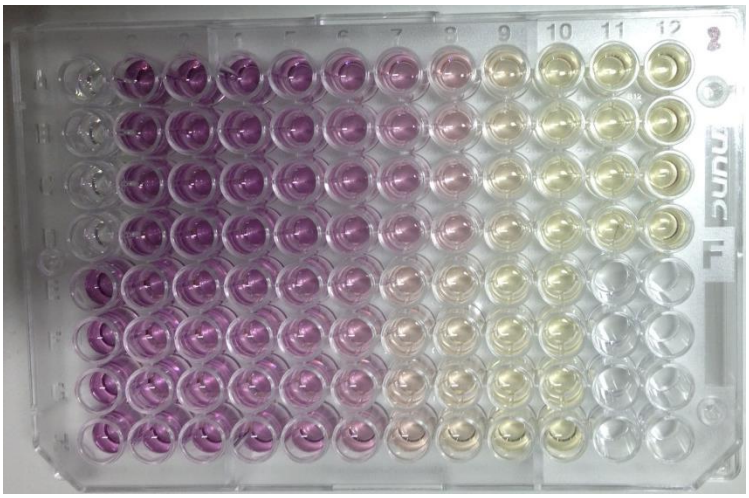


Figure 4.24: Picture of 96 wellplate of DPPH radical scavenging activity testing.

-Purple solution indicated free radical of DPPH presented.

-Yellow solution indicated the DPPH radicals are scavenged by sample.

Another antioxidant testing method was used in this study was ferric reducing antioxidant power (FRAP). In this study, if the test sample have reduction potential, it will react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}). The latter will then reacts with ferric chloride to form ferric potassium ferrocyanide

complex (Prussian blue) which has an absorbance maximum at 700 nm **(Figure 4.25)**. Increase absorbance of the reaction mixture indicates an increase in reduction capacity (Singhal et al., 2011). However, in this study reducing power was reported in quercetin equivalent, therefore, high value of quercetin equivalent indicate high reducing power.

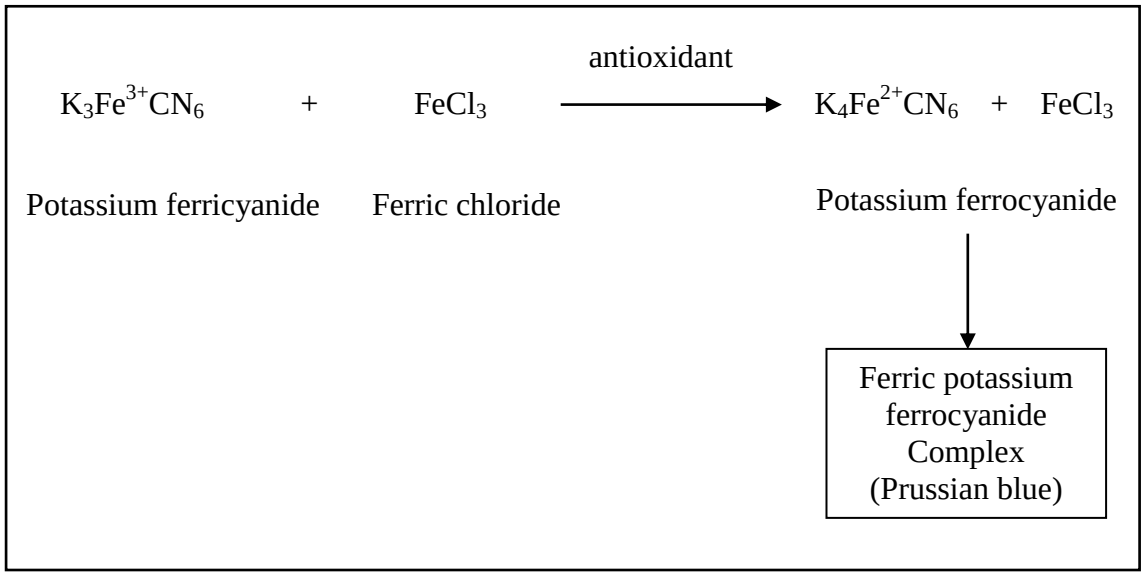


Figure 4.25: Reaction scheme for reducing power testing

The results **(Figure 4.26)** demonstrated that EtOAc extract gave the best reducing power (3.28 quercetin equivalent), followed by dichloromethane extract (2.18 quercetin equivalent), *n*-butanol extract (1.51 quercetin equivalent). Water and hexane extracts gave low reducing power, 0.31 and 0.38 quercetin equivalent, respectively. The obtained results were in accordance with the results of DPPH radical scavenging assay. Crude ethanol extract gave almost the same level of reducing power to that of quercetin, therefore, indicated the potential use of these extracts as supplement as antioxidant in diabetic patients.

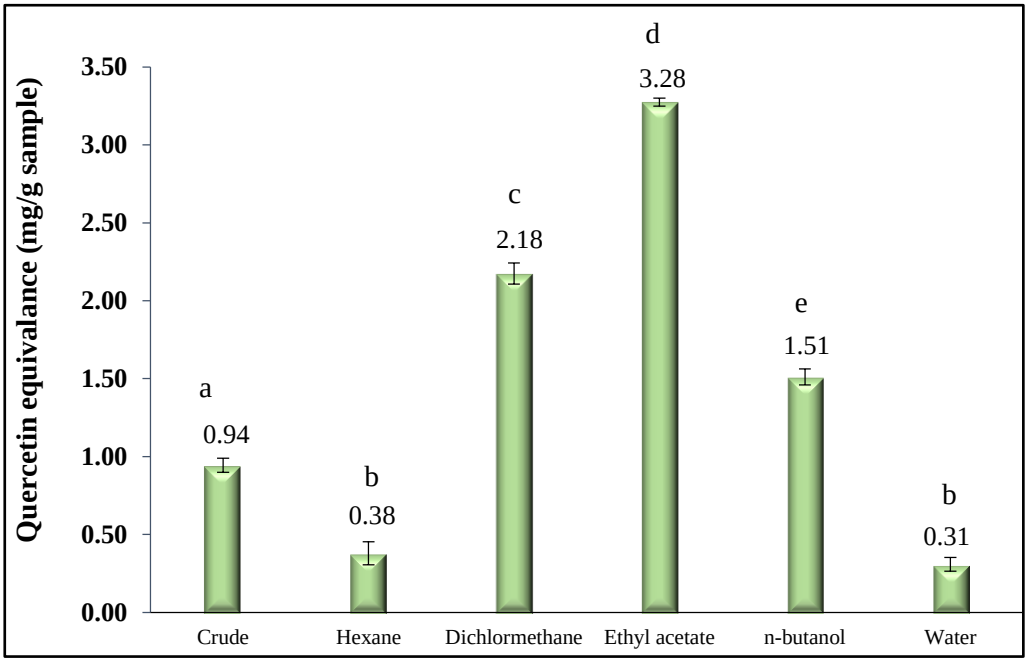


Figure 4.26: Quercetin equivalent from FRAP assay of crude EtOH extract and other extracts from partition

The different letters were shown the statistically significant different data

CHAPTER 5

CONCLUSION

The current study reported antidiabetic activity of various extracts (ethanol, hexane, dichloromethane, ethyl acetate, *n*-butanol, and water) from rhizome of *Etlingera elatior* against α-glucosidase and α-amylase enzymes. Evaluation of the crude ethanol extract (concentration 25 µg/mL) revealed high inhibitory activity against α-glucosidase (95.34%) and α-amylase (45.39%) compared to those of acarbose (11.12% and 43.39%, respectively). However, IC_{50} of ethanol extract against α-glucosidase and for α-amylase were 1.22 µg/mL and 68.13 µg/mL, respectively compared to those of acarbose, 139.85 and 5.67 µg/mL. Moreover, the kinetic study of the crude ethanol extract revealed a competitive inhibition mode against α-glucosidase while that of acarbose was a non competitive inhibition mode.

The separation processes demonstrated that known compounds are obtained, a mixture of β-amyrin and fixed oil and a mixture of β-sitosterol and stigmasterol. First mixtures displayed higher activity and the second mixture showed lower activity than acarbose. Another five pure compounds were also achieved and under investigation for their structure. Furthermore, for antioxidant activity of ethyl acetate, *n*-butanol and dichloromethane extracts demonstrated as good scavengers of DPPH radical with IC_{50} is 12.60, 24.77 and 26.61 µg/mL, respectively as compared to ascorbic acid, a standard having IC_{50} 4.22 µg/mL. Ferric reducing antioxidant power (FRAP) assay showed good antioxidant profile for dichloromethane, *n*-butanol and ethyl acetate extracts reported as quercetin equivalent in 1.51, 2.18 and 3.28 mg quercetin/g sample, respectively. Good antioxidant activity may be due to the presence of tannin, phenolic and flavonoid in these extracts. Therefore, these results could support the potential utilization in of *Etlighera elatior* rhizome in diabetic treatment.

This research is the first report of *Etlingera elatior* rhizome for inhibitory activity against α-glucosidase and α-amylase enzymes, hence it has potential to be developed as anti-diabetic agent. Further research should be explored to find active compounds of α-glucosidase and α-amylase inhibitor to support the biomarker study.

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APPENDIX

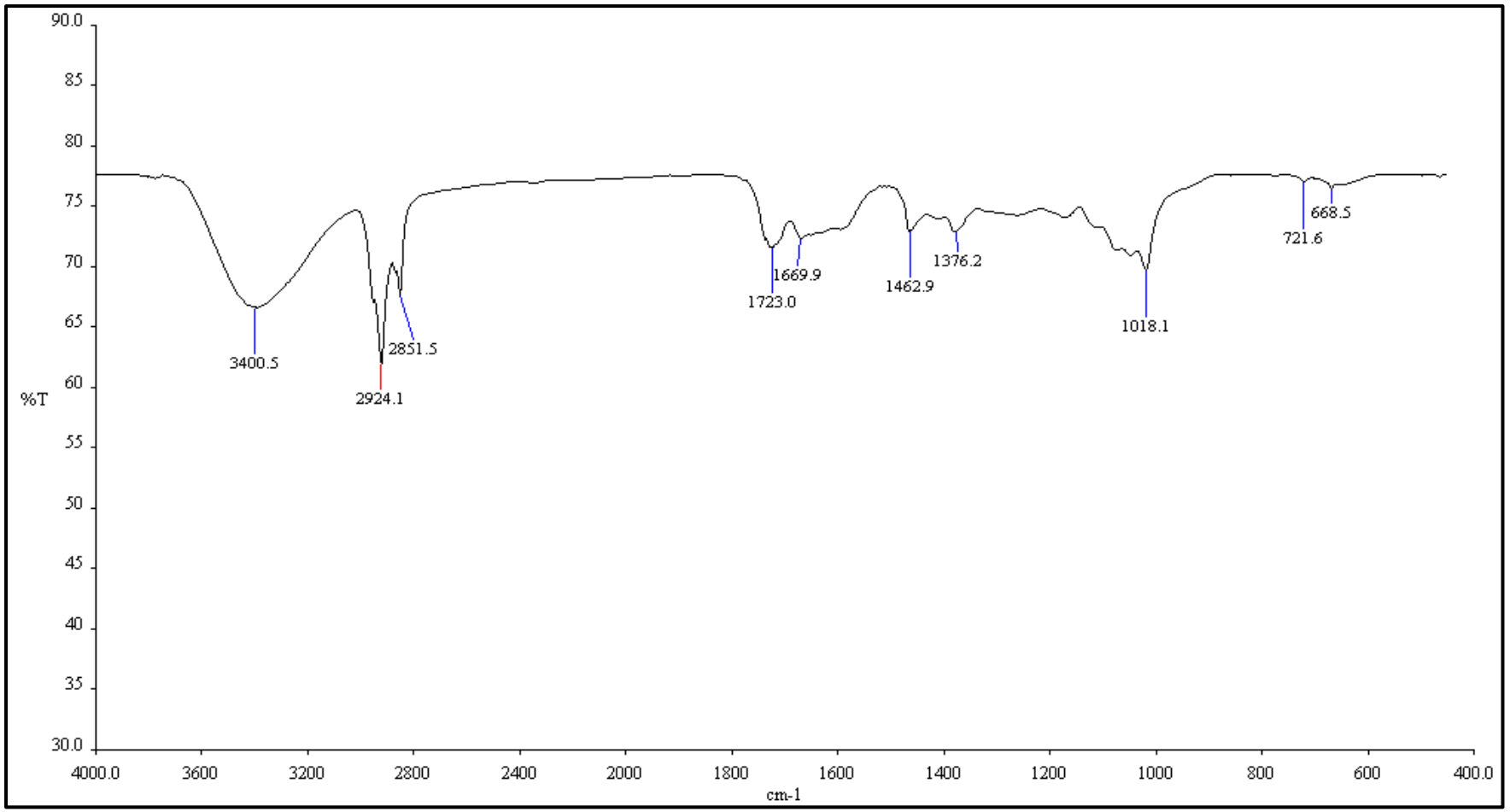

Figure A.1: FT-IR (KBr) Spectrum of a mixture of *β*-amyrin and fixed oil

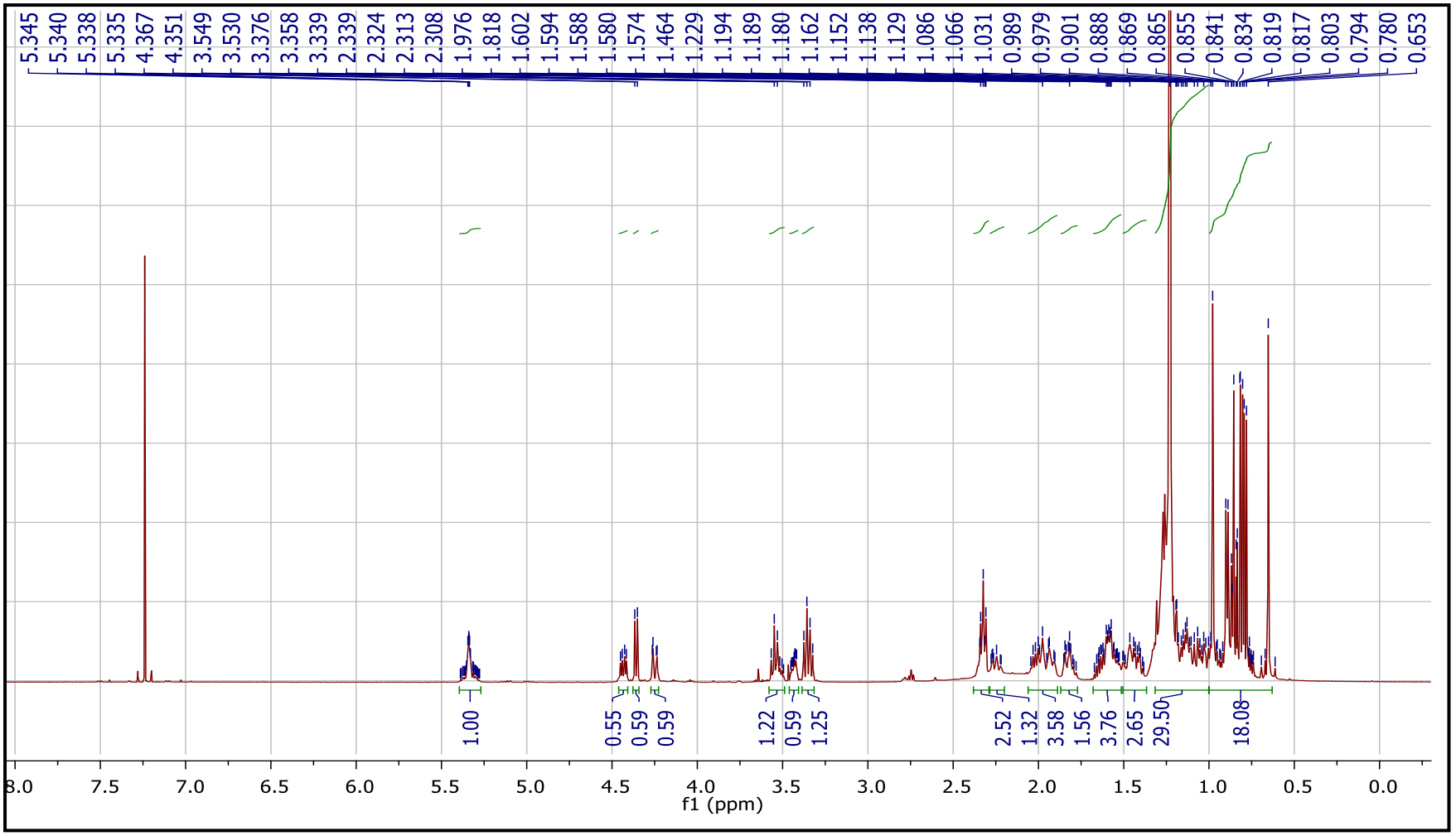

Figure A.2: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of *β*-amyrin and fixed oil

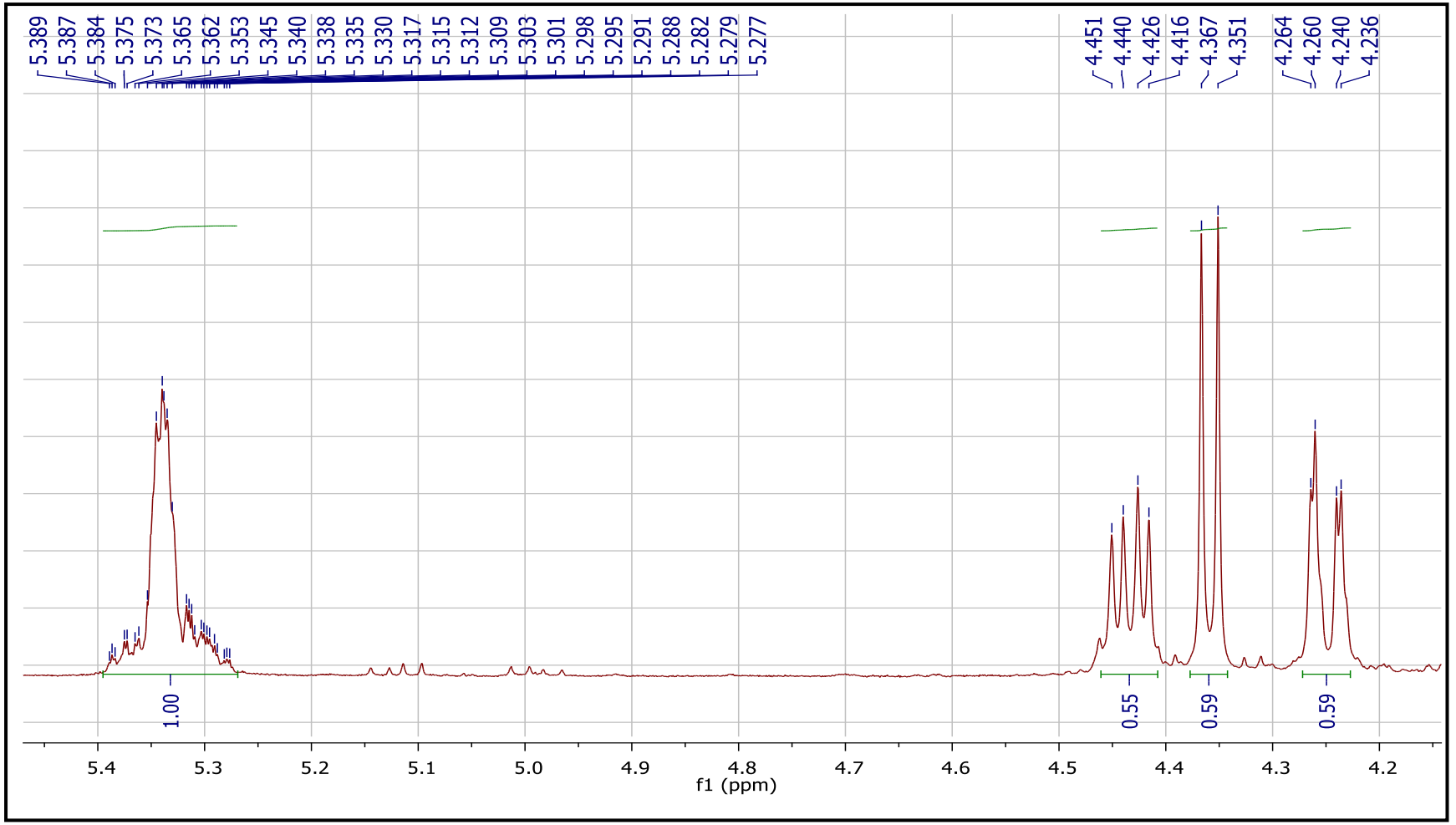

Figure A.3: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of *β*-amyrin and fixed oil (Expansion)

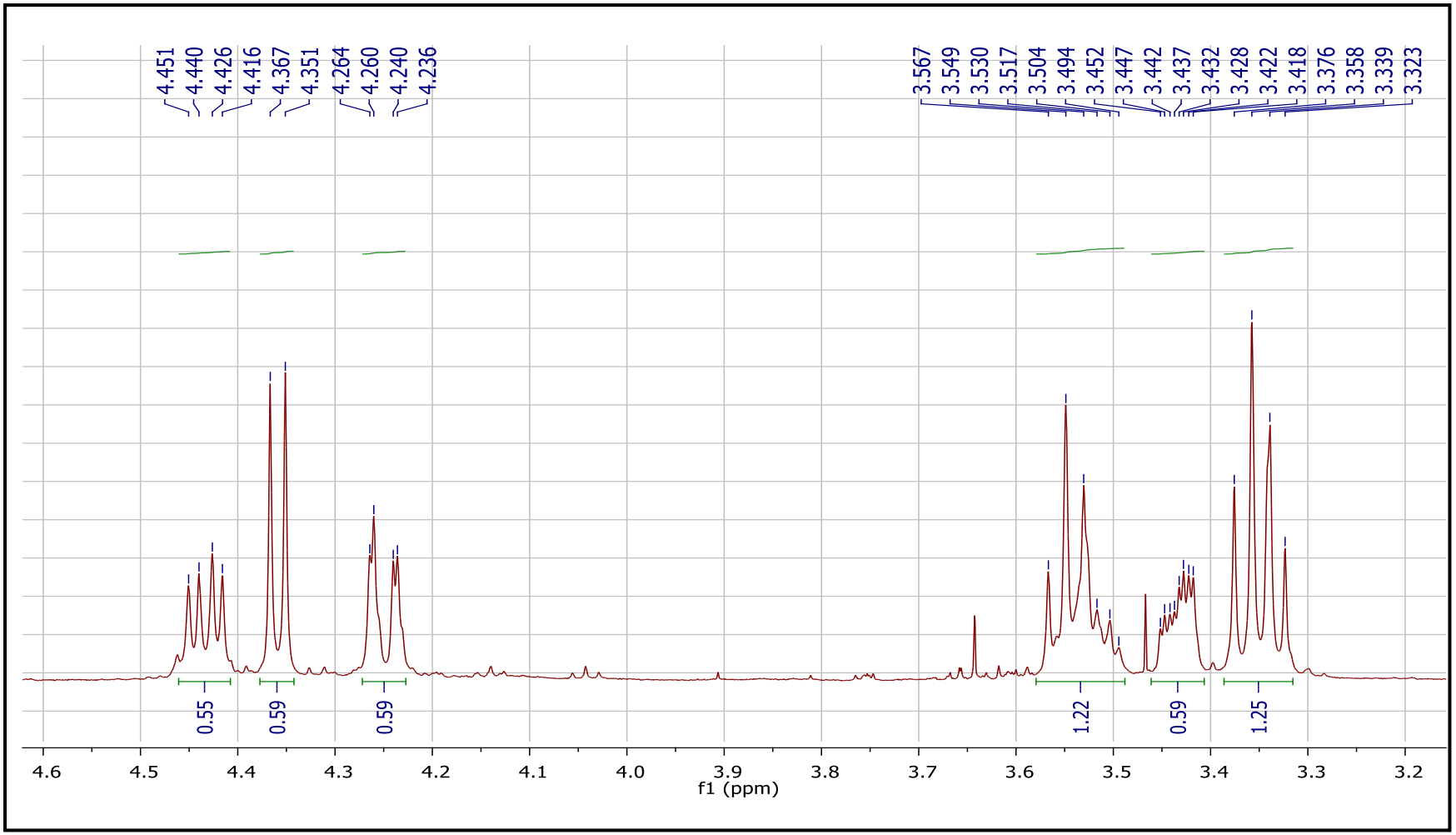

Figure A.4: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of *β*-amyrin and fixed oil (Expansion)

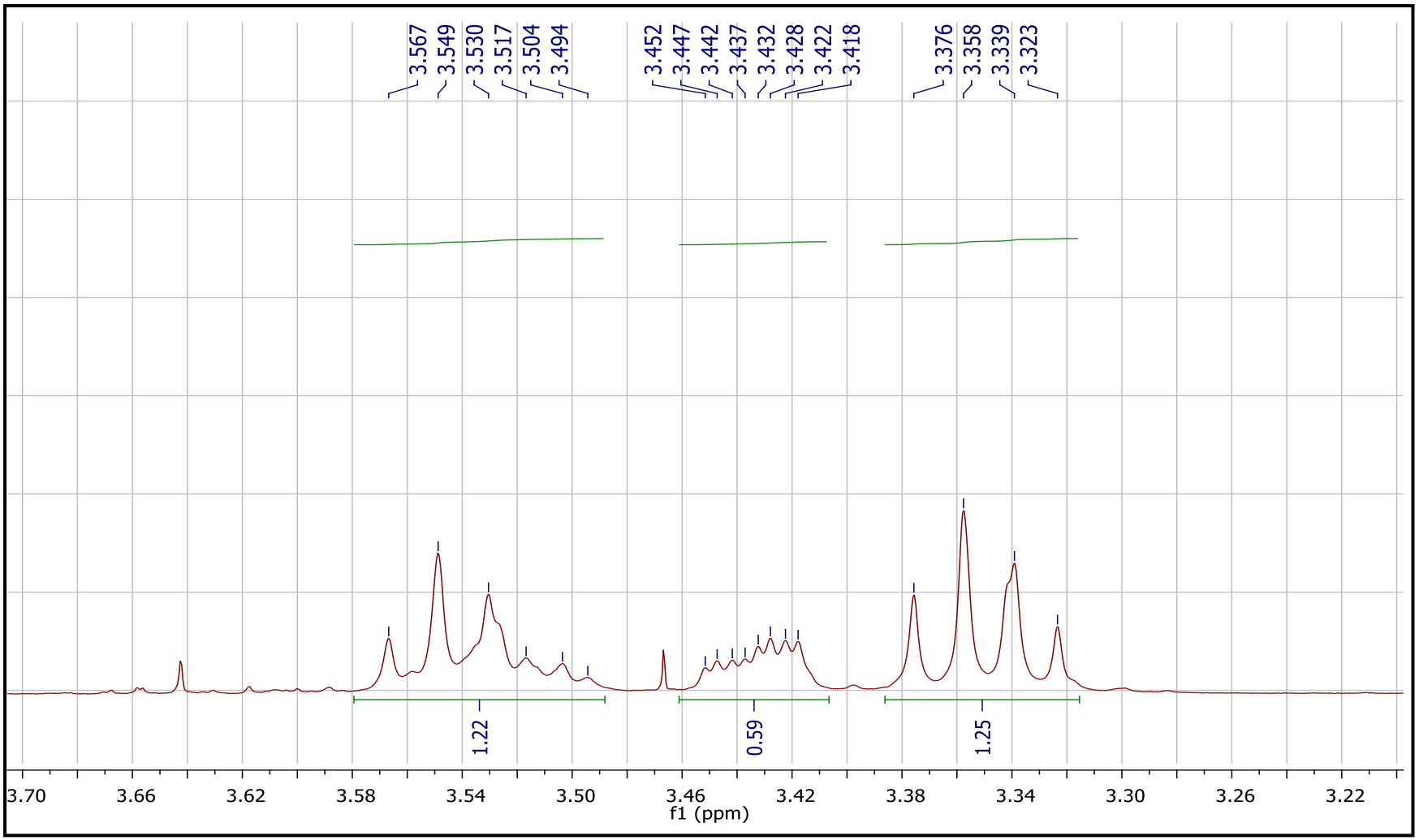

Figure A.5: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of *β*-amyrin and fixed oil (Expansion)

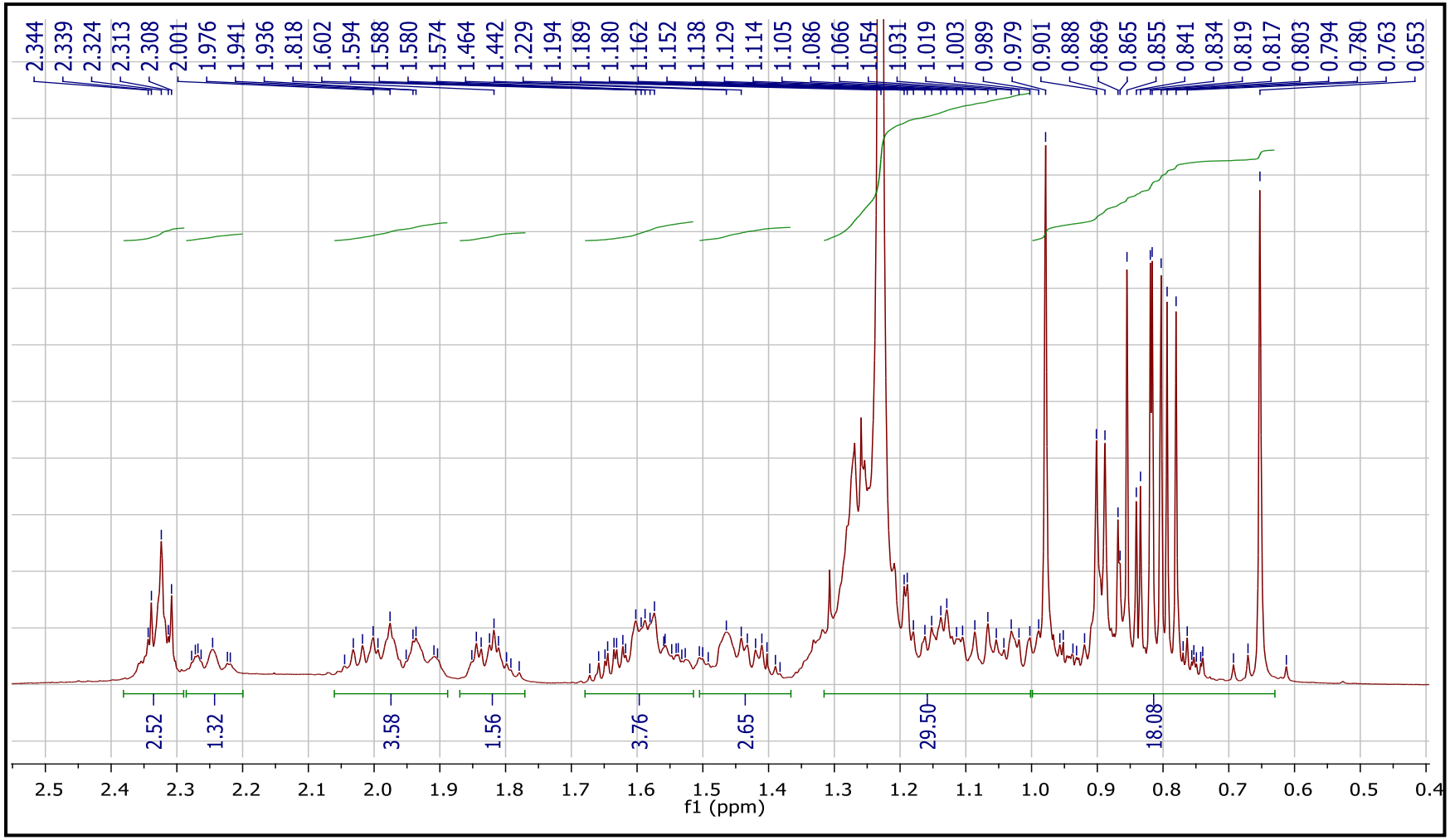

Figure A.6: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of β-amyrin and fixed oil (Expansion)

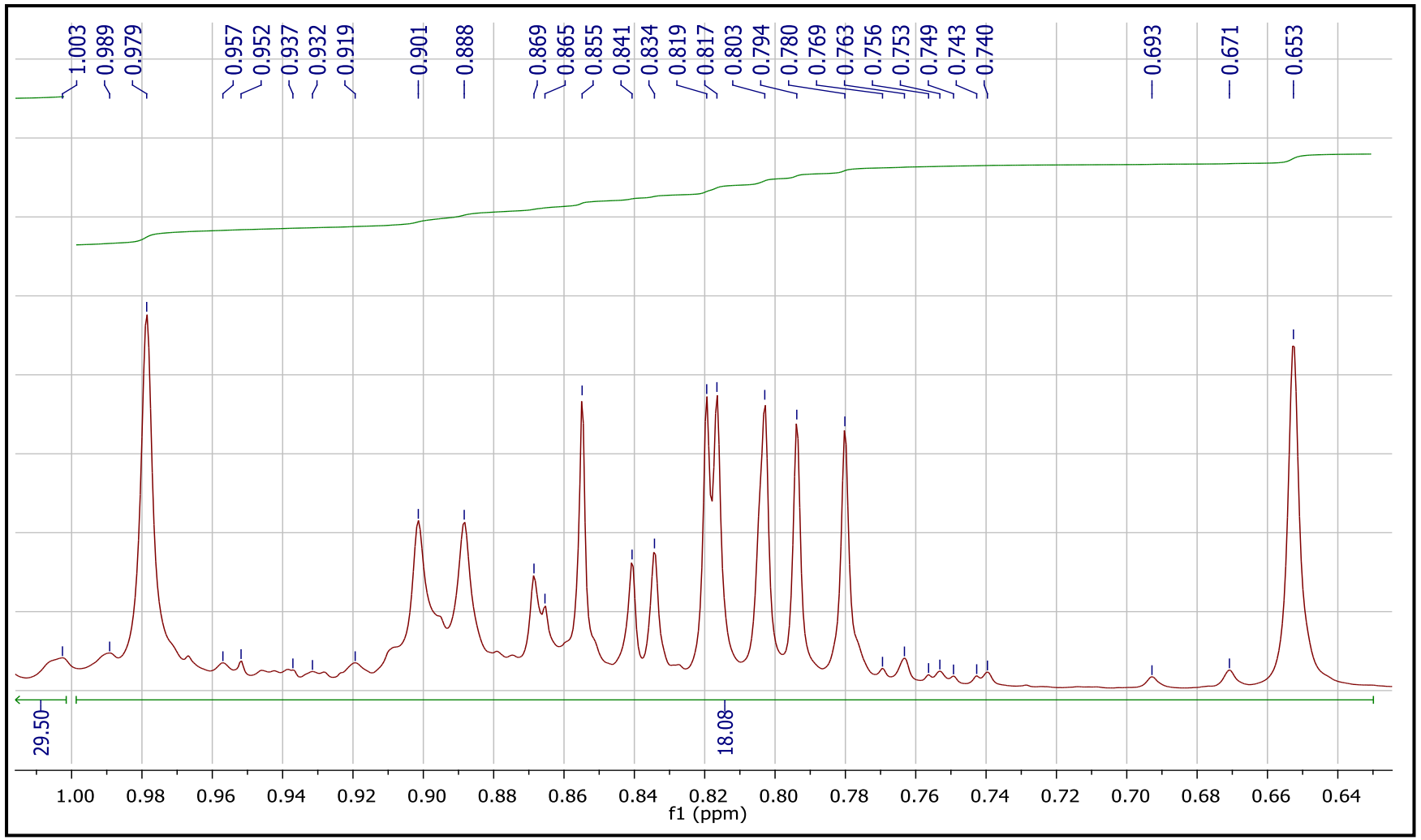

Figure A.7: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of *β*-amyrin and fixed oil (Expansion)

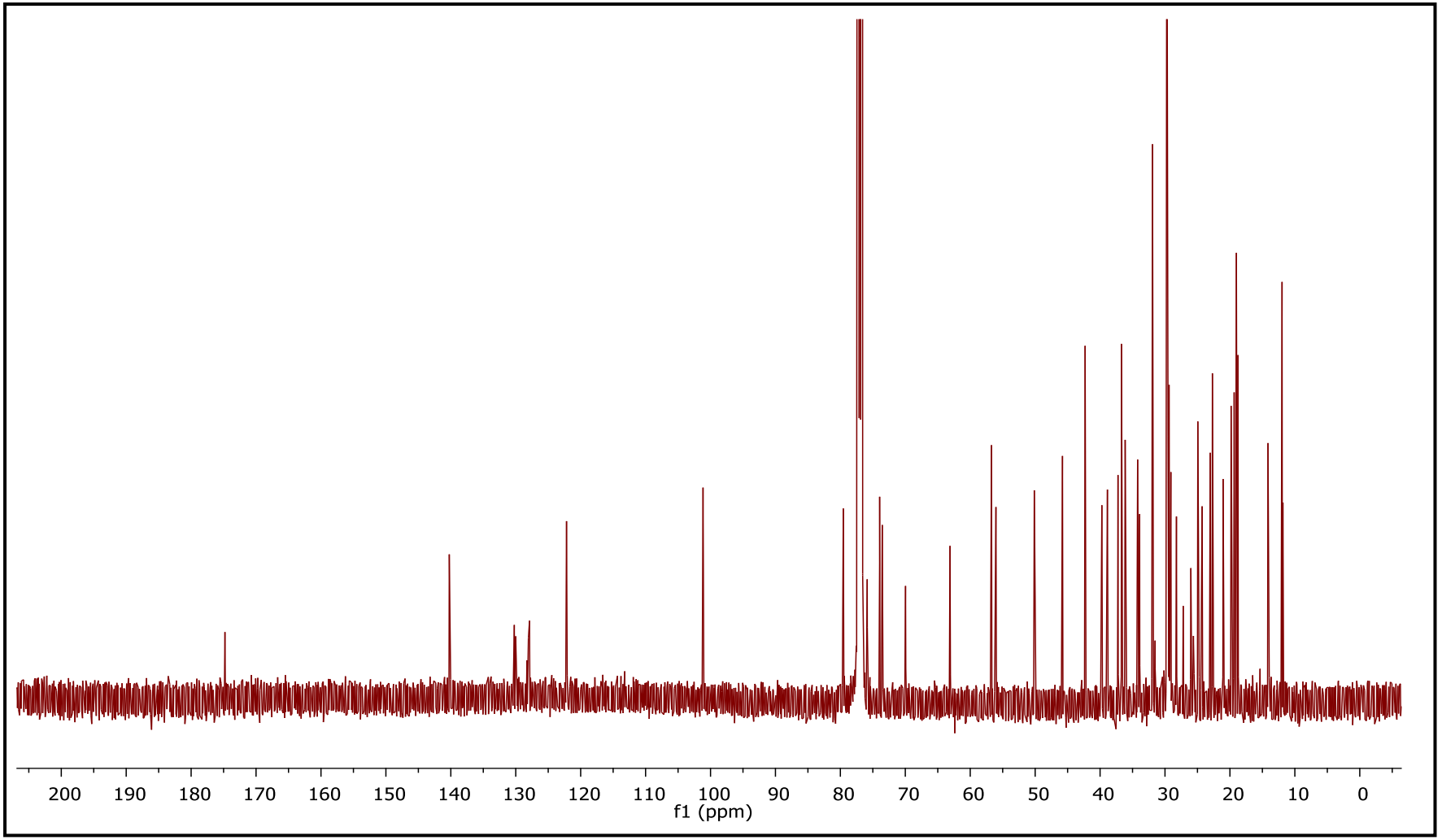

Figure A.8: ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of a mixture of *β*-amyrin and fixed oil

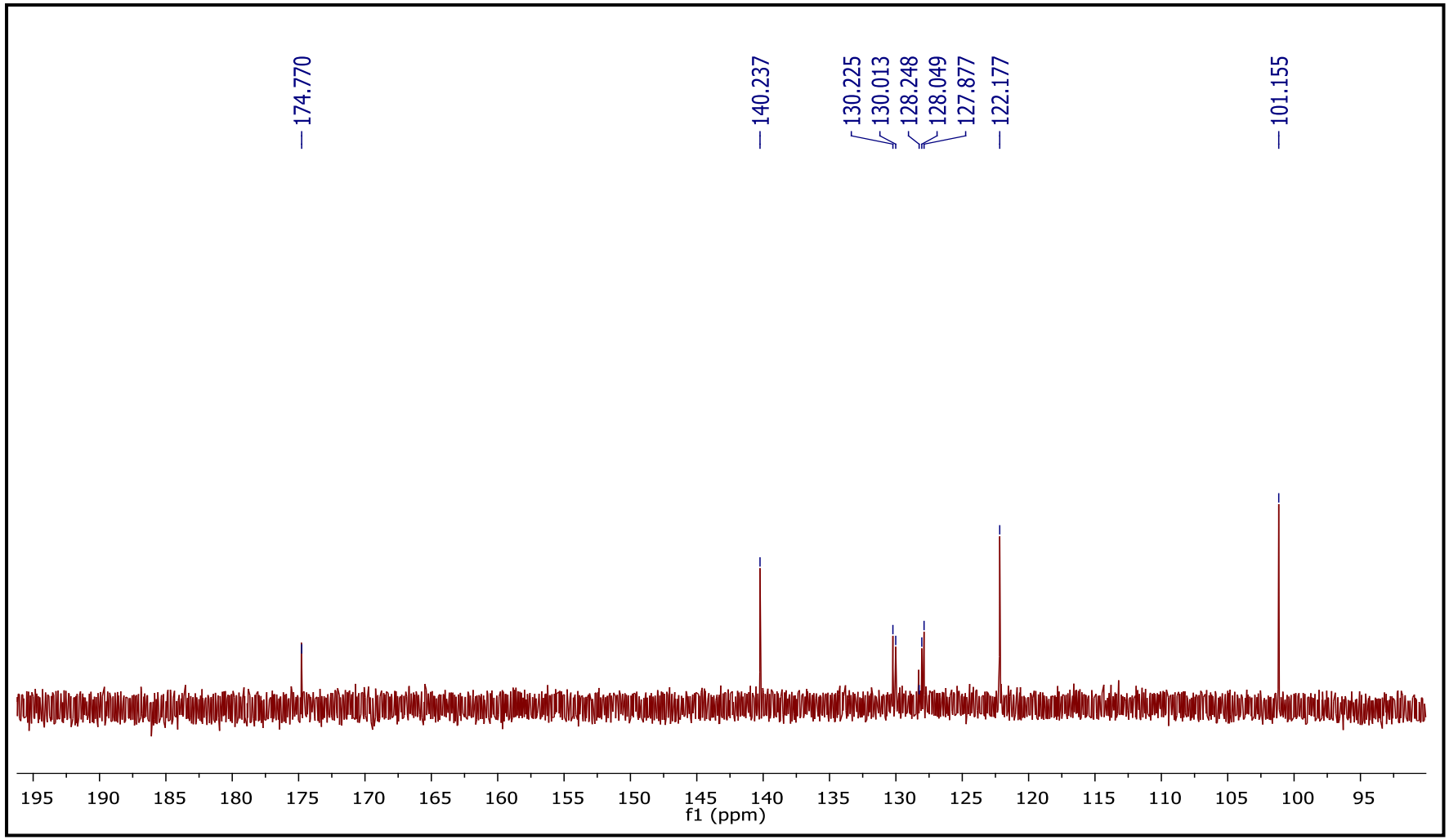

Figure A.9: ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of a mixture of *β*-amyrin and fixed oil (Expansion)

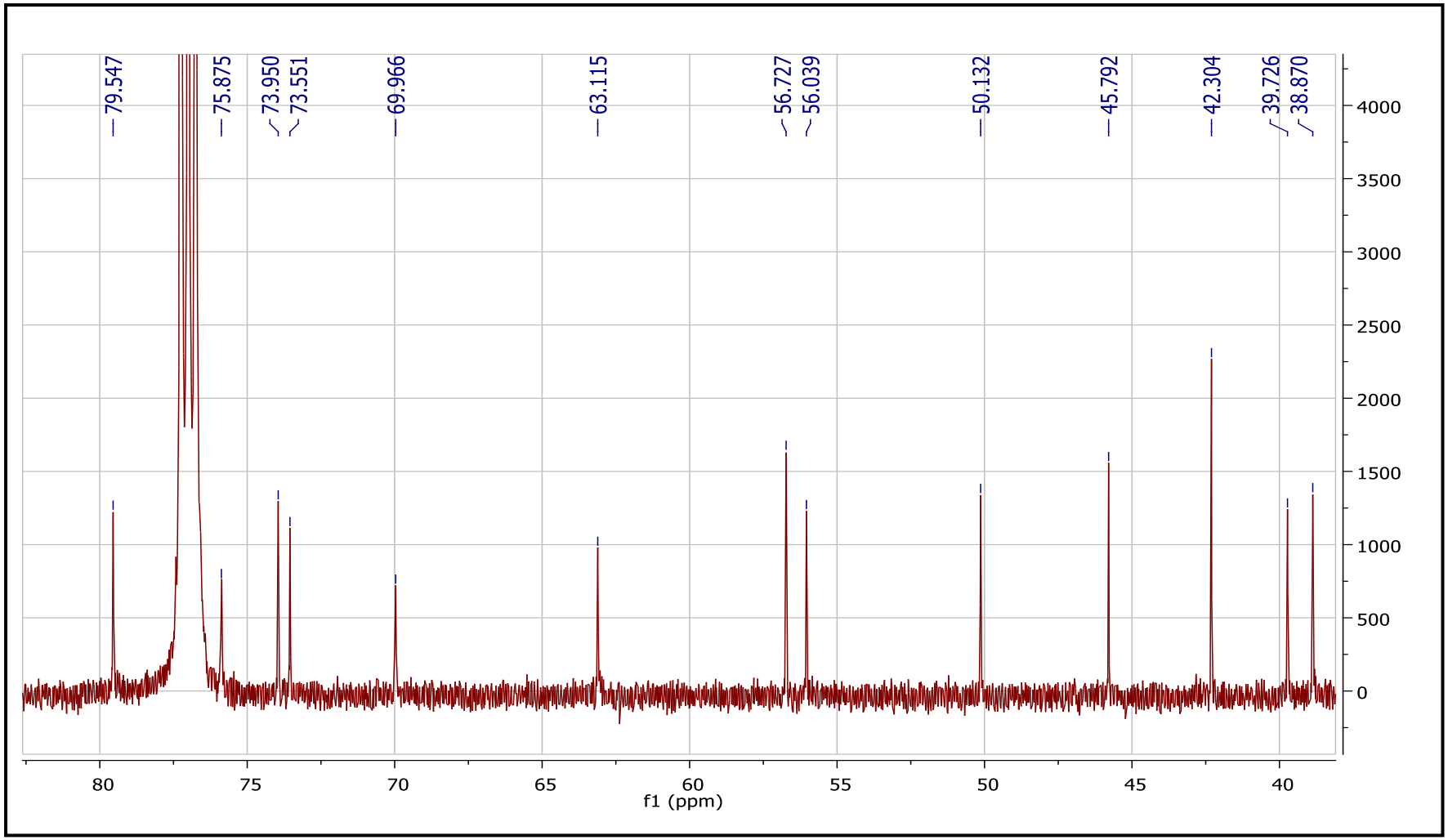

Figure A.10: ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of a mixture of *β*-amyrin and fixed oil (Expansion)

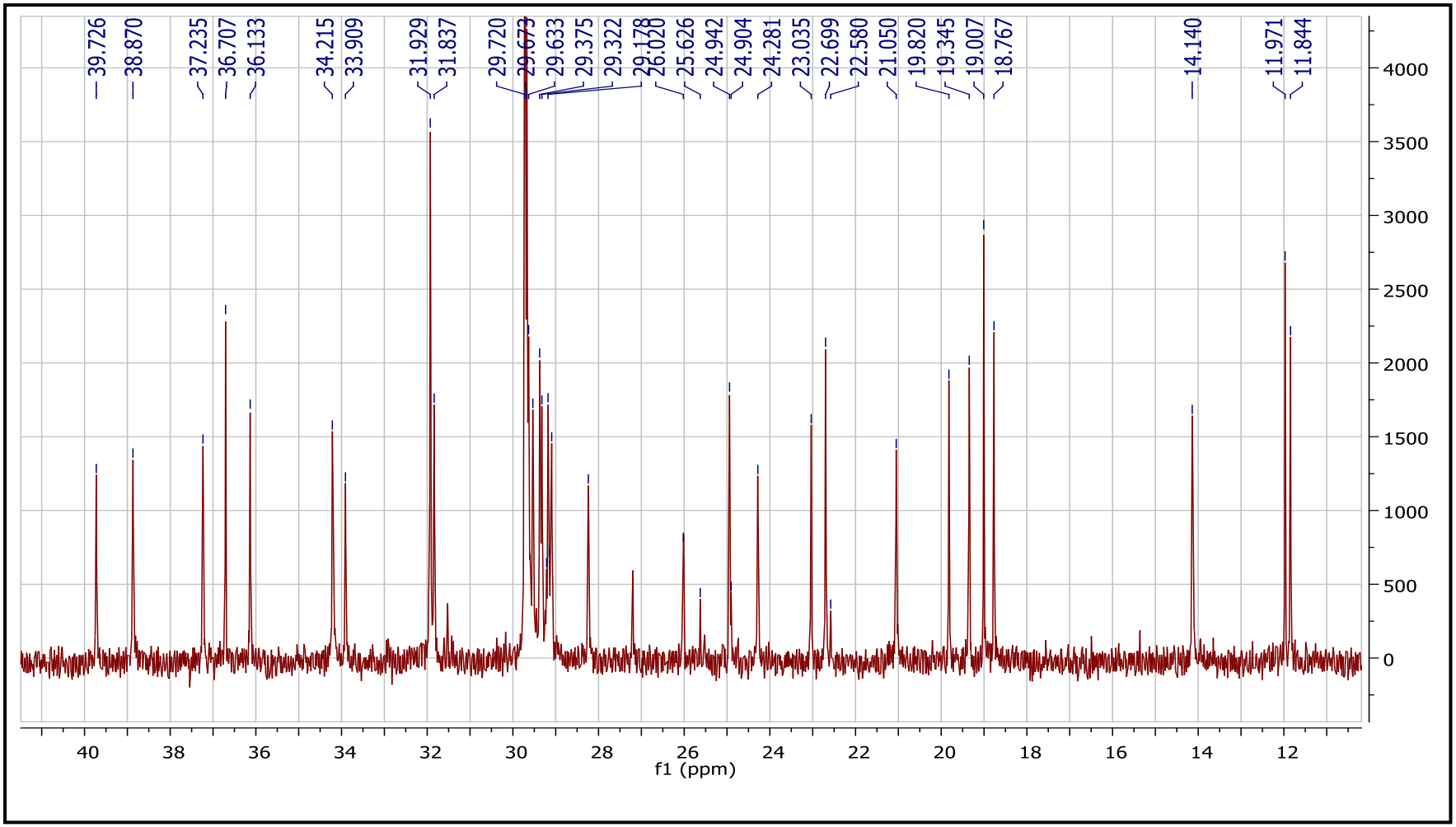

Figure A.11: ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of a mixture of β-amyrin and fixed oil (Expansion)

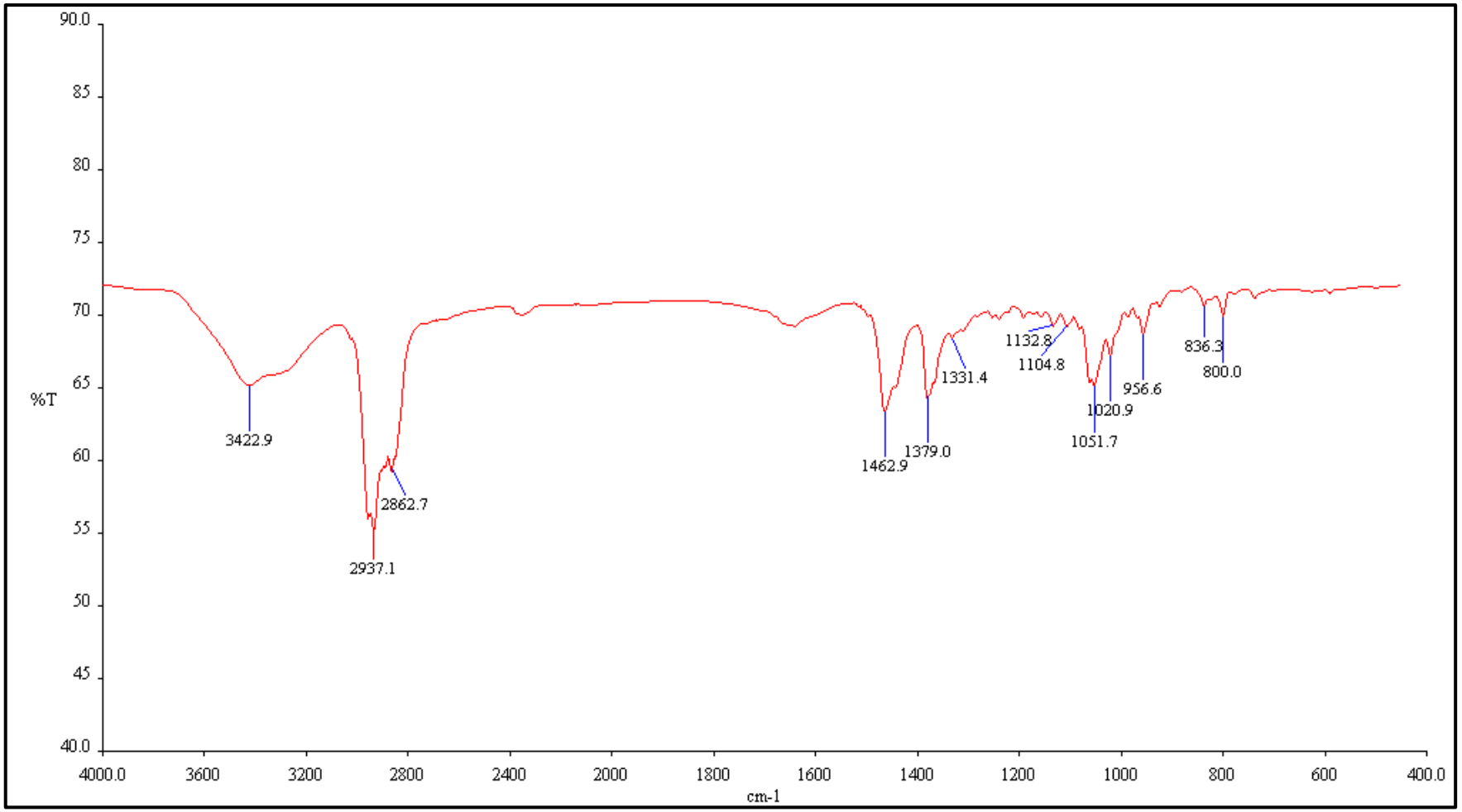

Figure A.12: FT-IR (KBr) Spectrum of a mixture of *β*-sitosterol and stigmasterol

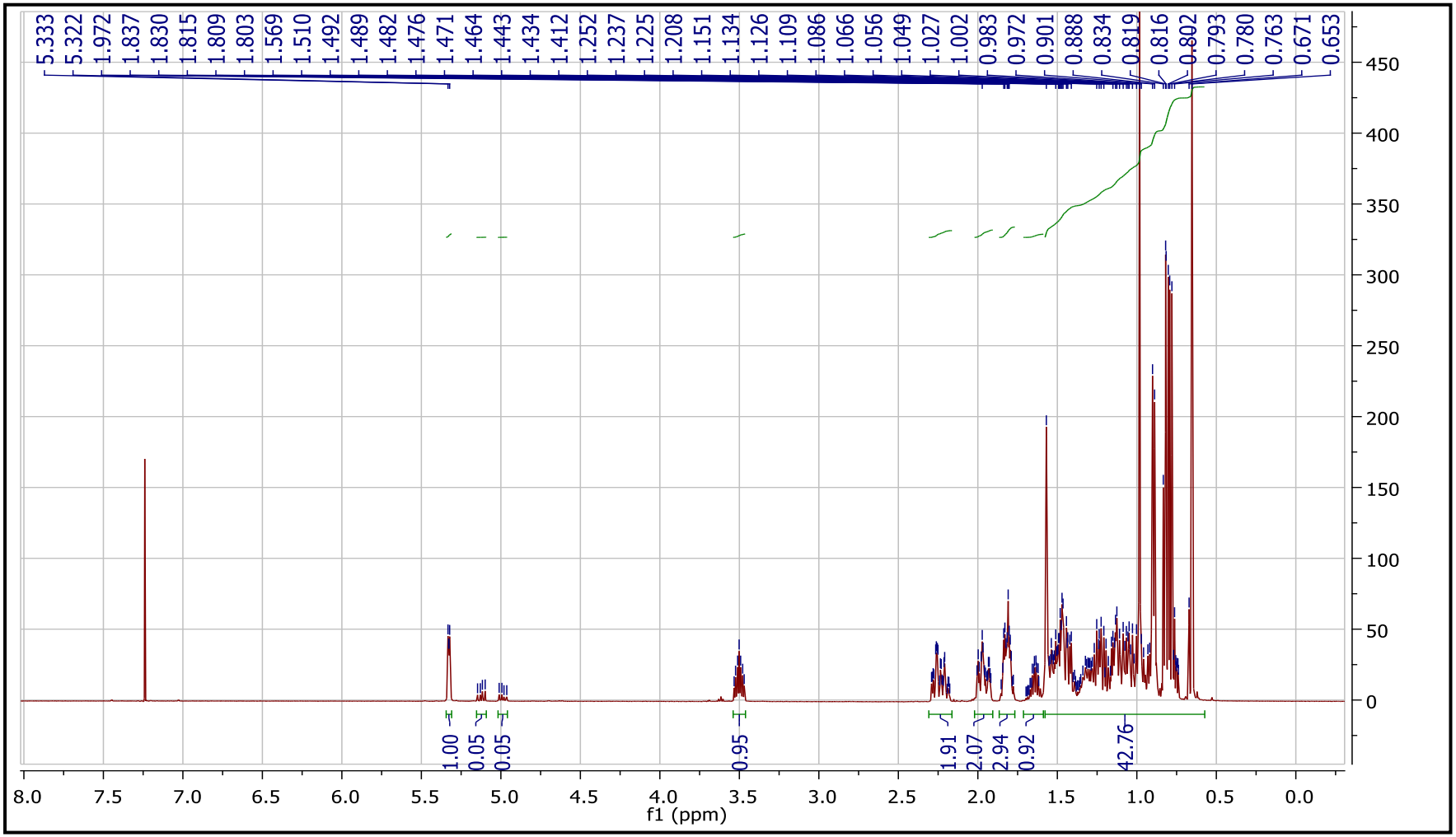

Figure A.13: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of *β*-sitosterol and stigmasterol (Expansion)

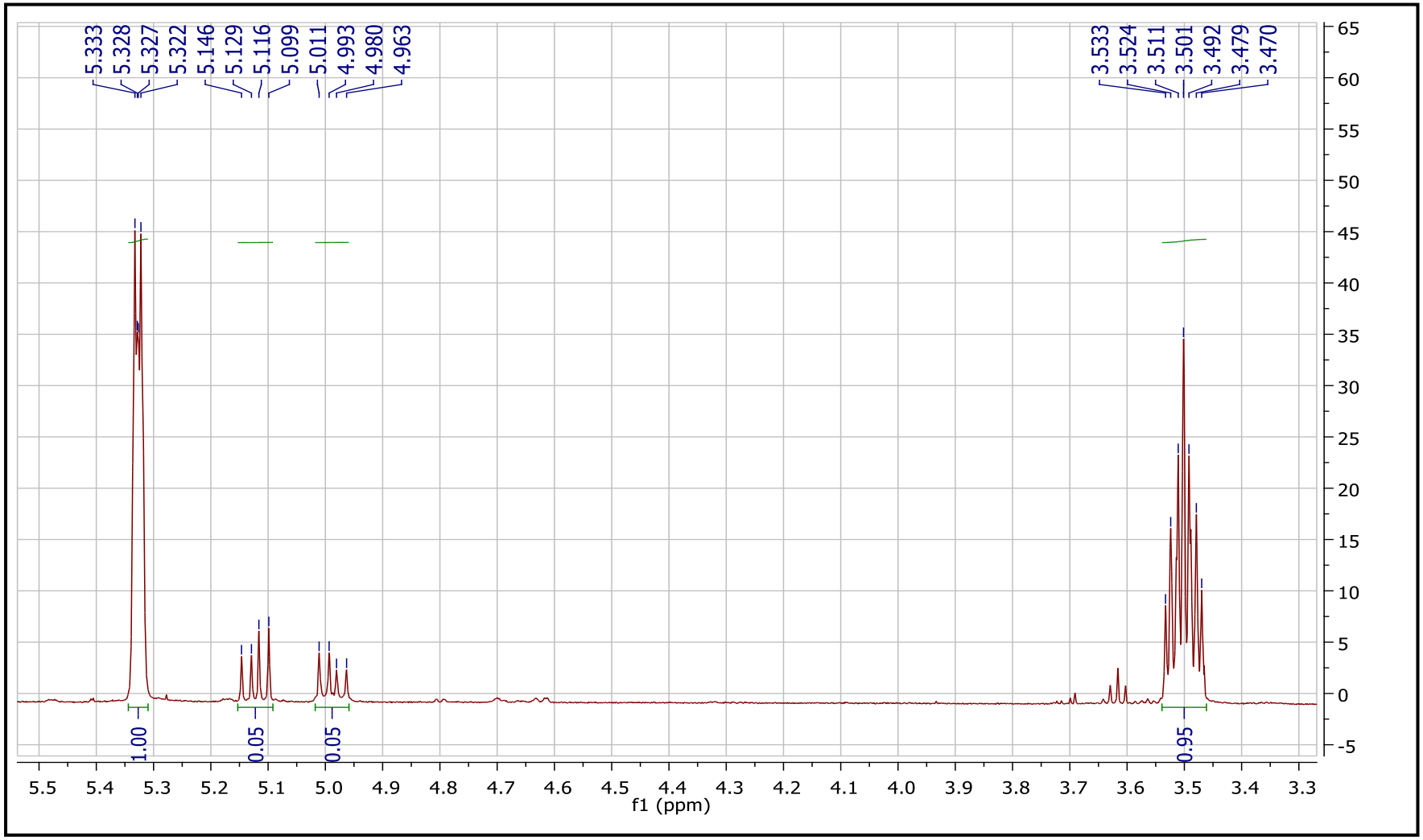

Figure A.14: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of *β*-sitosterol and stigmasterol (Expansion)

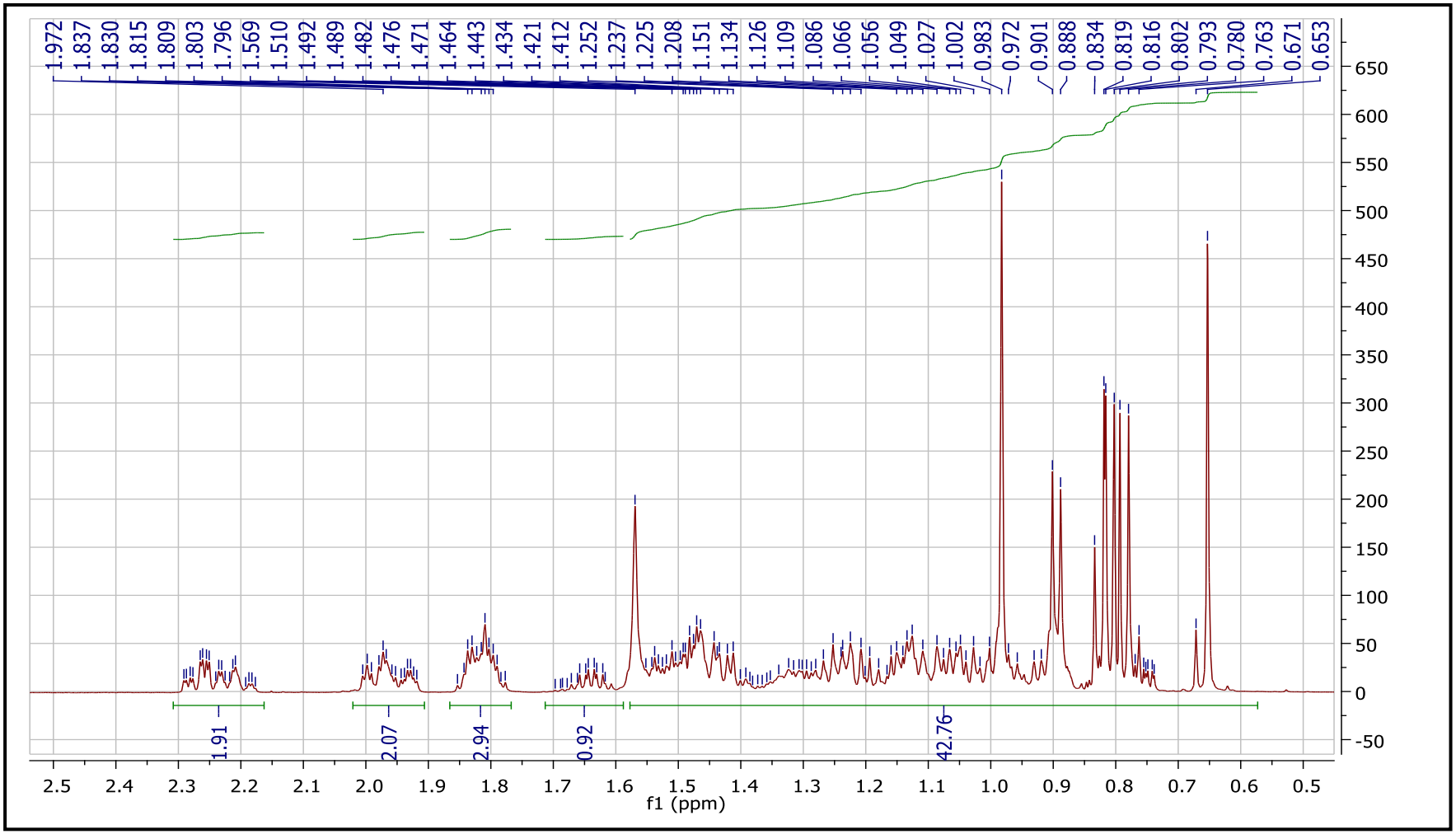

Figure A.15: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of *β*-sitosterol and stigmasterol (Expansion)

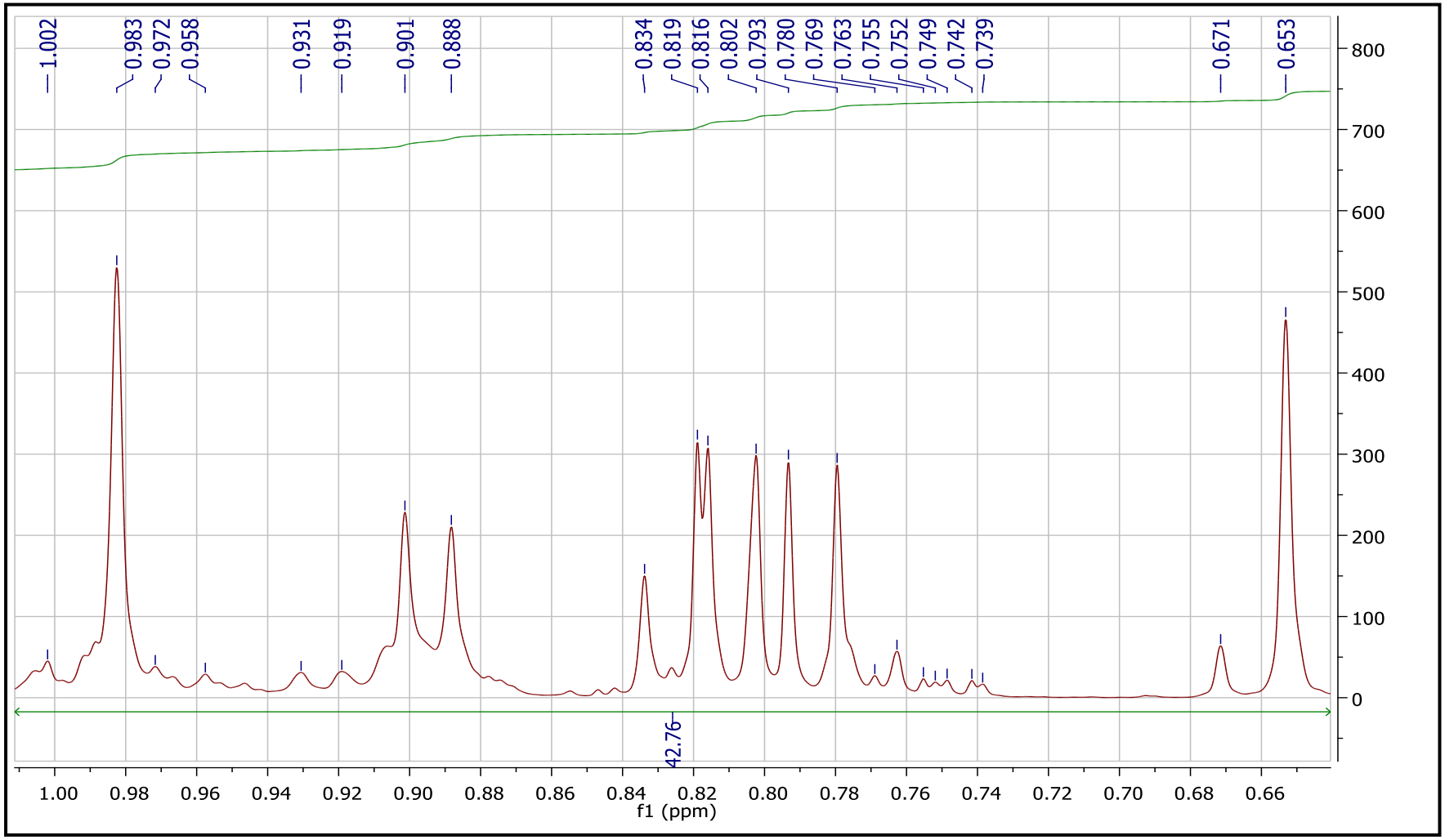

Figure A.16: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of *β*-sitosterol and stigmasterol (Expansion)

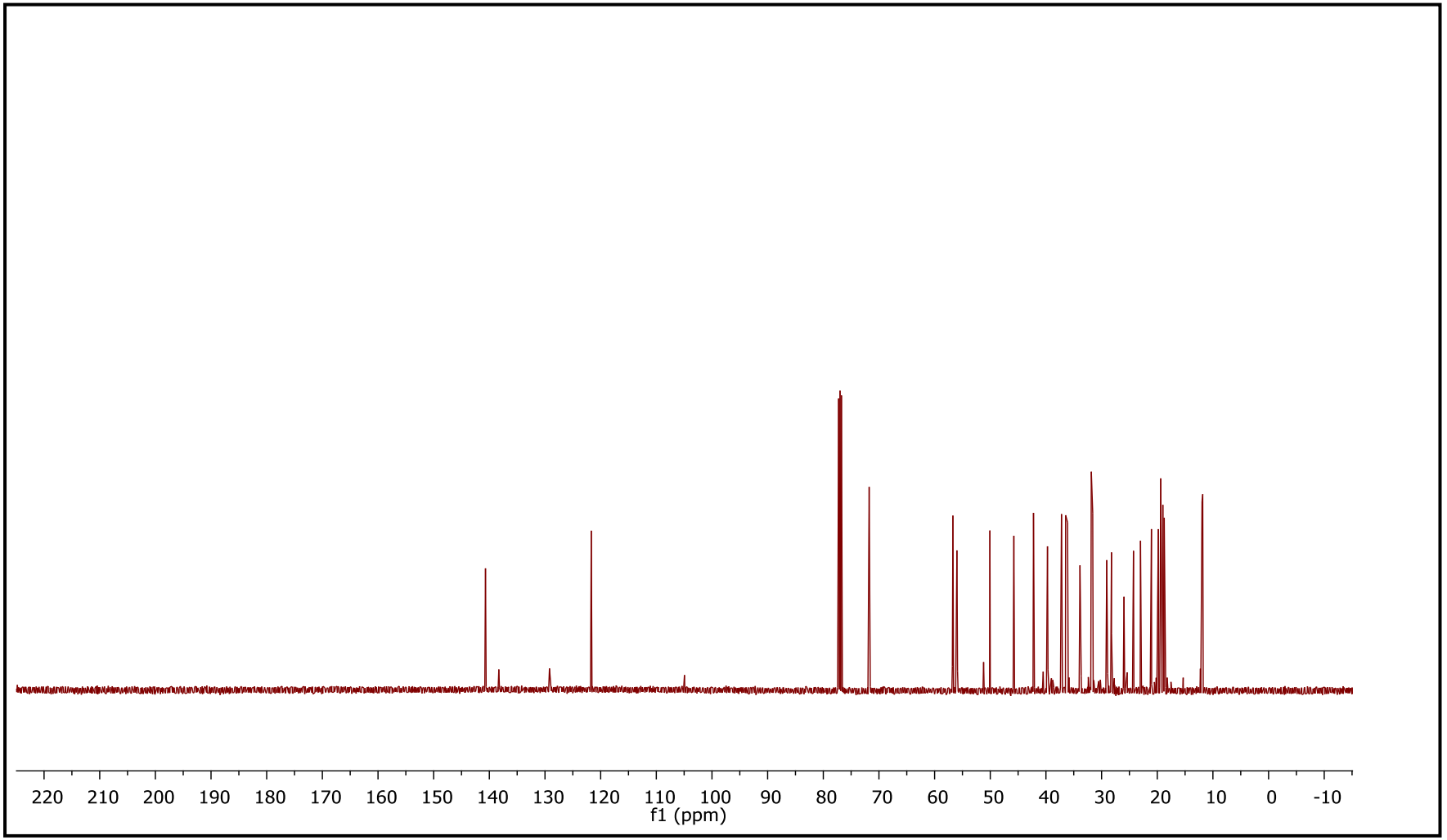

Figure A.17: ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of a mixture of β-sitosterol and stigmasterol

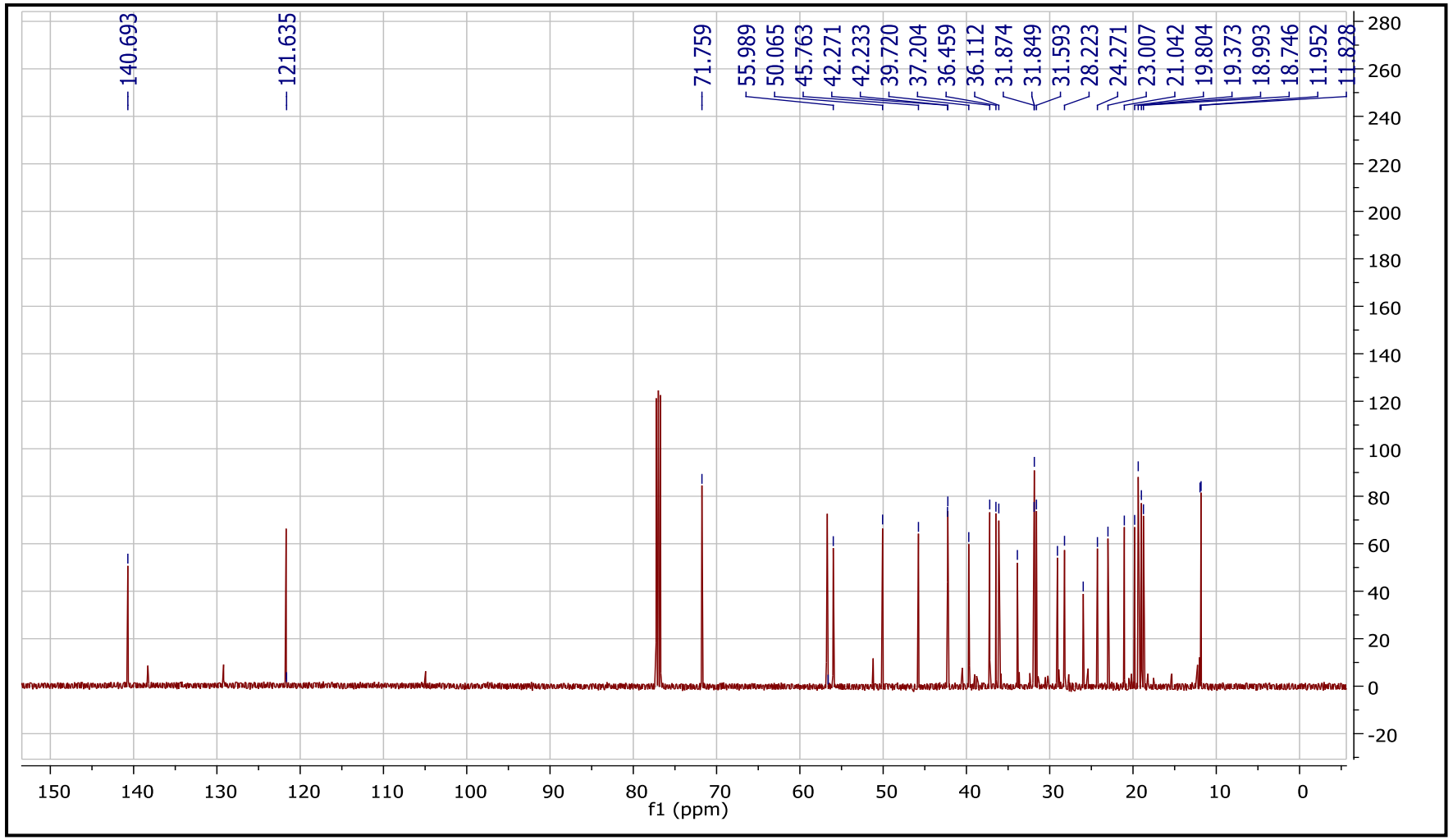

Figure A.18: ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of a mixture of β-sitosterol and stigmasterol

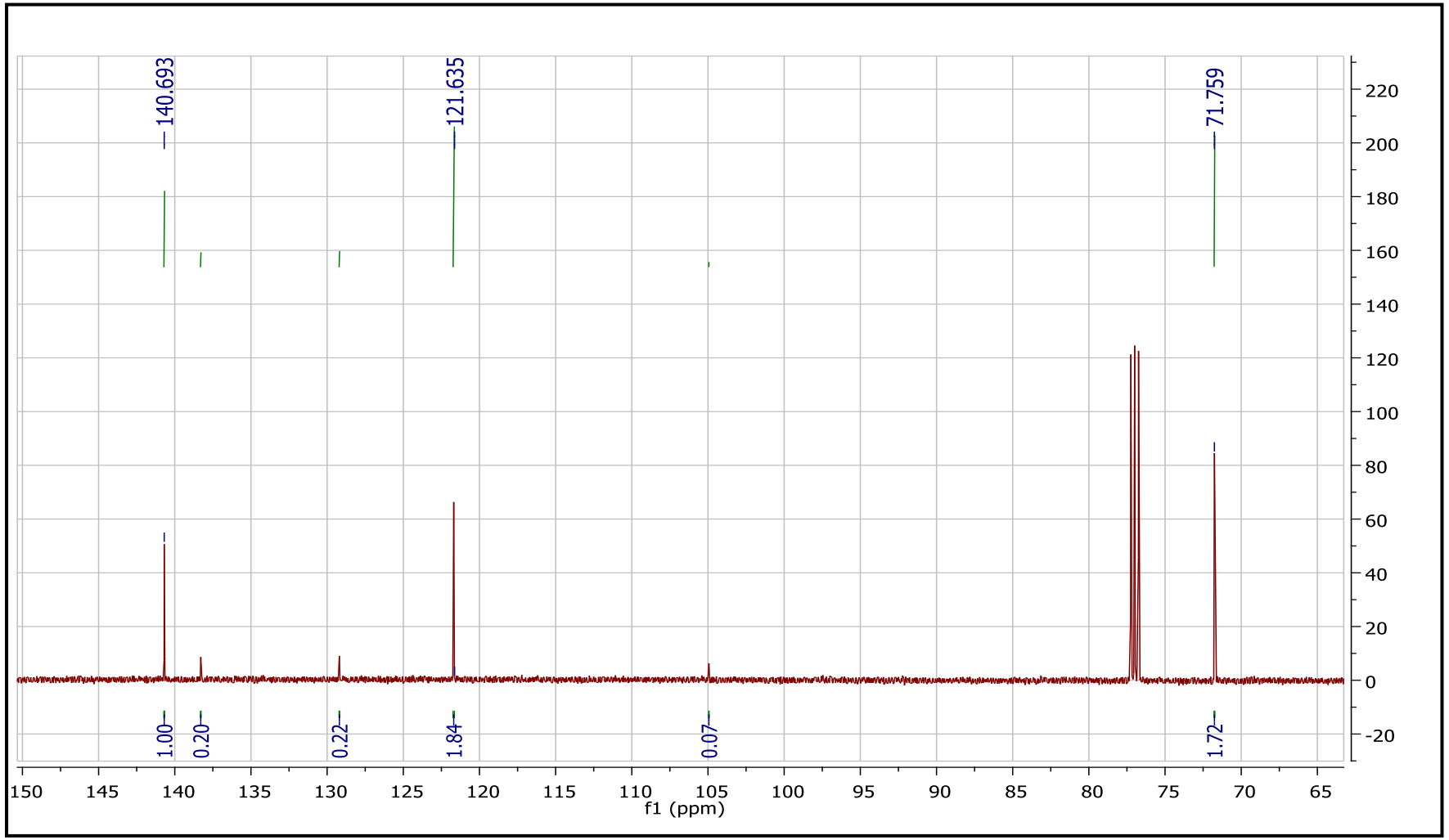

Figure A.19: ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of a mixture of β-sitosterol and stigmasterol (Expansion)