Anti-hyperglycemic effects of holybasil Ocimum sanctum and Japanese maple Acer palmatum leaves extracts

Master's Thesis

ANTI-HYPERGLYCEMIC EFFECTS OF HOLYBASIL *Ocimum sanctum* AND JAPANESE MAPLE *Acer palmatum* LEAVES EXTRACTS

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修士学位論文内容要旨
Abstract

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論文題目 Title	Anti-hyperglycemic effects of holybasil *Ocimum sanctum* and Japanese maple *Acer palmatum* leaves extracts ホーリーバジル及びノムラモミジの葉のエキスの血糖値上昇抑制作用		

Diabetes mellitus (DM) is one of several lifestyle-related diseases. It is characterized by high blood glucose level called hyperglycemia, which leads to the development of Type 2 DM, and is often associated with some complications. Understanding the mechanism of carbohydrate metabolism could provide possible ways to suppress postprandial hyperglycemia through the inhibition of digestive enzyme and glucose absorption in the small intestine. It is reported that ethanolic extract of *Ocimum sanctum* has showed anti-diabetic effects through the reduction of blood glucose level in diabetic-induced rats and the isolated bioactive compound was elucidated to have a tetracyclic triterpenoid structure, while the anti-hyperglycemic mechanism is still unclear. On the other hand, some studies have presented the anti-hyperglycemic effects of some *Acer spp.* plants, apple and peach leaves; and among those *Acer spp.*, *Acer palmatum cv.* Nomura (APN) has an anti-hyperglycemic effect in sucrose-loaded and glucose-loaded mice. Therefore, it can be presumed that there is a possibility to inhibit the glucose transportation in the small intestine.

This study aims for the prevention of diabetes, through the use of natural products from plant extracts, *Ocimum sanctum* (BPK1) and APN, for the suppression of blood glucose level. In addition, confirmation of the compound potency will be tested with the use of the isolated active compounds. Furthermore, this study also aims to elucidate the inhibitory mechanism for hyperglycemia. The methodological summary is as follows, processed extract with solvents were applied on an assay-guided fractionation by *in vitro* and *in vivo* experiments to further separate the extract. *In vitro* α-amylase inhibitory activity test, ethanol was used for BPK1 extraction determined that it has inhibitory activity. Comparing to separated fractions, hydrophobic layers (ethyl acetate and hexane) have stronger α-amylase inhibitory activity. From HPLC analysis in hexane layer, the strongest inhibitory activity, Fr.6, compound A, its IC_{50} value (142.8 μg/mL) is one eighth of acarbose. Compound A is estimated that hydrophobic fraction contains ester-like constituents.

In vivo glucose-loading test, the results showed that the group which contains 1000 mg/kg methanol extract of APN decreased blood glucose level at 30 minutes. At the first step separation, both ethyl acetate and water layer have activity. Then the second step separation, hexane layer was showed significant difference comparing to control group. After HPLC fractionation in hexane layer, Fr.A10&12 suppressed blood glucose level. On the other hand, Fr.B5 in water layer also suppressed blood glucose level and showed significant difference. It is estimated that hydrophilic fraction, compound B contain triterpenoid-like constituents, and hydrophobic fraction, compound C, contain sugar moiety with quinic acid or rosmarinic acid, respectively.

In summary, the inhibitory effects of potential leaves on hydrolysis of α-amylase and postprandial elevation of the blood glucose level and the properties of their possible active components were elucidated, respectively. Improvement of the hyperglycemic condition is considered as an effective way for preventing diabetes. These two kinds of leaves may prevent diabetes or metabolic syndrome by inhibiting hyperglycemia, provided as functional foods for people who have awareness in the suppression or delay of high blood glucose state in life. Through this study, people could have more chances to prevent disease with their daily meals before the disease progresses and becomes difficult to manage and treat. This study could as well contribute to the enhancement of the quality of life and to help build a healthier society.

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Chapter 1

General Introduction

Chapter 1 General introduction

1.1 Diabetes mellitus (Diabetes, DM)

Nowadays living in modern life, people do not only pursue eating for satiation, but as well as paying attention on the important values of convenience, delicacy, nutrition, and health benefits of food. There are three basic food functions, which are: for nutrition, sensory satisfaction, and disease prevention by modulation of physiological systems.[1] Among these, the third function has the most important effect on health, which makes it possible to prevent lifestyle-related diseases.

'Diabainein' is a Greek word for diabetes which means 'something passes through. This term was derived from a kind of symptom experienced by diabetic people, which is the excessive amounts of urine excretion. Diabetes is a kind of lifestyle-related disease, but can also occur to some people with metabolic syndrome which is associated with high risk of visceral obesity which in turn could lead to abnormal carbohydrate metabolism. In addition, diabetes as a chronic disease can occur either when the pancreas could not produce enough insulin, which is a hormone that helps the body use glucose for energy, or when the body cannot effectively use the insulin it produces and causes abnormal conditions.[2] Hyperglycemia is one of the common symptoms of uncontrolled diabetes, which could lead to developed disability and/or life threatening health complications in the heart, blood vessels, eyes, kidneys and nerves. There are several factors that could promote rapid increase of blood glucose level such as: excessive food intake, lack of exercise, stress, poor and inadequate sleep quality, smoking, and alcohol drinking; which could result to increased insulin secretion and later on compromise the pancreas condition.

Data from the National Health and Nutrition Survey by the Ministry of Health, Labor and Welfare in Japan in 2014, showed that there are 3.2 million diabetic patients as presented in Fig.1-1. The percentage of male having diabetes showed higher ratio when compared to female patients, as well as the percentage of both are increasing with the age as presented in Fig.1-2. Trends in the percentage of subjects strongly suspected of having diabetes was shown in Fig.1-3. On the other hand, from the data of the Ministry of Health and Welfare in Taiwan in 2014, presented that the prevalence of diabetes reached to 9.2%, and it is considered to be the 5th leading cause of death in Taiwan.[3]

Survey data from World Health Organization (WHO), showed that there are 422 million people with diabetes in 2014, and the global prevalence of diabetes among adults over 18 years of age has risen to 8.5% in 2014. Furthermore, it is estimated that diabetes will be the 7th leading cause of death by 2030 around the world, as shown in Fig.1-4. [4, 5]

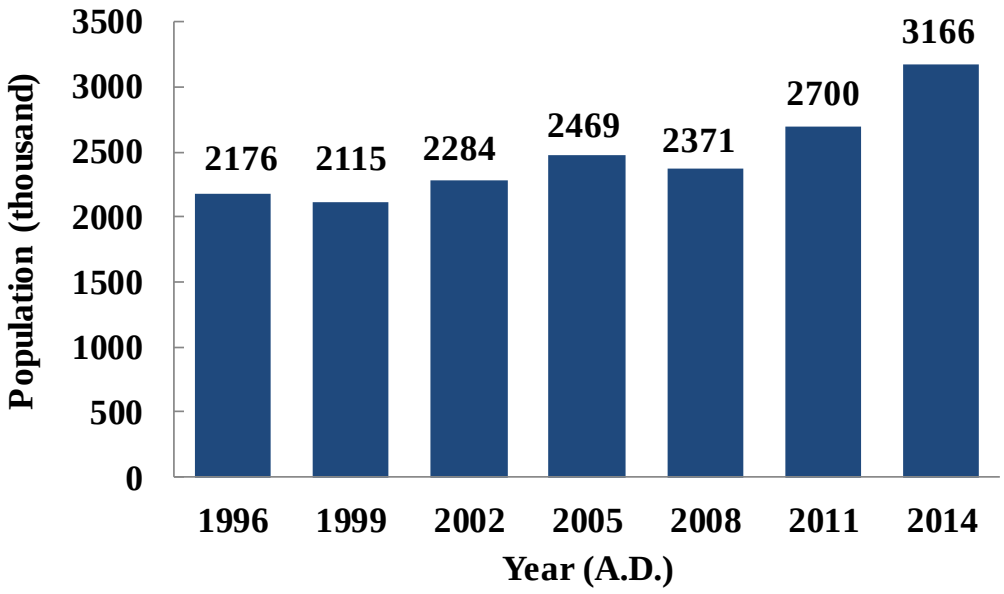


Fig.1-1 Number of population of diabetes patients in Japan. [6]

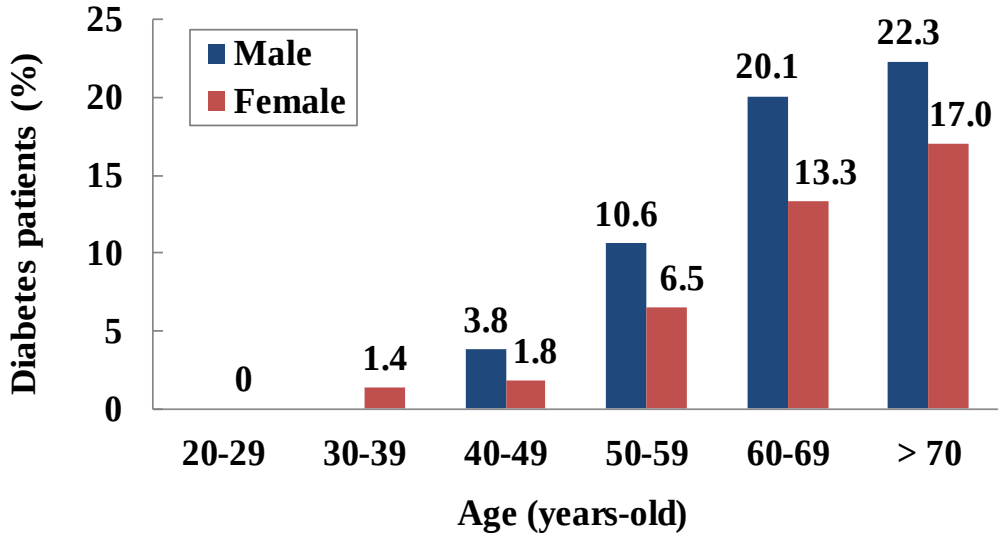


Fig.1-2 Percentage of male and female diabetic patients in Japan in 2014. [6]

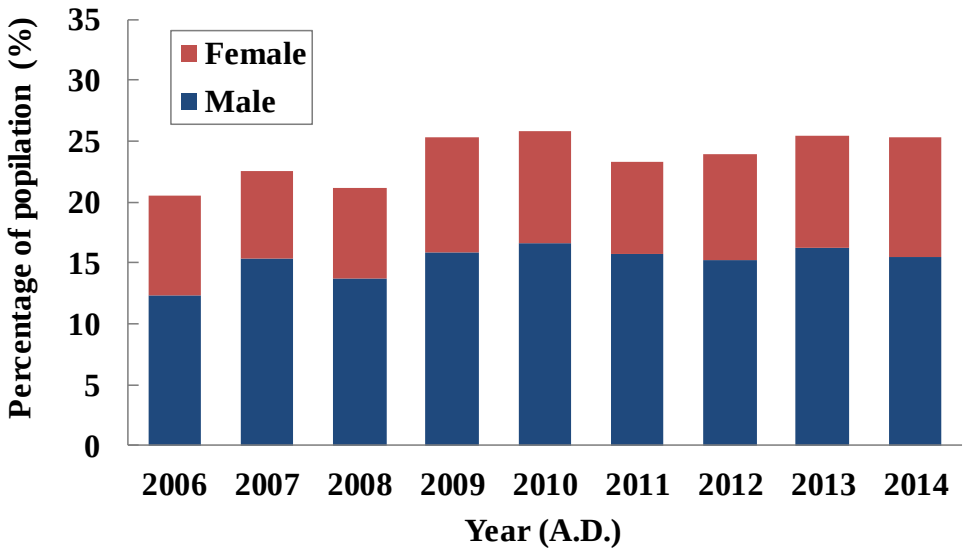


Fig.1-3 Trends in the percentage of "subjects strongly suspected of having diabetes" in Japan. [6]

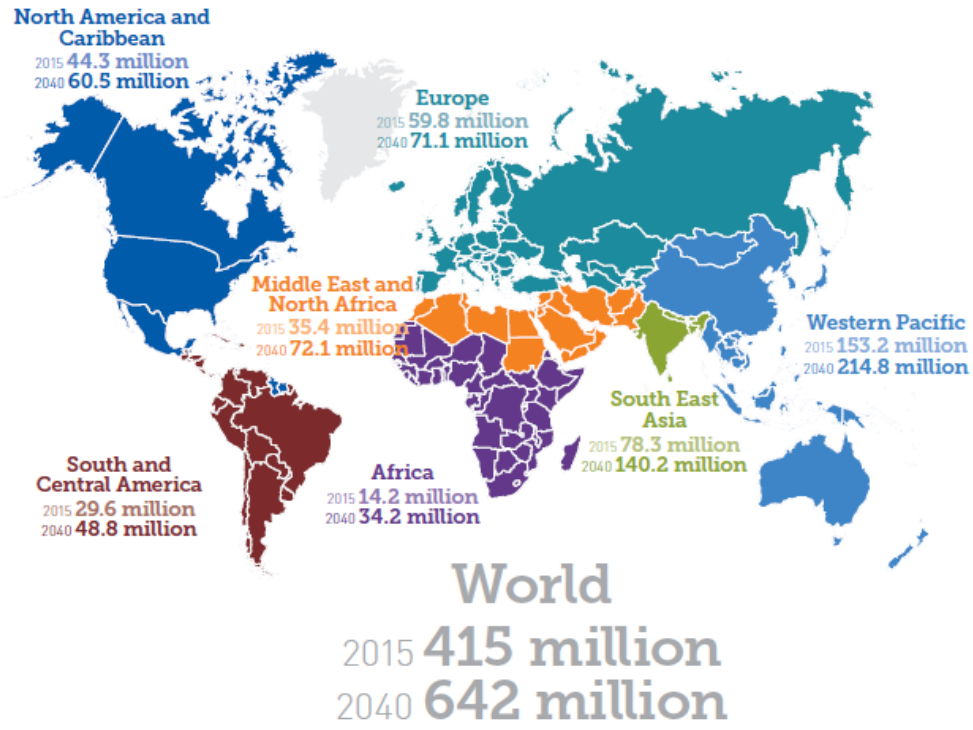


Fig.1-4 Estimated number of people with diabetes worldwide and per region in 2015 and 2040. [7]

1.1.1 Classification of diabetes mellitus

Diabetes is commonly classified into three types: Type 1 diabetes, Type 2 diabetes and gestational diabetes, which will be discussed in details in the succeeding parts of this chapter.

1) Type 1 diabetes (insulin-dependent diabetes):

The type 1 diabetes is characterized by the deficient insulin production of the body. Patients commonly acquire this condition from childhood and requires daily administration of insulin to regulate the amount of glucose in their blood, which dictates their survival. Symptoms may include excessive urination and thirst, constant hunger, weight loss, vision changes and fatigue. The cause of type 1 diabetes can be related to family history diabetes, genetics, infections and other environmental factors; however, it is not clearly understood and currently not preventable. [4,5]

2) Type 2 diabetes (non-insulin-dependent diabetes):

Type 2 diabetes results from the body's ineffective use of insulin. This type of diabetes accounts for the vast majority of affected population and grows rapidly around the world. This rise is associated with aging, economic development, increasing urbanization, less healthy diets and reduced physical activities. Symptoms may be similar to those of type 1 diabetes, such as excess body weight, physical activity, poor nutrition, family history diabetes, genetics, past history of gestational diabetes and old age, but are often less marked or absent. As a result, the disease may go undiagnosed for several years, until complications have already arisen. For many years, type 2 diabetes was seen only in adults but it has begun to occur in children as well. [4,5]

3) Gestational diabetes

Gestational diabetes is a temporary condition that occurs during pregnancy and carries long-term risk of type 2 diabetes. The condition is present when blood glucose levels are above normal but still below the diagnosis of diabetes. Women with gestational diabetes are at increased risk of some complications during pregnancy and childbirth, which could also affect their infants. Gestational diabetes can be diagnosed through prenatal screening, rather than reported symptoms. [8]

1.1.2 Diagnosis of diabetes mellitus

Diabetes can be diagnosed and characterized based on the following criteria on blood glucose level: (**1**) fasting blood glucose level ≥ 126 mg/dL (7.0 mmol/L); (**2**) blood glucose level ≥ 200 mg/dL (11.1 mmol/L) 2 hours after administration of 75 g glucose in oral glucose tolerance test (OGTT); (**3**) symptoms of hyperglycemia and casual blood glucose ≥ 200 mg/dL (11.1 mmol/L); and (**4**) glycated hemoglobin ($HbA1_C$) ≥ 6.5% (The standard value is 6.1% in Japan).

The recommending criteria for diagnosis of intermediate hyperglycemia from WHO is shown in Table 1-1. Impaired glucose regulation refers to blood glucose level between diabetes criteria and of normal people with fasting blood glucose level from 100 to 125 mg/dL (5.6 to 6.9 mmol/L) are considered as impaired fasting glucose (IFT); and patients with blood glucose level at 140 to 200 mg/dL (7.8 to 11.1 mmol/L), 2 hours after a 75 g glucose in OGTT are considered as impaired glucose intolerance (IGT).

The value of $HbA1_C$ above 6.5% can be used as a parameter for diagnosing diabetes; however, $HbA1_C$ below 6.5% should not be recommended as there are no risk of suffering from diabetes. [9]

Table 1-1 Current World Health Organization (WHO) recommendations for the diagnostic criteria for diabetes and intermediate hyperglycemia in 2006. [4]

Condition	**After 2h blood glucose mg/dL (mmol/L)**	**Fasting blood glucose mg/dL (mmol/L)**
Normal	< 140 (< 7.8)	< 110 (< 6.1)
Impaired Fasting Glucose (IFG)	< 140 (< 7.8)	110 – 125 (6.1 – 7.0)
Impaired Glucose Tolerance (IGT)	119 – 140 (6.6 – 7.8)	< 126 (< 7.0)
Diabetes mellitus (DM)	**≥ 200 (≥ 11.1)**	**≥ 126 (≥ 7.0)**
Gestational Diabetes (GDM)	153 – 199 (8.5 – 11.1) *after 1h blood glucose ≥ 180 (≥ 10.0)	92 – 125 (5.1 – 6.9)

1.2 Diabetes complications

Diabetes has an increased risk of inducing serious complications such as hyperglycemia, hypoglycemia, ketoacidosis and hyperosmolar hyperglycemic non-ketotic coma. Diabetic complications are classified into two types: the microvascular and macrovascular complications. Microvascular complications include retinopathy leading to blindness, nephropathy leading to renal failure, and neuropathy leading to severe infections or lower limb amputation. Macrovascular complications include cardiovascular diseases such as heart attacks, strokes, and peripheral vascular diseases. Maintaining blood glucose, blood pressure, and cholesterol levels at or close to normal value can help relieve or prevent diabetes complications. [4, 7]

The pathological mechanism can be classified to impairment of polyol pathway, activation of protein kinase C, increase in oxidative stress, and other similar or related factors. It is hypothesized that aldose reductase is one of the key factors which causes diabetic complications. According to the National Health and Nutrition Research by the Ministry of Health, Labor and Welfare in Japan, describes diabetic patients having complications which includes but not limited to a combination of diabetic neuropathy, diabetic retinopathy and/or diabetic nephropathy. [6]

1.2.1 Diabetic neuropathy

Diabetic neuropathy is a kind of nerve damage causing injury to the nerve fibers throughout the body due to high blood sugar condition. Common symptoms may include tingling sensation, numbness, burning sensation, and pain often experienced on feet and legs. The incidence is highest during diabetic complications, which develops at an early phase. Based on the distribution of disorders development pattern, diabetic neuropathy is classified as hyperglycemic neuropathy, symmetric polyneuropathy, focal and multifocal neuropathy, and could be in mixed forms.

Blood glucose control, aldose reductase inhibitor such as Epalrestat, antioxidants reported by α-lipoic acid, incretin (glucagon-like peptide-1, GLP-1 and glucose-dependent insulinotropic polypeptide, GIP), and regeneration therapy are some of the approaches to treat neuropathy. [10, 11]

1.2.2 Diabetic retinopathy

Diabetic retinopathy is a major cause of vision loss in many industrial countries worldwide, which is the second largest cause of the blindness in adults. It can affect many parts of the eye, including the retina, macula, lens and the optic nerve. The cause of diabetic retinopathy is pointed to hyperglycemia which is associated with changes to the tiny blood vessels in the retina, a light-sensitive tissue at the back of the eye, swells and leaks fluid or blood which follows an abnormal growth of a new blood vessels on the surface of the retina. Patients would see some spots floating, experience blurred the vision, or could worsen and develop into blindness. A more serious consequence than diabetic retinopathy is diabetic macular edema (DME) which symptom is characterized of swelling in an area on the retina called the macula.[12]

Multiple cellular pathways and potential molecular mechanisms have been proposed to explain diabetes induced complications. In diabetic retinopathy, four mechanisms are involved: the activation of protein kinase C (PKC) pathway, increased flux of glucose through the polyol pathway or hexosamine pathway, and increased advanced glycation end product (AGE) formation. [13]

1.2.3 Diabetic nephropathy

Diabetic nephropathy is the leading cause of chronic kidney disease in patients with renal replacement therapy and it is related to increased cardiovascular mortality. Almost a third of the number of total dialysis patients in Japan have diabetic nephropathy. The nephrons, which is accounted to be approximately one million units in the kidney, thickens and scars, which makes them less able to filter waste and remove fluid from the body. Changes of tubular basement membrane thickening and mesangial expansion, results in increased urinary albumin excretion (UAE).

It occurs with metabolic factors, such as increased oxidative stress, renal polyol formation, activation of intracellular renin angiotensin II, endothelin, second messenger protein kinase C and MAP kinase, activated mitogen protein kinase (AMPKs), nuclear transcription factors such as NF-κB, growth factors like prosclerotic cytokine, TGF-β, and VEGF, and accumulation of advanced glycation end-products (AGEs). [14-17]

1.3 Insulin resistance

Insulin is an anabolic hormone composed of 51 amino acids and has a molecular weight of 5,807 Da. It is produced by β-cells of the pancreatic islets. Insulin, is often described as a "key", unlocks the cell to allow sugar to enter from carbohydrates to glucose storage and be used for energy, regulating glucose homeostasis.

Insulin resistance is the cells fail to respond to the normal actions of the insulin, or the body produces insulin, but the cells in the body become resistant to insulin and are unable to use it effectively, leading to hyperglycemia. [18, 19]

Insulin resistance plays a major role in the development of type 2 diabetes. It is associated with a number of health disorders, including obesity, glucose tolerance, dyslipidaemia and hypertension clustering in the so-called metabolic syndrome. The most important factor is obesity, which visceral adipose tissue resistant to the anti-lipolytic effect of insulin and release excessive amounts of free fatty acid.

1.4 Carbohydrate metabolism

The small intestine plays a central role in the digestion and absorption of nutrients from diets of animals. Among the nutrients, glucose derived from carbohydrates supplies as the most common sugar form which is liable to be absorbed and digested in the body.

This can be explained in two mechanisms, the hydrolysis by digestive enzymes and glucose transportation in the small intestine, as presented in Fig.1-5; wherein, when glucose nears intestinal cell, GLUT2 will go to basolateral side to assist glucose transport to enter epithelial cell.

1.4.1 Hydrolysis by digestive enzymes

Food products such as rice and wheat which is considered to be staple in some countries contain long-chain carbohydrates such as starch, which is hydrolyzed by α-amylase secreted by salivary or pancreas, and produces oligo- and/or disaccharides maltose, sucrose, etc. These disaccharides are hydrolyzed by another enzyme: maltase or sucrose called α-glucosidase secreted by the small intestine, which breaks down complex carbohydrates to monosaccharide such as glucose or fructose.

1.4.2 Glucose transportation in the small intestine

Increased glucose concentration in the blood will result to insulin secretion in the body. Blood glucose level is controlled by digestion, absorption, and metabolism of carbohydrates in the body. Since glucose is a polar molecule, it is necessary for a particular and specific transport proteins to be present to assist its passing through the cell membrane. The specific transport proteins are called glucose transporter (GLUT) or solute carrier 2A (SLC2A) in gene symbol. There are 13 GLUTs (GLUT1-GLUT12) with a H^+-myo inositol symporter (HMIT), and 2 Na^+-coupled glucose co-transporter (SGLT1 and SGLT2) in a family of membrane proteins in mammals.

There are some glucose transporters on the surface of small intestine, such as SGLT1 and GLUT2 that assist glucose in transporting into blood vessel.

1) Glucose transporter 2 (GLUT2):

It is located in the plasma membrane of the liver, pancreatic β-cells, intestinal, kidney cells as well as in the portal and the hypothalamus areas. Glucose is transported from the intestinal epithelia cells into the blood vessels by GLUT2 in basolateral and apical membranes, which has a high capacity and low affinity.

2) Na^+-coupled glucose co-transporter 1 (SGLT1):

It is located mainly in small intestinal cells and is also present in kidney, heart, the parotid and submandibular salivary glands. SGLT1 is a low capacity and high affinity transporter, and mainly mediates the transport of glucose or galactose, only slightly transport mannose or 2-deoxy-D-glucose.

It is known that there are numerous approaches to suppress hyperglycemia, besides the two mentioned above, promotion of glucose transportation in the blood, promotion of insulin secretion, insulin-like effect, insulin signal tissue activation effect, inhibition of glucose reabsorption in renal tubule, promotion of carbohydrate metabolism, catabolism or storage, etc.

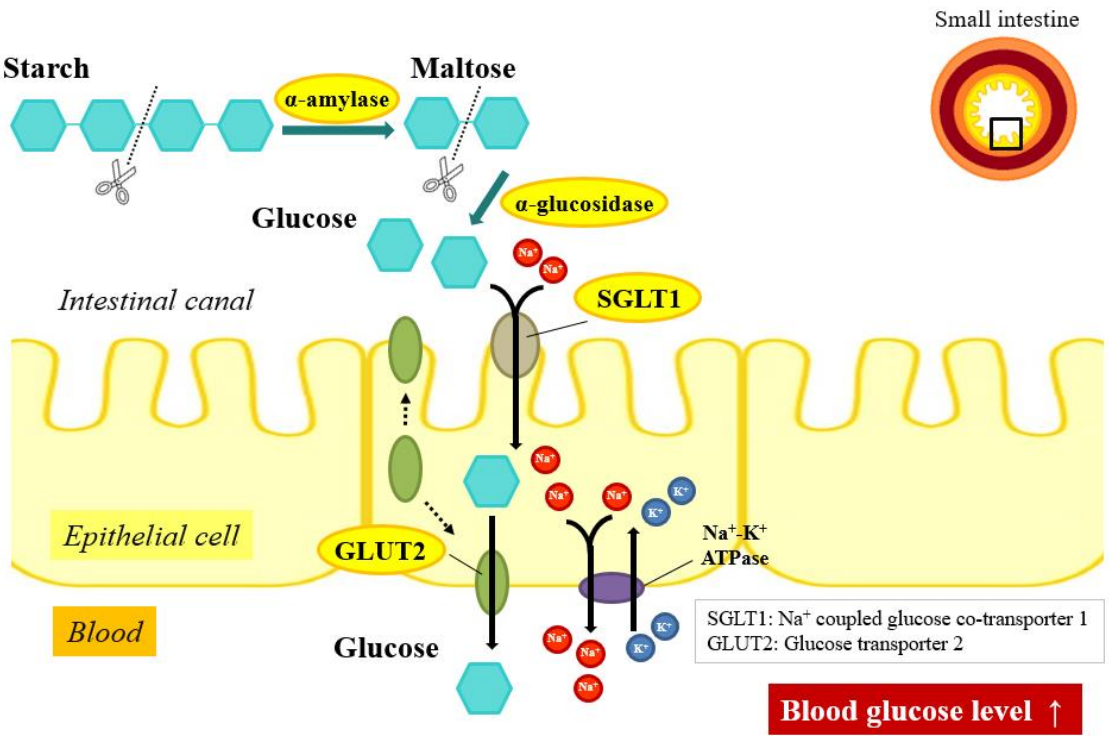


Fig.1-5 The process of elevation of postprandial blood glucose level.

1.5 Treatment and prevention of diabetes

To date, there have been a variety of approaches developed to treat or prevent diabetes. The simplest and direct way is insulin injection to balance high blood sugar to normal level after meals. Anti-diabetic agents are based on some targets such as the β-cell stimulation, inhibition of aldose reductive enzyme, and inhibition of digestive hydrolytic enzyme, α-glucosidase. In addition, functional foods in both Japan and Taiwan, reported studies with anti-diabetic constituents from natural plants which will be introduced below.

1.5.1 Anti-diabetic agents

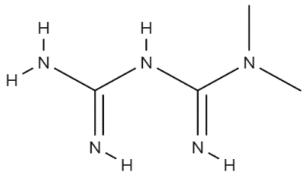
There are some famous anti-diabetic medications currently used in the market today, which are classified into different inhibitory mechanisms.

Biguanides which includes Metformin (850 mg/day) is a well-established and one of the most effective medication which could decrease insulin resistance and do not cause weight gain or low sugar level. Gliclazide (40-80 mg/day) is a sulfonylurea, which increases insulin secretion in type 2 diabetes. Both medications are on the WHO list of essential medicines for diabetes. Chemical structures are shown in Fig.1-6 (a-b).

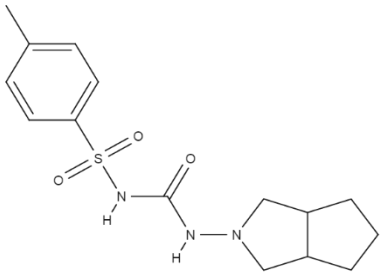
Other sulfonylureas like glimepiride, glipizide, glibenclamide, or glitinide, such as repaglinide or nateglinide are some of the commonly used treatments that could increase insulin secretion. GLP-1 analogues such as exenatide, liraglutide are injectable treatments, and DPP4 inhibitors which are usually used with metformin, sitagliptin (100 mg/day), vildagliptin, saxaglipitin or linagliptin. chemical structures of representative constituents are shown in Fig.1-6 (c-e).

The α-glucosidase inhibitor like miglitol (50-100 mg/three times a day) or acarbose could prevent the absorption of sugars from the stomach and intestines. Chemical structures are shown in Fig.1-6 (f-g).

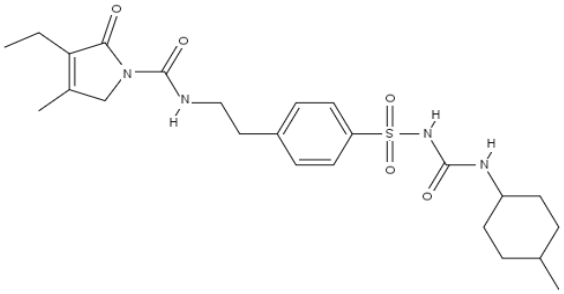
These treatments both enhance the body's natural response to ingested food, reducing blood glucose levels after eating. In addition, people may need access to medications to control blood pressure and blood cholesterol levels in either types of diabetes.



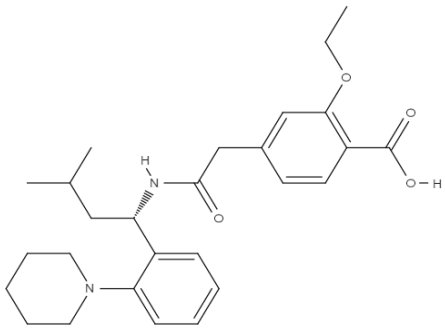
(a) Metformin



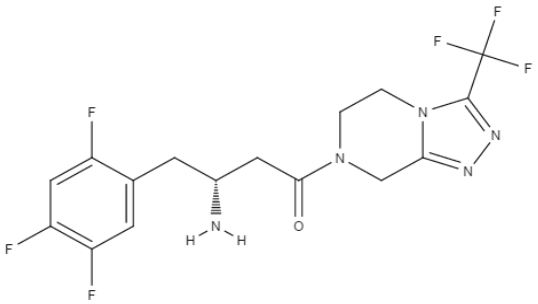
(b) Gliclazide



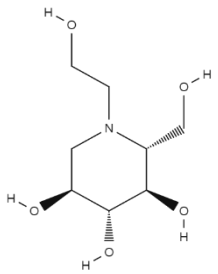
(c) Glimepiride



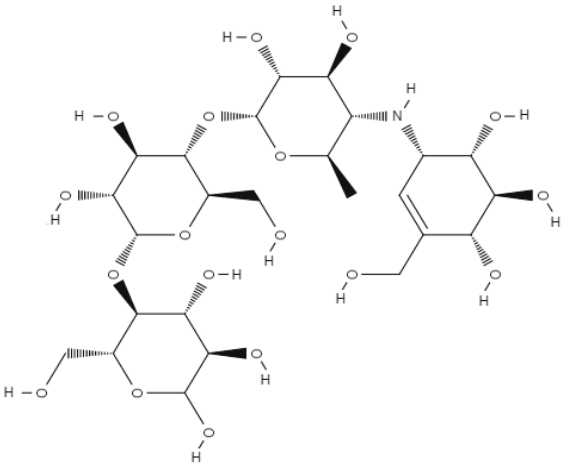
(d) Repaglinide



(e) Sitagliptin



(f) Miglitol



(g) Acarbose

Fig.1-6 Anti-diabetic agents.

1.5.2 Functional foods

1) Health foods and foods with Health Claims in Japan

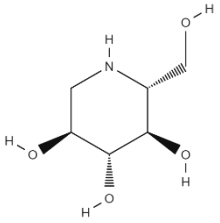
The term ‘health foods’ is commonly used to describe what may be called ‘functional foods’ or dietary or food supplements’ in the USA or other countries. The ‘Foods for Specified Health Uses’ (FOSHU) concept is described as it evolved from functional foods which was first developed and published for the health benefits of foods in Japan in 1984. In 1991, the Ministry of Health and Welfare (MHW), now known as the Ministry of Health, Labor and Welfare (MHLW), officially presented the FOSHU system as a basis for making the functional claims of food with reference to the increase in the occurrence of lifestyle-related disease. In 2001, a new regulatory system, ‘Foods with Health Claims’ (FHC) was established and consisted of a new concept of ‘Foods with Nutrient Function Claims’ (FNFC) and innovated FOSHU. In 2005, the MHLW additionally changed the existing FOSHU system. [20, 21]

FOSHU refers to foods containing ingredient with functions for health and officially approved to claim its physiological effects on the human body. FOSHU is intended to be consumed for the maintenance and/or promotion of health or special health uses by people who wish to control health conditions, including 9 specific health uses. In April 2016, there are 1250 food products have been granted the status of FOSHU, approximately 200 items approved as ‘food related to the regulation of blood sugar levels’, which suppress postprandial elevation of the blood glucose level to inhibit α-glucosidase or delay glucose absorption.

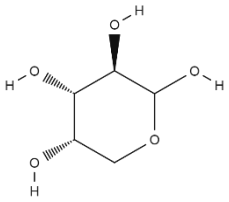
In health foods, the active ingredients such as 1-deoxynojirimycin derived from mulberry leaves, L-arabinose, guava tea polyphenol, wheat albumin, salacinol derived from *Salacia reticulata*, indigestiable dextrin, gymnema are shown in Fig.1-7 (a-d). [21]

2) Health food in Taiwan

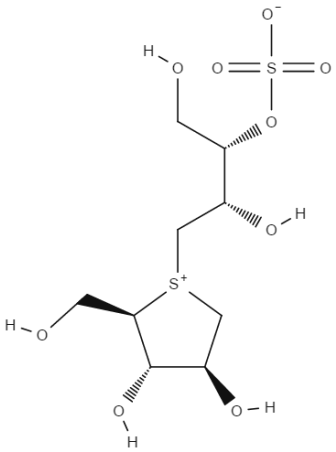
In Taiwan, there are16 products that have been permitted the status of health food which includes β-glucan, mulberry alkaloids, trivalent chromium, *Lactobacillus reuteri,* isoflavones (daidzein, glycitein, genistein), ρ-tyrosol with salidroside, indigestiable dextrin, monacolin K with quercetin, α-linolenic acid from cynobacteria, chlorella, and ginsenoside Rb2 as shown in Fig.1-7 (e-j). [22]



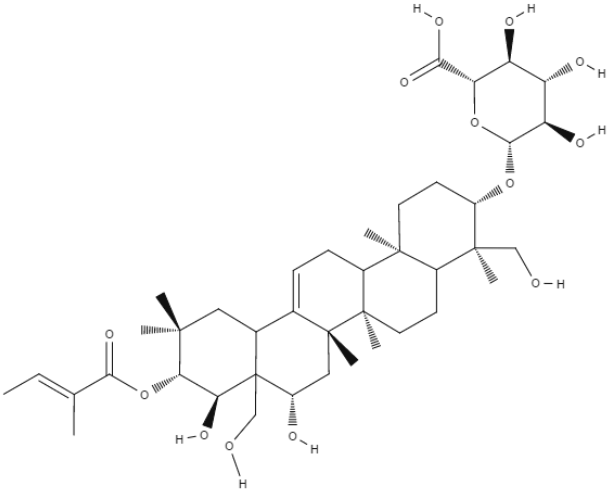
(a) 1-Deoxynojirimycin



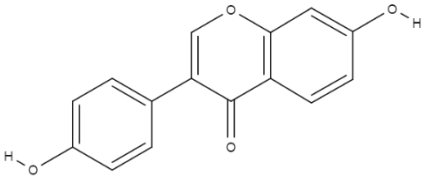
(b) L-arabinose



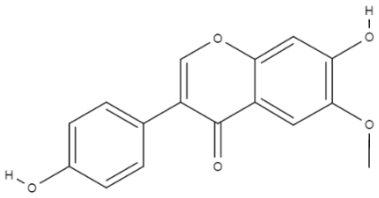
(c) Salacinol



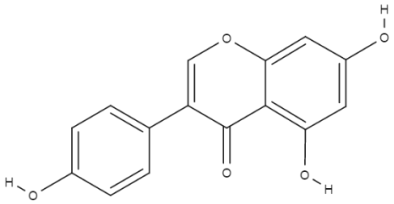
(d) Gymnema



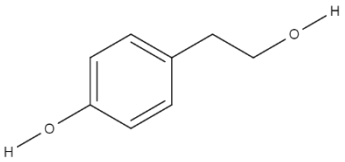
(e) Daidzein



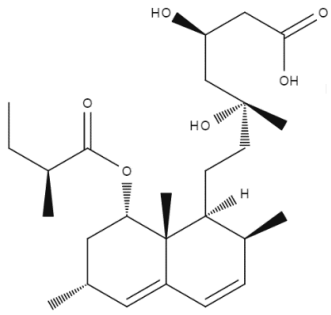
(f) Glycitein



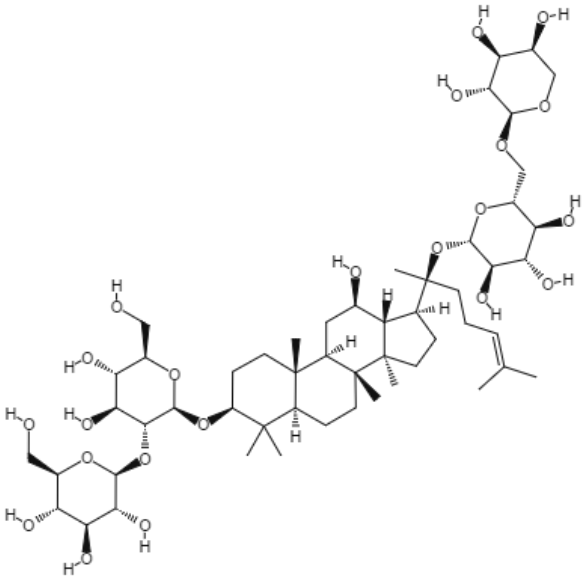
(g) Genistein



(h) ρ-Tyrosol



(i) Monacolin K



(j) Ginsenoside Rb2

Fig. 1-7 Main decisive ingredients of FOSHU in Japan and Health Food in Taiwan.

1.5.3 Antidiabetic constituents from natural plants

Consumers are aware of the fact that medical drugs can treat diseases more quickly, however there are some disadvantages such as serious side-effects, high cost, etc. In recent years, a large amount of researches focused on studying anti-diabetic constituent from plants. Some common antidiabetic constituents which classified as α-amylase or α-glucosidase inhibitor, and glucose transportation in the small intestine includes the following.

1) α-Amylase inhibitor

Arjunolic acid and Asiatic acid, a kind of triterpenes, were derived from kiwifruit leaves (*Actinidia chinensis*) as presented in Fig.1-8 (a-b). [23]

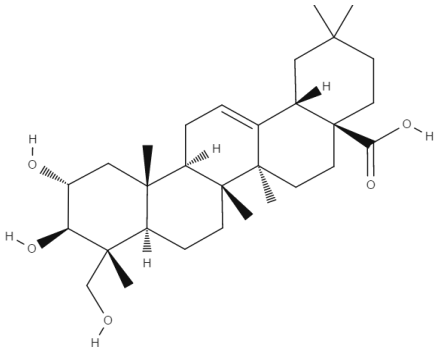
2) α-Glucosidase inhibitor

The most popular one is 1-deoxynojirimycin (DNJ) as presented in Fig.1-7 (a), which was extracted from mulberry leaves (*Morus alba* L.). The oxygen atom in the ring of monosaccharide is replaced by nitrogen (this kind of ring called azasugars). [24]

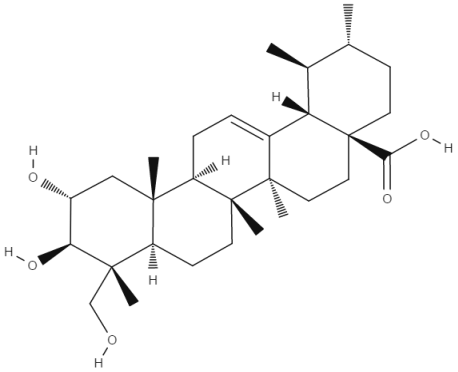
Others are isolated from different maple leaves, corilagin (β-2-*o*-galloyl-3,6-(R)-hexahydroxy diphenoyl-D-glucose) from *Acer amoenum Carr. var. matsumurae Ogata*, ginnalin A (2,6-gallyol-1-deoxyglucose) from *A. saccharum* Marsh, ginnalin B (6-gallyol-1-deoxyglucose) and ginnalin C (2-gallyol-1-deoxyglucose) from *A. pycnanthum* K Koch as shown in Fig.1-8 (c-f). These compounds have sugar structure as base and connect to galloyl structure (3 hydroxy group attach to the benzene ring). The sucrase activity is stronger when it has more galloyl group in chemical structure.[25–27]

3) Glucose transportation inhibitor in the small intestine

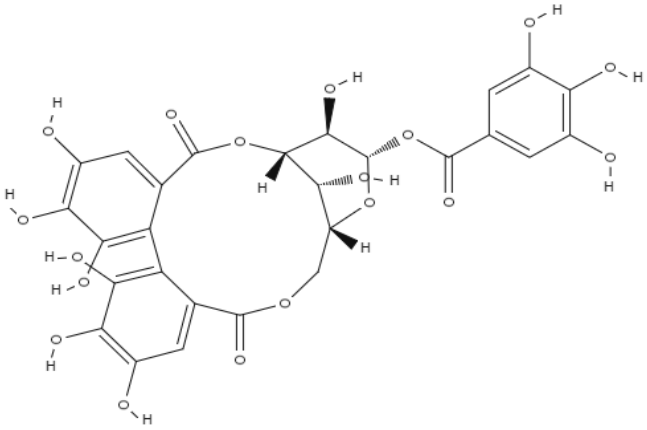
Phlorizin (62.5 mg/kg), as shown in Fig.1-8 (g), a kind of chalcone, which was derived from apple leaves (*Malus pumila)*, and is reported to potential SGLT2 inhibitor. [28] Multiflorin A (MFA) (8.5 mg/kg), Fig.1-8 (h), a kind of kampferol, extracted from peach leaves (*Prunus percica)*, a type of flavonoid, which was found in a variety of plants and plant-derived foods. [29, 30]



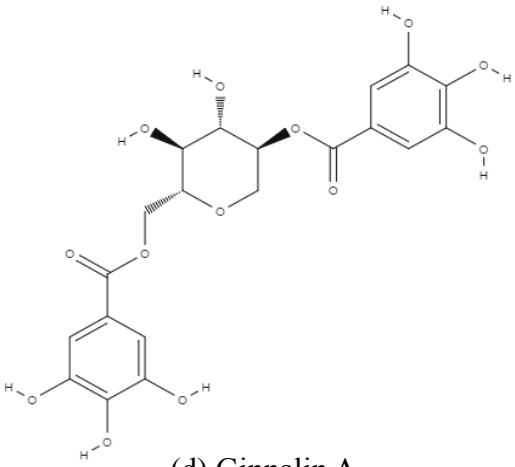
(a) Arjunolic acid



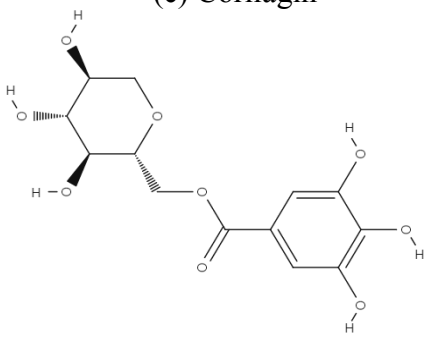
(b) Asiatic acid



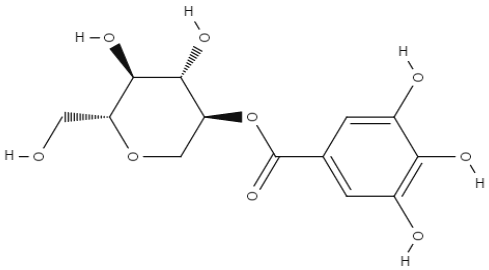
(c) Corilagin



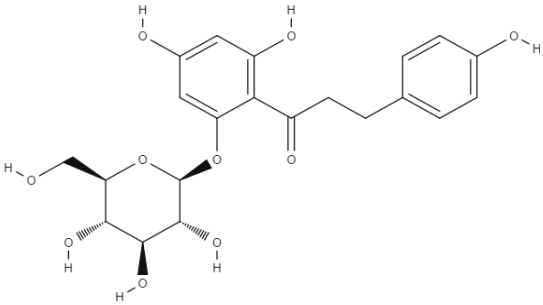
(d) Ginnalin A



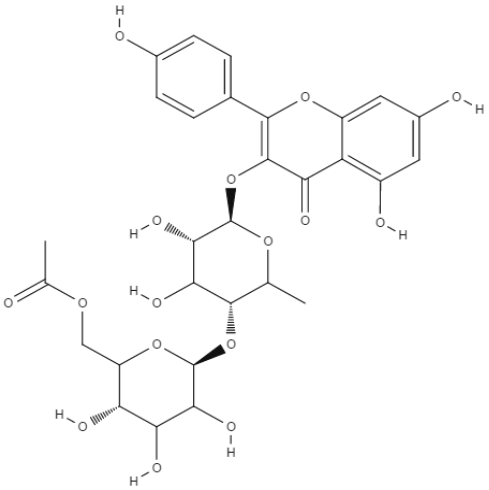
(e) Ginnalin B



(f) Ginnalin C



(g) Phlorizin



(h) Multiflorin A

Fig.1-8 Anti-diabetic constituents in natural plants.

1.6 Objective of present study

The purpose of this study is to determine the postprandial elevation inhibition of blood glucose level which plays an important role in Type 2 diabetes. Plants were screened for potential of preventing Type 2 diabetes. Active constituents were isolated with assay-guided fractionation and their chemical structures were identified by instrumental analysis. Furthermore, to elucidate two kinds of decreasing postprandial hyperglycemia mechanisms responsible in the inhibition of hydrolytic enzyme α-amylase activity (*in vitro)* and glucose transportation in the small intestine (*in vivo)*. Inhibition of enzyme activity or postprandial blood glucose elevation, reduction of the rate of glucose decomposition, and suppression of high blood glucose level may maintain elevation of blood glucose level to be stable in normal value after meal. From these, it could prevent precipitous insulin secretion to improve insulin resistance to keep normal state of blood glucose.

Through this study, it is aimed to further understand the inhibitory mechanisms of hyperglycemia; and to isolate active constituents which are expected to be applied in functional foods in the future. In addition, it is aimed to be capable of contributing to the global decrease in the acquisition rate of Type 2 diabetes. Moreover, to be able to extend healthy life expectancy and relieve the pain caused and coupled with the disease.

Chapter 2

Materials and Methods

Chapter 2 Materials and methods

2.1 Reagents

A glucose C-II test kit, soluble starch, disodium hydrogen-phosphate, sodium dihydrogen-phosphate dehydrate, hydrochloric acid, iodine (beads) and potassium iodide were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Heparin sodium was purchased from Mitsubishi Tanabe Pharma Corp. (Tokyo, Japan). α-amylase (EC 3.2.1.1) from porcine pancreas (A3176) was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). *D*-(+)-Glucose, methanol, ethanol, ethyl acetate, hexane, *p*-anisaldehyde and sulfuric acid used in the study were high performance liquid chromatography (HPLC)-grade chemicals purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Acetic acid was purchased from Kokusan Chemical Co., Inc. (Tokyo, Japan). Deuterium oxide (100% D), methanol-d_4 (99.8% D) and chloroform-d_1 (stabilized with silver foil 99.8% D) of NMR spectroscopy were purchased from Kanto Chem. Co., Inc. (Tokyo, Japan). Acarbose tablets were purchased from Bayer Co. (Leverkusen, Germany).

2.2 Experimental animals

Male 6 week-old ddY mice purchased from SLC Inc. (Shizuoka, Japan) were used as experimental subject of this study. The mice room (individual animal breeding system LP-80 CCFL-6ARSS, NK system) was maintained at 24 ± 1 ^{o}C and 50 ± 10% humidity under a 12 h light / dark cycle. The animals had free access (*ad libitum*) to water and food which was purchased from Nosan Corp. (Yokohama, Japan) for 1 week to accommodate the surroundings.

Animal studies were conducted according to the 2006 guidelines entitled: “Notification No.88 of the Ministry of the Environment in Japan and Guidelines for Animal Experimentation of Tokyo University of Marine Science and Technology” with the approval of the Animal Care and Use Committee of Tokyo University of Marine Science and Technology.

2.3 Preparation of sample extract

2.3.1 *Ocimum sanctum*

The dried powders and fresh leaves of *Ocimum sanctum* were supplied from Ishigaki island Modama workshop (Okinawa, Japan) and Kanazu Giken Co., Ltd (Fukui, Japan) in 2015. Dried powders from former supplier were extracted with 10-fold volume of three kinds of solvents: methanol, ethanol and hot water for 1 hr and soaked for 1-3 weeks. Extracts were separately filtered 3-5 times, and subsequently concentrated using rotary vacuum evaporator, then freeze dried overnight to obtain the crude extract which were dark green to brown powders. On the other hand, fresh leaves and dried powders from latter supplier were extracted with 10-fold volume of methanol and ethanol, respectively. Extracts were treated as the same way as the former one. The recovery rates were recorded as weight of crude extract/weight of powder % (w/w). These powdered extracts were stored at -20 ^{o}C prior to experimental schedule.

2.3.2 *Acer palmatum cv.* Nomura

Fresh leaves of *Acer palmatum cv.* Nomura were collected from the campus of university, Tokyo in 2014. The leaves were extracted in a similar method with that of *O. Sanctum* samples, with 10-fold volume of methanol and soaked for 1-3 weeks. Extracts were separately filtered 3-5 times, and subsequently concentrated using rotary vacuum evaporator, then freeze dried overnight to obtain the crude extract which were dark red powders. The recovery rates were recorded as weight of crude extract/weight of powder % (w/w). These powdered extracts were stored at -20^{o}C prior to experimental schedule.

2.4 Inhibitory activity against α-amylase assay

α-amylase inhibitory activity was assayed based on Xiao's method [31] with a slight modification. Briefly, a 2.5 mg/mL soluble starch solution was prepared by heating for 20 min at 100 ºC until completely dissolved, then cooled down to room temperature, and stored in 4 ºC. The enzyme, α-amylase (EC 3.2.1.1) 0.2 units solved in 0.1 M sodium phosphate buffer saline (PBS), iodine solution of 5 mM I_2 and 10 mM KI with 0.1 HCl was prepared. The evaluation was based on the residual starch which were not decomposed reacted and with iodine solution after reaction time. The assay was carried out in 96-well microtiter plates in a total volume of 300 μL.

Firstly, 50 μL sample of 2-fold serial dilution with PBS and 50 μL of α-starch (pre-gelatinized starch) solution were added into each well. Then the contents were incubated for 5 min at 37 ºC (Electronic low temperature chamber THS020DB model, Advantec). The reaction started with the addition of 100 μL of α-amylase and incubated for 10 min at 37 ºC. After second incubation, 100 μL iodine solution was added and the absorbance was measured at 590 nm wavelength. Test blanks for substrate were done without enzyme and another without starch as 0% and 100% of α-amylase activity, respectively. IC_{50} value was expressed by 50% of α-amylase inhibitory activity. The schematic flow of the process was shown in Fig. 2-1.

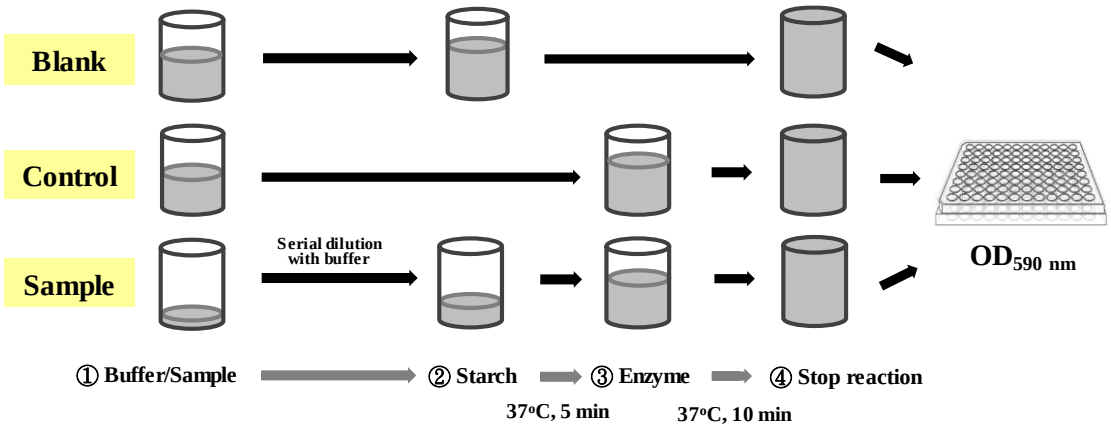


Fig.2-1 The schematic procedure of α-amylase inhibitory activity assay.

2.5 Glucose-loading tests in mice

Mice were fasted for 18-24 hours and divided into groups according to body weight (n=8) in advance. Each sample was dissolved in appropriate solvents and administered orally. In control group, mice were given glucose (1000 mg/kg body weight) dissolved in distilled water or triglyceride. In the sample-treated group, mice were given glucose and a sample with tested dosage.

Oral administration was performed by ingestion through to stomach with feeding needle. Blood was sequentially collected from the tail at 0 (before administration), 30 and 60 min after administration. Blood was collected in sodium heparin-containing micro-tubes and centrifuged at 4,500 *g* for 1 min (Centrifuge MCD-2000 model, Hsiangtai). After collecting the serum from the supernatant, the following assay was carried out in 96-well microtiter plates. Two μL of each serum was dispensed into the well (n=2) and the blood glucose concentration was measured by 300 μL of Glucose C-II Test Wako Kit on the absorbance at 490 nm wavelength. Blood glucose levels were calculated based on a calibration curve. The flow path was showed in Fig. 2-2.

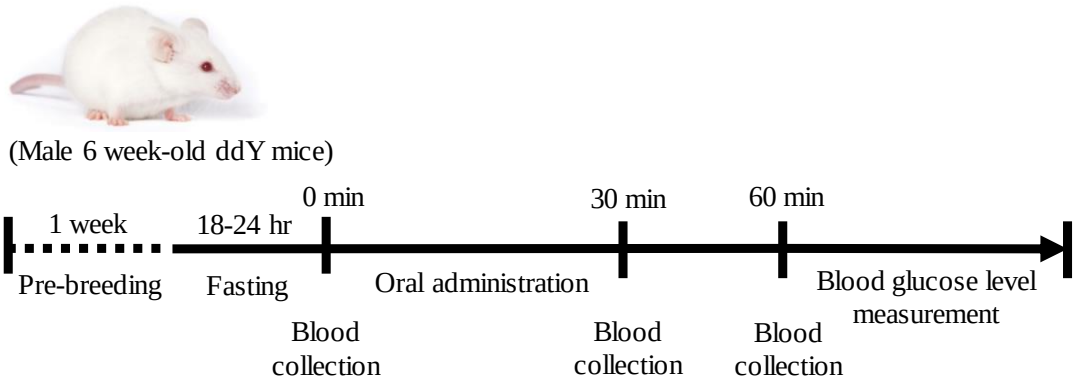


Fig.2-2 The flow path of glucose-loading test in mice.

2.6 Isolation and purification

2.6.1 The basic operation of isolation and purification

The solvent in the extracts were concentrated by the rotary vacuum evaporator (N-1100 type, Eleya), solvent recovery units (DPE-2120 type, Eleya), with pressure less than 30-50 mmHg controlled by diaphragm type dry vacuum pump (DTC-41 type, Eleya) and vacuum controller units (NVC-2100 type, Eleya). The temperature of water bath was maintained 25-35^{o}C. (SB-1100 type, Eleya). The sample was then subsequently lyophilized using a freeze dryer (FDU-1200, Eleya), with manifold for flask (PHM-4, Eleya) and Chemistry Hybrid pump (RC-6, Vacuubrand). Ultrasonic cleaner (VS-150, As One) was used for homogenization.

2.6.2 Column chromatography (CC)

TSK-Gel G3000S column packing (Tosoh) was used for this chromatographic method. The condition of CC was noted with the following details.

[column packing (weight), developing solvent]

2.6.3 Thin layer chromatography (TLC)

TLC plate of Silica 60 F_{254} (Cat.No. 1.05715) and ODS RP-18WF_{254S} 0.5 mm × 1.5 × 10 cm (Cat.No. 1.13124) purchased from E. Merck were used. The detection methods are exposure to UV (254 nm), immersed in *p*-anisaldehyde solution (500 mL of 95% ethanol, 13.7 mL of *p*-anisaldehyde, 18.4 mL of sulfuric acid and 5.6 mL of acetic acid) then heat on electronic griddle until the color appeared and turn to purple.

2.6.4 Preparative layer chromatography (PLC)

PLC plate of Silica gel 60 F_{254} 1 mm × 20 × 20 cm (Cat.No. 1.13895.0009) purchased from E. Merck were used. The detection methods are exposure of UV (254 nm), immersed in *p*-anisaldehyde solution (500 mL of 95% ethanol, 13.7 mL of *p*-anisaldehyde, 18.4 mL of sulfuric acid and 5.6 mL of acetic acid) then heat on electronic griddle until the color appeared and turn to purple.

2.6.5 High performance liquid chromatography (HPLC)

The master flex pump (PU-2080 plus, JASCO) and UV Spectrophotometer (UV-2075 plus, JASCO), column (Develosil Silica 30-5 and ODS HG-5, Nomura Chemical) were used for analysis and isolation of compound. The condition of HPLC noted with the following details.

[column (diameter (mm) × length (mm)), developing solvent, flow rate, detection]

2.7 Statistical analysis

Data were expressed as mean ± S.E. (standard error), and the statistical significant of differences between means was evaluated by Student's *t*-test, and the results were considered to be statistically significant when p values < 0.05. Thompson rejection test was used to find which was one outlier at a time.

2.8 Instrumental analysis

2.8.1 UV-Visible spectrophotometer

Molecular Devices Thermo Max Microplate Reader (31139, USA) was used for the measurement of absorbance. The condition was recorded as the following details.

UV: λ_{max} the maximum absorbance wavelength (nm)

2.8.2 Proton nuclear magnetic resonance (^{1}H NMR)

The machine of NMR was used for measuring (600 MHz, Bruker). The condition was recorded with the following form.

^{1}H NMR (measuring frequency, measuring solvent), δ chemical shift (multiplicity, spin coupling, proton).

Chemical shift was based on the residual proton signal of internal standard [$^{13}CDCl_3$ (7.26 ppm) and $^{13}CH_3OD$ (3.31 ppm)] in the measuring solvent. Multiplicity was stated as *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet) and *m* (multiplet). Spin coupling *J* was described in Hz.

2.8.3 Carbon nuclear magnetic resonance (^{13}C NMR)

The machine of NMR was used for measuring (600 MHz, Bruker). The condition was recorded with the following form.

^{13}C NMR (measuring frequency, measuring solvent), δ chemical shift (multiplicity).

Chemical shift was based on the residual proton signal of internal standard [$^{13}CDCl_3$ (77.16 ppm) and $^{13}CH_3OD$ (49.00 ppm)] in the measuring solvent. Multiplicity of *s* (quaternary carbon), *d* (methine carbon), *t* (methylene carbon) and *q* (methyl carbon) were evaluated from HMQC spectrum.

2.8.4 Two-Dimension nuclear magnetic resonance (2D NMR)

The machine of NMR was used for measurement (600 MHz, Bruker). The condition was recorded as the following.

(1) COSY: Data point (2048 x 128)

(2) HMQC: Data point (1024 x 128), $\Delta 1 = 1/2\,J = 180$ ms [$^{1}J_{CH} = 145$ Hz]

(3) HMBC: Data point (1024 x 128), $\Delta m = 1/2\,J = 83$ ms [$^{2,3}J_{CH} = 6$ Hz]

2.8.5 High resolution mass spectrometry

The machine of mass spectrometry was used for measurement (MicrOTOF II, Bruker). The ionization method of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were used. The condition was recorded as estimated value of molecular weight (m/z) and calculated value of molecular formula ($C_xH_yO_z$).

Chapter 3

Effects of *Ocimum sanctum* Leaf Extracts on α-amylase Activity

Chapter 3 Effects of *Ocimum sanctum* leaf extracts on α-amylase activity

3.1 Introduction to *Ocimum sanctum* (OS)

Ocimum sanctum, syn. *Ocimum tenuiflorum*, also known as holybasil or Tulsi in Sanskrit name, is an aromatic plant which belongs to the family of *Lamiaceae*. It is native to India and widespread as a cultivated plant throughout the Southeast Asia and other tropical regions. It is an erect, multiple-branched subshrub, which normally grows up to 75-90 cm tall, has many hairs all over, leaves size are on the average around 2-4 cm in length, and holds a characteristic green color with purple in part of ovate shape, has close whorls reddish flower with strong scent in oil constituent.

OS is usually cultivated for religious and medicinal purposes. It is widely known as herbal tea in India and called as 'Queen of Herbs'. And it is used for its special flavor and aroma when cooked with oil in Thai dish 'Pad Kra Prao'.

It has been commonly used to treat diabetes in the Indian system of medicine since time immemorial. Some studies reported that OS possess hypoglycemic, anti-oxidative [32, 33], hypotensive [34], anti-ulcer [35], anti-inflammatory [36], anti-asthmatic [37], anti-stress [38], anti-bacterial, anti-fungal [39], anti-cataract activity.[40, 41]

A study in diabetic-induced rats showed that treatment with isolated fraction of OS was a potent antidiabetic agent by the reduction of blood glucose level. The isolated compound elucidated as a tetracyclic triterpenoid structure ($C_{27}H_{46}O_2$). [42] Other study suggested that 70% ethanol extract of OS leaves showed a significant blood sugar lowering effect in normal and streptozotocin-induced diabetic model rats at a dose dependent manner. It improved oral glucose tolerance and potentiated the activity of exogenously injected insulin.[43]

OS leaf extracts exert prominent stimulatory effects on insulin secretion from the β-cells via physiological pathways. *In vivo* studies also indicate that ethanol extract decreased blood glucose and increased plasma insulin in type 2 diabetic rats.[44]

In one of the clinical trials, noninsulin dependent diabetes mellitus (NIDDM) patients take dried powder 2.5 g/day orally on empty stomach could reduce the fasting glucose level of up to 21 mg/dL and the postprandial blood glucose by 15.8 mg/dL.[45]

3.2 Preparation of OS

One of OS was donated by Modama workshop (Ishigaki island) which came in 2 kinds of powder: the Krishna (BPM1) and Kapool (BPM2). These two types of leaves were extracted with three different solvents: methanol, ethanol and hot water at 10-fold volume. Crude extracts were obtained with a characteristic appearance of dark green (methanol), dark green (ethanol), brown (hot water) color, respectively.

Another supporter Kanazu Giken Co. (Kanazawa) supplied fresh leaves of OS with two kinds of leaves, Krishna (BLK1), Kapool (BLK2) and powder of Krishna (BPK1) respectively. The leaves are of the same species as the previous one (BPM1 and BPM2), but were cultivated in different locations. The two types of fresh leaves were extracted with methanol at a 10-fold volume. BLK1 and BLK2 were obtained with the appearance of dark green color. The powder form (BPK1) was extracted with ethanol at a 10-fold volume. BPK1 extracts were obtained with the appearance of dark green color. After this process, these five extracts were kept in -20 ^{o}C prior the experiment. All the extracts are referred with abbreviated names as reflected in Table 3-1.

Table 3-1 Different type and species of *Ocimum sanctum*.

Supporter	Type	Species	Leaves color	Abb.
Modama workshop (Ishigaki island)	Powder	Krishna	purple	BPM1
	Powder	Kapool	green	BPM2
Kanazu Giken Co. (Fukui)	Leaves	Kapool	green	BLK1
	Leaves	Krishna	purple	BLK2
	Powder	Krishna	purple	BPK1

Fig.3-1 Fresh leaves of BLK1 (left) and BLK2 (right).

3.3 Anti-hyperglycemic effects of BPM1 and BPM2 in α-amylase inhibitory activity test

The two leaves were weighed (3.0 g) and extracted with three kinds of solvents: methanol, ethanol and hot water at a 10-fold volume. The recovery rate (%) of each extract were at 22.28 % (methanol; MeOH), 13.62 % (ethanol; EtOH) and 27.31 % (hot water; HW) in BPM1; 19.65 % (MeOH), 14.66 % (EtOH), and 23.62 % (HW) in BPM2.

The effect of six extracts of BPM1 and BPM2 (200-1600 μg/mL) on the α-amylase activity was examined in enzymatic inhibitory activity test *in vitro* in Fig.3-2.

It is shown that only MeOH and EtOH extracts have concentration-dependent α-amylase activity. The IC_{50} value of extracts are at: 1018.9 μg/mL (MeOH), 692.4 μg/mL (EtOH) in BPM1, 746.7 μg/mL (MeOH), 394.4 μg/mL (EtOH) μg/mL in BPM2. There was no detection found in hot water extracts. The IC_{50} value of positive control acarbose is at 14.1 μg/mL. In addition, EtOH extracts of both BPM1 and BPM2 have 1.5 times higher α-amylase inhibitory activity when compared with the MeOH extracts.

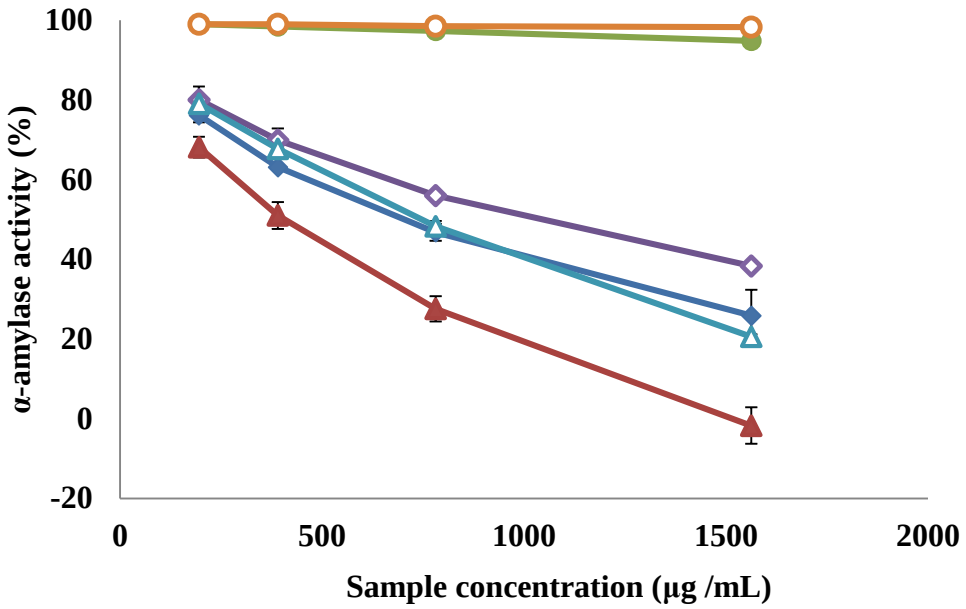


Fig. 3-2 Inhibitory effects on α-amylase activity. Extracted with MeOH (filled and open diamonds), EtOH (filled and open triangles), and HW (filled and open circles) from BPM1 and BPM2, respectively. α-amylase activity was expressed as % of control without samples. Data was represented as the mean ± S.E. (n=3).

3.4 Anti-hyperglycemic effects of BLK1 and BLK2 in α-amylase inhibitory activity test

Fresh leaves of BLK1 and BLK2 were collected, weighed (41.35 g and 36.89 g, respectively), and subsequently extracted with methanol at a 10-fold volume. The recovery rate (%) of each extract is at 5.83 % and 3.90 %. Dried extracts were obtained at 2.41 g and 1.44 g.

The effects of the two extracts of BLK1 and BLK2 (39.1-1250 μg/mL) on the α-amylase activity was examined in enzymatic inhibitory activity test *in vitro*, and results are presented in in Fig.3-3.

It is shown that two of extracts have a concentration-dependent activity on α-amylase. The IC_{50} value of the extracts are: 1525.9 μg/mL and 1053.7 μg/mL, respectively. The IC_{50} value of positive control: acarbose is at 7.0 μg/mL. In addition, MeOH extract of BLK1 has 1.4 times higher α-amylase inhibitory activity when compared to BLK2.

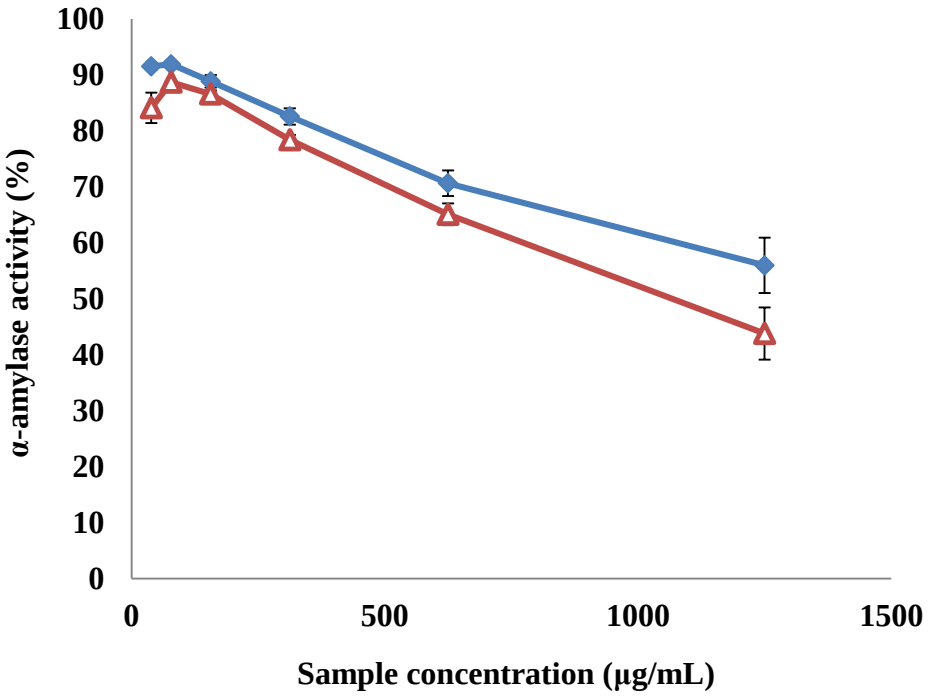


Fig.3-3 Inhibitory effects of methanol extract of BLK1 (filled diamonds) and BLK2 (open triangles) on α-amylase activity. α-amylase activity was expressed as % of control without samples. Data was represented as the mean ± S.E. (n=3).

3.5 Anti-hyperglycemic effects of BPK1 in α-amylase inhibitory activity test

The powder (BPK1) was weighed (20.24 g) and extracted with ethanol at a 10-fold volume, and was able to obtain 1.49 g extract with 7.40 % recovery rate. The extraction scheme is shown in Fig.3-4.

The effects of powder extracts of BPK1 (24.4-781.2 μg/mL) on the α-amylase activity was examined in an enzymatic inhibitory activity test *in vitro*, and results are presented in in Fig.3-5.

It is shown that BPK1 extract has a concentration-dependent activity on the α-amylase. The IC_{50} value of extract is at 363.0 μg/mL. The IC_{50} value of positive control acarbose is at 16.8 μg/mL.

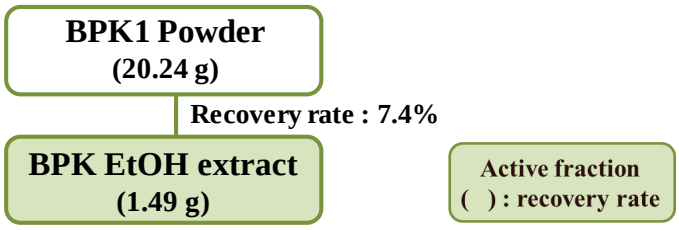


Fig.3-4 Extraction scheme of BPK1 in EtOH solvent.

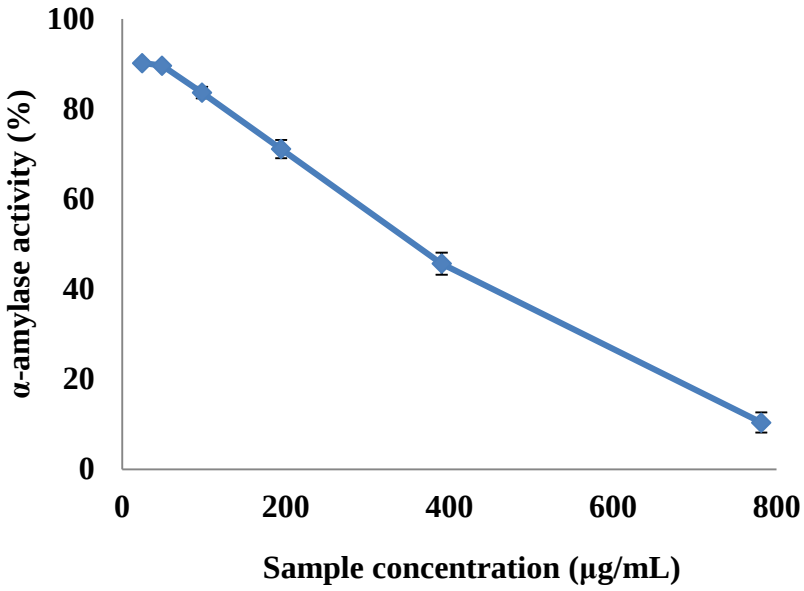


Fig.3-5 Inhibitory effects of EtOH extract (filled diamonds) of BPK1 on α-amylase activity. α-amylase activity was expressed as % of control without samples. Data was represented as the mean ± S.E. (n=3).

3.6 Anti-hyperglycemic effects of ethyl acetate and water layers of BPK1 in α-amylase activity test

Since BPK1 ethanol crude extract has a potential to suppress α-amylase activity from previous results. The extract was separated into two layers from the succeeding part of the procedure. Firstly, BPK1 extract was dissolved in water and the same amount of ethyl acetate was added. The two layers were mixed, let to stand for a while, were separately collected and concentrated by rotary evaporator. The recovery rates of ethyl acetate and water are at 40.9 % and 59.0 %, respectively. Isolation scheme is shown in Fig.3-6.

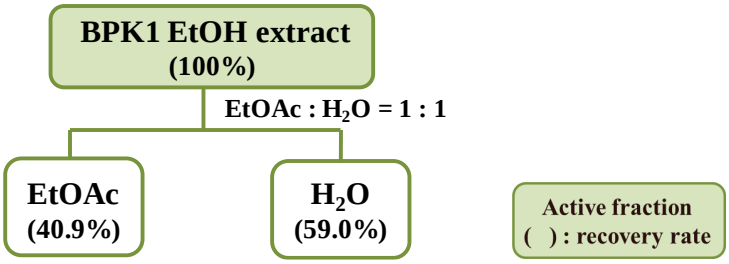


Fig.3-6 Isolation scheme of BPK1 from EtOH extract.

The effects of ethyl acetate and water layers of BPK1 (39.1-312.5 μg/mL and 10.9-350 μg/mL, respectively) on α-amylase activity was examined in an enzymatic inhibitory activity test *in vitro* and results are presented in Fig.3-7.

It is shown that only EtOAc layer of BPK1 extract has a concentration-dependent activity on α-amylase. The IC_{50} value of EtOAc layer BPK1 is at 174.1 μg/mL. There was no detection of activity in the water layer of BPK1. The IC_{50} value of positive control acarbose is 15.3 μg/mL.

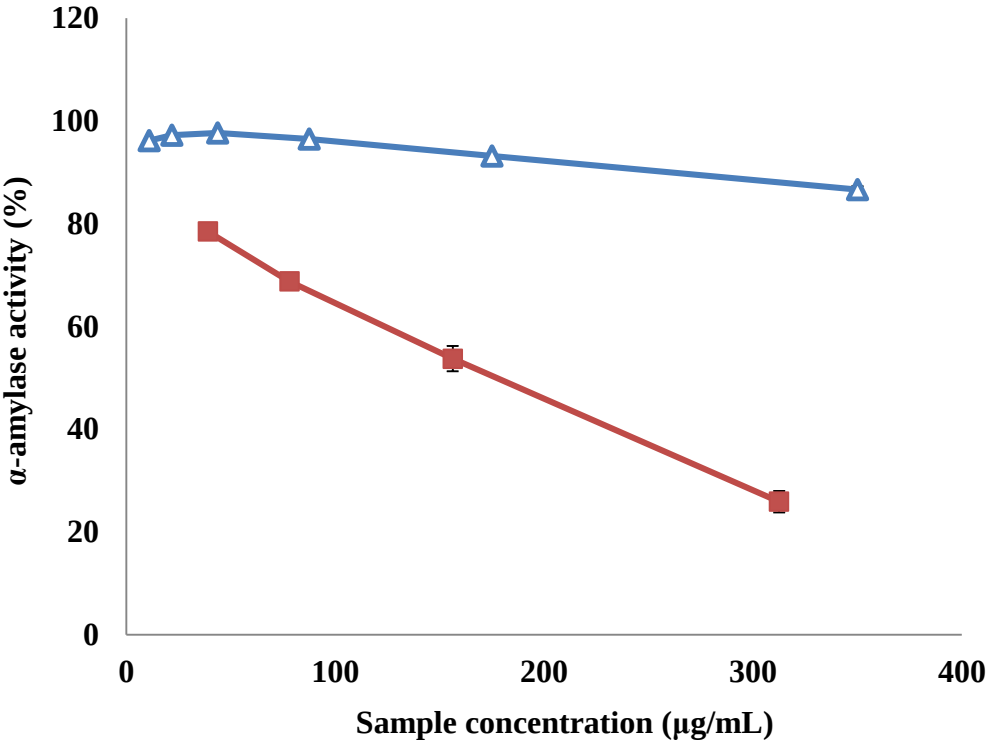


Fig.3-7 Inhibitory effects of EtOAc layer (filled squares) and water layer (open triangles) of BPK1 on α-amylase activity. α-amylase activity was expressed as % of control without samples. Data was represented as the mean ± S.E. (n=3).

3.7 Anti-hyperglycemic effects of 90%MeOH and hexane layers of BPK1 in α-amylase activity test

Since ethyl acetate layer of BPK1 has a potential to suppress α-amylase activity based from previous results. The extract was separated into two layers on the succeeding part of the procedure. Firstly, EtOAc layer extract of BPK1 was dissolved in 90% MeOH, and the same amount of hexane solvent was added. The two layers were mixed, let to stand for a while, separately collected, and concentrated by rotary evaporator. The recovery rates of two layers are at 24.0% and 16.4%, respectively. Isolation scheme is shown in Fig.3-8.

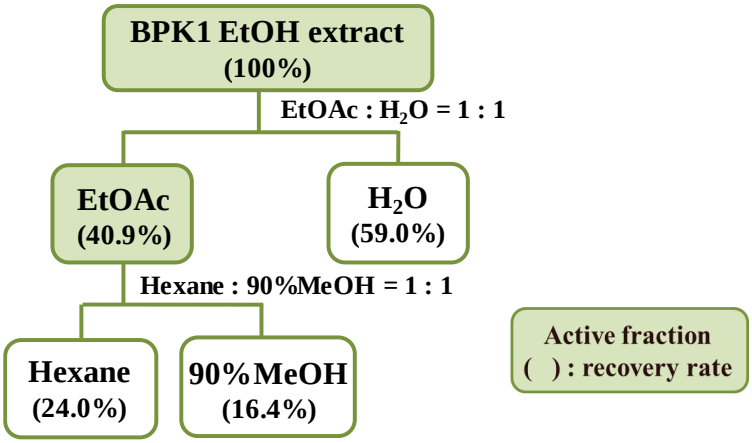


Fig.3-8 Isolation scheme of BPK1 from EtOAc extract.

The effects of hexane and 90% MeOH layers of BPK1 (39.1-312.5 μg/mL) on α-amylase activity was examined in an enzymatic inhibitory activity test *in vitro* anf results are presented in Fig.3-9.

It is shown that both hexane and 90% MeOH layer of BPK1 extract have a concentration-dependent activity on α-amylase. The IC_{50} values of hexane and 90 % MeOH layer of BPK1 are at 231.7 μg/mL and 993.8 μg/mL respectively. The IC_{50} value of positive control acarbose is at 15.3 μg/mL. It is known that hexane layer has 4 times stronger α-amylase inhibitory activity when compared to 90 % MeOH layer of BPK1. Therefore, it is considered that hexane layer of BPK1 extract has a potential to suppress α-amylase activity.

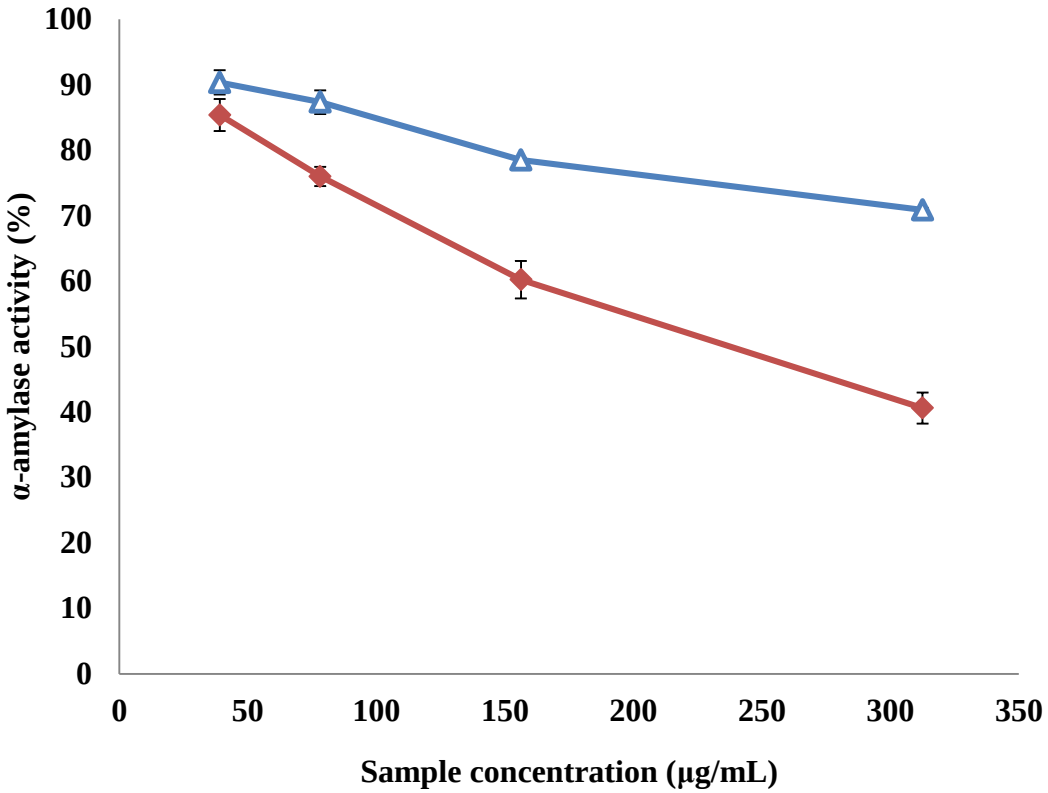


Fig.3-9 Inhibitory effects of hexane layer (filled diamonds) and 90%MeOH layer (open triangles) of BPK1 on α-amylase activity. α-amylase activity was expressed as % of control without samples. Data was represented as the mean ± S.E. (n=3).

3.8.1 Separation of hexane layer of BPK1 by HPLC fractionation

Hexane layer of BPK1 was separated into ten fractions by HPLC fractionation. Representative HPLC chromatogram is shown in Fig.3-10. After which, TLC was used to confirm the difference in each fraction, and were divided into four groups for glucose-loading test (Fig.3-11). R_f value of Fr.1-10 are 0.90, 0.90, 0.78, 0.71 and 0.56, 0.51, 0.53 and 0.44, 0.44, 0.42, 0.42, 0.26 and 0.0, respectively. The recovery rates of Fr.1-10 are at 0.30%, 2.32%, 0.49%, 0.36%, 0.66%, 0.33%, 0.51%, 3.11%, 5.47%, and 10.46% respectively. Fr.10 was collected after 20 min and washed with EtOAc and MeOH solvent. The isolation scheme is shown in Fig.3-12.

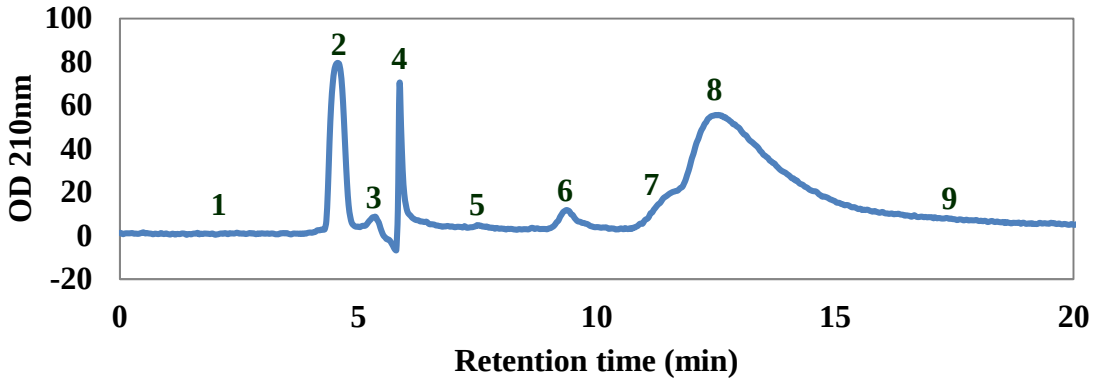


Fig.3-10 Representative HPLC chromatograph of hexane layer of BPK1 with the following chromatographic conditions: Develosil 30-5 (20 x 250 mm) column, mobile phase hexane:EtOAc (3:1), λ = 210 nm, 10 mL/min flow rate. 1.0 mL injection of 10 mg/mL sample.

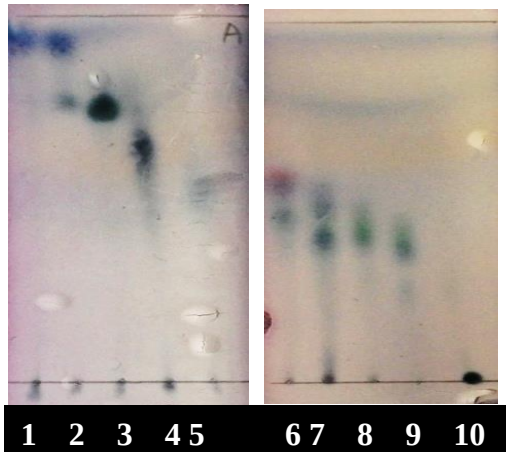


Fig.3-11 TLC chromatograph of each HPLC fractions after with the following chromatographic conditions: silica plate, mobile phase hexane:EtOAc (3:1), *p*-anisaldehyde in ethanol:sulfuric acid = 27:1 solution stain visualized after heat treatment.

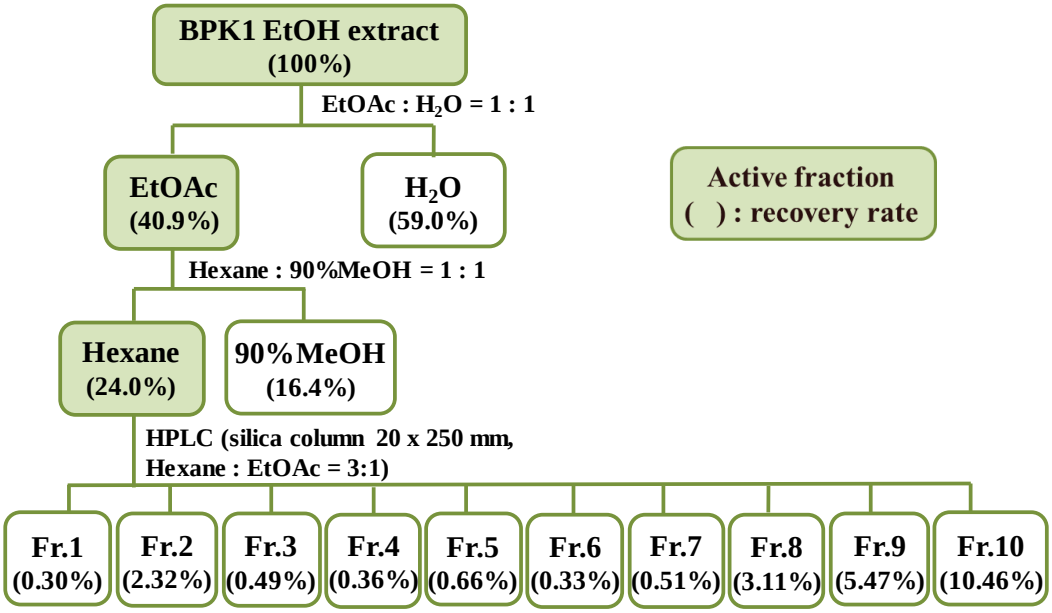


Fig.3-12 Isolation scheme of BPK1 after HPLC fractionation.

3.8.2 Anti-hyperglycemic effects of the HPLC fractions from the hexane layer of BPK1 in α-amylase activity test

The effects of each group of hexane layer of BPK1 after HPLC fractionation on α-amylase activity was examined in an enzymatic inhibitory activity test *in vitro* and results are presented in Fig.3-13. From Fig.3-12, seven fractions (Fr.2, Fr.3, Fr.4, Fr.6, Fr.8, Fr.9, Fr.10) were selected to be examined the α-amylase inhibitory activity test.

It is shown that all the fractions of BPK1 extract have a concentration-dependent activity on α-amylase. The IC_{50} value of each fraction are at 258.1 μg/mL, 337.8 μg/mL, 219.5 μg/mL, 142.8 μg/mL, 303.3 μg/mL, 613.9 μg/mL and 235.1 μg/mL respectively. The IC_{50} value of positive control acarbose is at 18.2 μg/mL. It is determined that the top three potential fractions which have stronger α-amylase activity are Fr.6, Fr.4 and Fr.10, respectively. It is considered that Fr.6 of BPK1 extract has strongest potential to suppress α-amylase activity among the different fractions.

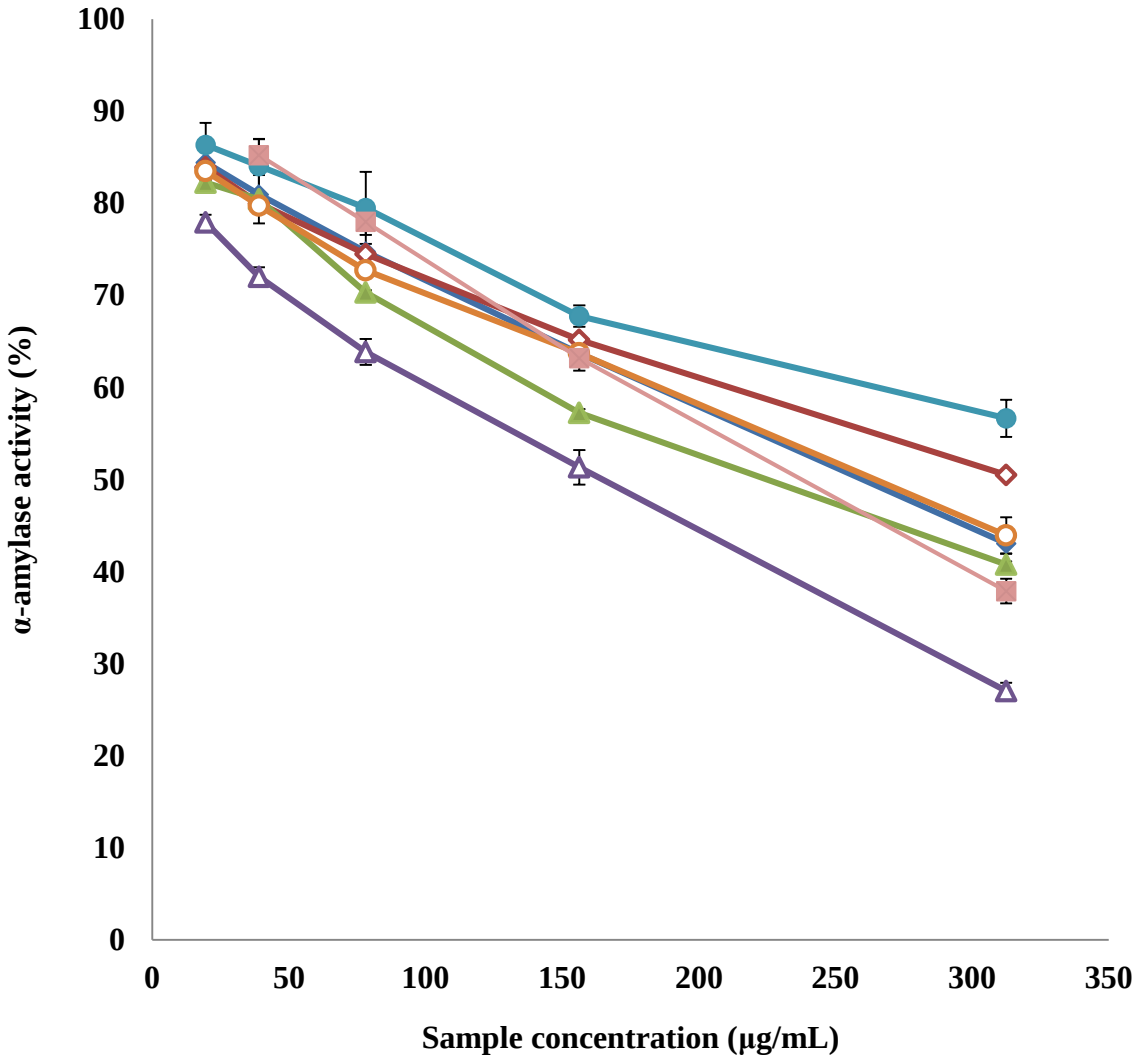


Fig.3-13 Inhibitory effects of Fr.2 (filled diamonds), Fr.3 (open diamonds), Fr.4 (filled triangles), Fr.6 (open triangles), Fr.8 (filled circles), Fr.9 (open circles) and Fr.10 (filled squares) of BPK1 after HPLC analysis on α-amylase activity. α-amylase activity was expressed as % of control without samples. Data was represented as the mean ± S.E. (n=3).

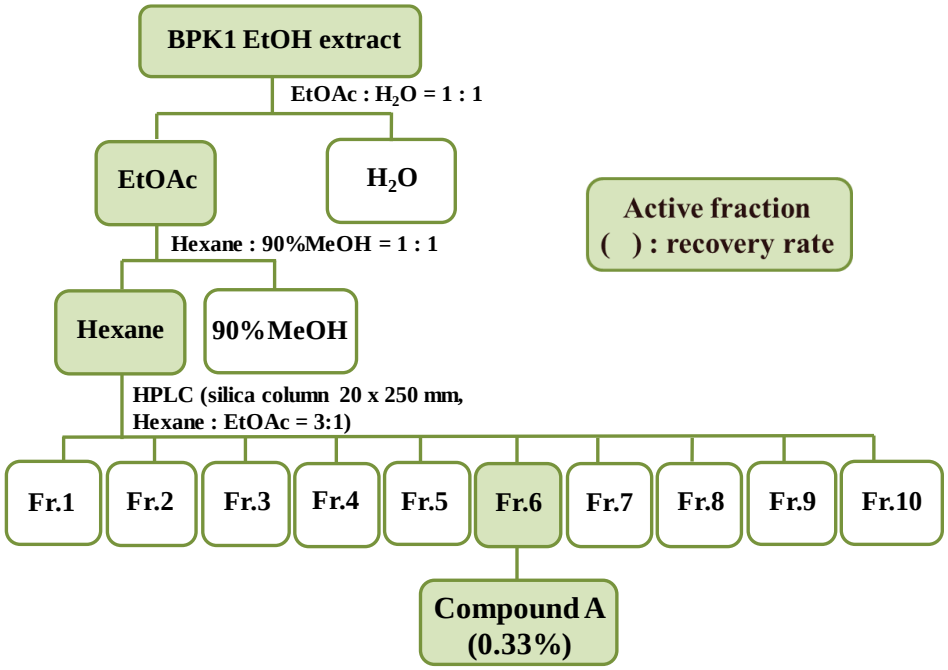


Fig.3-14 Isolation scheme of BPK1 from ethanol crude extract to hexane layer of HPLC fractionation.

3.9 Structure estimation of compound A in BPK1

Compound A from Fr.6 of hexane layer in BPK1 was speculated to inhibit α-amylase activity (Fig.3-13), with a single spot observed on the TLC plate (Fig.3-11). The chemical structure was analyzed using NMR. All the spectra data were shown in the Appendix (^{1}H NMR, ^{13}C NMR, COSY, HMQC and HMBC).

The extract from which compound A was isolated has light green color, which is given from hexane-soluble fraction. And the molecular weight was measured by electrospray ionization mass spectra (ESIMS) and required the molecular peak at [m/z 360.32 $(M+Na)^{+}$], the molecular formula was intended to $C_{21}H_{28}O_5$.

In ^{1}H NMR spectra, at low field (δ_H around 5.3 ppm) ethylene group were observed, and at δ_H 4.8 and 2.8 ppm showed few hydroxyl groups. Furthermore, in ^{13}C NMR spectra, from DEPT and HMQC, carboxylic acid (δ_C 176.8 ppm), and (δ_C 130, 128, 127 ppm) ethylene group were existed. Besides the spectra mentioned above, the chemical structure of compound A could be speculated in COSY and HMBC spectra.

The chemical structure of compound A is under consideration, other way to certify the integrated structure is needed.

3.10 Discussion

In this study, the anti-hyperglycemic activity of holybasil leaves *Ocimum sanctum* (OS) was determined using an α-amylase activity assay. To elucidate operation mechanism, the following experiments *in vitro* were performed. The effects of BPM1 and BPM2 extracted in three different solvents in α-amylase inhibitory activity test is showed in Fig.3-2. BPM1 showed the strongest activity among them, which is implicated that the species Krishna has a more potent α-amylase activity than Kapool, and ethanol is more preferred to be use as the extraction solvent.

The effects of BLK1 and BLK2 in α-amylase inhibitory activity test is shown in Fig.3-3. BLK2 has higher α-amylase inhibitory activity compared with BLK1. With the comparison between Fig.3-2 and Fig.3-3, powder holds more α-amylase inhibitory components than fresh leaves. One of reason is considered that fresh leaves contain more moisture even after extraction with organic solvent. In contrast, between BPM1 and BPK1, with different source but the same species (Krishna) and organic solvent (ethanol), the inhibitory activity of BPK1 in Kanazu was higher than BPM1 in Ishigaki according to IC_{50} value (363.0 and 692.4 μg/mL, respectively).

BPK1 extract was chosen for further bioassay-guided fractionation but not only the species Krishna in powder form, but also the extract from ethanol. Result presented in Fig.3-5 shows that ethanol extract of BPK1 has the inhibition activity on α-amylase. The results of the fractions from ethyl acetate and hexane layer in α-amylase inhibitory test are shown in Fig.3-7 and Fig.3-9, respectively. The results revealed that the hydrophobic component has a potential to suppress glucose absorption by inhibiting α-amylase activity.

Results of the HPLC fractions from hexane layer of BPK1 are shown in Fig.3-13. We found that compound A separated from Fr.6 which has promising effects of suppressing α-amylase activity. Compound A is to be confirmed by ESIMS and NMR. In this study, compound A was approximately 0.33 % of the BPK1 extract.

The IC_{50} values of each sample and the comparison with acarbose is shown in Table 3-2. It is reported that OS leaf has anti-diabetic effect in diabetic-induced rats. However, there are no previous studies that reported on the possible inhibitory mechanism in glucose metabolism of the OS extract, and the characteristics of compounds present in the extract. This is the first research about the anti-hyperglycemic effects of α-amylase activity from OS ethanol extract.

In the prospective study, the pharmacological actions and toxic reaction of compound A from OS extract should be investigated and compared with those of other anti-diabetic drugs present in the market with similar mechanisms of α-amylase inhibitions during long-term administration in diabetic animal models.

Table 3-2 The IC_{50} values of each sample and the comparison with acarbose.

Sample	Extracted solvents / fractions	Sample IC_{50} value (μg/mL)	Acarbose IC_{50} value (μg/mL)	Sample / Acarbose
BPM1	MeOH	1018.9	14.1	72
	EtOH	692.4	14.1	49
	Hot water	-	14.1	-
BPM2	MeOH	746.7	14.1	53
	EtOH	394.4	14.1	28
	Hot water	-	14.1	-
BLK1	MeOH	1525.9	7.0	218
BLK2	MeOH	1053.7	7.0	151
BPK1	EtOH	363.0	16.8	22
	EtOAc	174.1	15.3	11
	Water	-	15.3	-
	Hexane	231.7	15.3	15
	90%MeOH	993.8	15.3	65
	Fr.2	258.1	18.2	14
	Fr.3	337.8	18.2	19
	Fr.4	219.5	18.2	12
	Fr.6	142.8	18.2	8
	Fr.8	303.3	18.2	17
	Fr.9	613.9	18.2	34
	Fr.10	235.1	18.2	13

Chapter 4

Effects of *Acer palmatum* Leaf Extracts on Postprandial Elevation of the Blood Glucose Level in Mice

Chapter 4 Effects of *Acer palmatum cv.* Nomura leaf extracts on postprandial elevation of the blood glucose level in mice

4.1 Introduction to *Acer palmatum* cv. Nomura (APN)

Acer palmatum cv. Nomura, syn. *Acer palmatum Thunb var. amoenum cv.* Sanguineum is a kind of Japanese maple, which is native to Japan, China, Korea, and commonly cultivated throughout the United States and Europe as an ornamental tree. *A. palmatum cv.* Nomura belongs to the family of *Sapindaceae*, a small tree up which grows up to 6-10 m in height with a spreading crown. Leaves are characterized to have 7-9 lobes, with double-tooth separated by deep narrow V-shaped notches. [46] The leaves hold an almost red to purple color, which turns to green during the hot season. In Shizuoka, Japan, leaves are being used as a coloring agent in sushi.

A study using hot water extract of APN has showed anti-hyperglycemic effects in glucose-loading test and sucrose-loading test in ddY male mice. [47] In another study, vitexin (apigenin-8-C-β-D-glucopyranoside) was isolated from ethyl acetate soluble fraction of *Acer palmatum* which may be used as antioxidative agent to prevent UV-induced adverse skin through free radical production which then causes skin cell damage. [48]

4.2 Preparation of APN

Fresh leaves of APN were collected from the campus of university, Tokyo during the month of April. Leaves were extracted using a 10-fold volume of methanol as solvent. The crude extract obtained has the characteristic appearance having red to purple color in powdered form. The powder was then kept in -20^{o}C prior the experiment.

Fig.4-1 Fresh leaves and dried methanol crude extract of APN.

4.3 Anti-hyperglycemic effects of APN in glucose-loaded mice

Fresh leaves of *Acer palmatum cv.* Nomura (referred to as APN below) were collected and weighed (523.39 g), cut into small pieces, and subsequently extracted using methanol as solvent while uniformly stirred using a mixer. The crude extract was evaporated using a rotary evaporator with vacuum, and 43.67 g of dry materials was obtained with a recovery rate of 10.4%. Extraction scheme is shown in Fig.4-2.

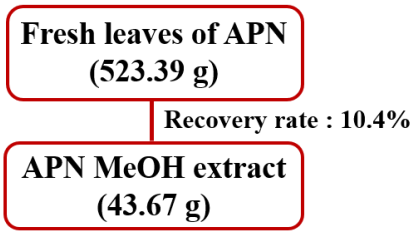


Fig.4-2 Extraction scheme of fresh leaves of APN.

The effects of APN on the postprandial blood glucose level was examined in glucose-loaded mice *in vivo*, as shown in Fig.4-3. In the control group (glucose administration only), the blood glucose level reached a maximum value 183 mg/dL at 30 min after ingestion to the mice. Then the level decreased gradually at 60 min. Phlorizin (150 mg/kg) was used as positive control. When APN extract (1,000 and 2,000 mg/kg) was orally administered simultaneously with glucose, the blood glucose level at 30 min after administration was significantly suppressed 25.6%and 30.2%, respectively, in a dose-dependent manner when compared with the control group.

Based on the results gathered as presented in Fig.4-3, it is considered that APN crude extract has potential to inhibit postprandial blood glucose level. After confirming the activity, APN was divided into two layers to further determine some active constituents present in APN.

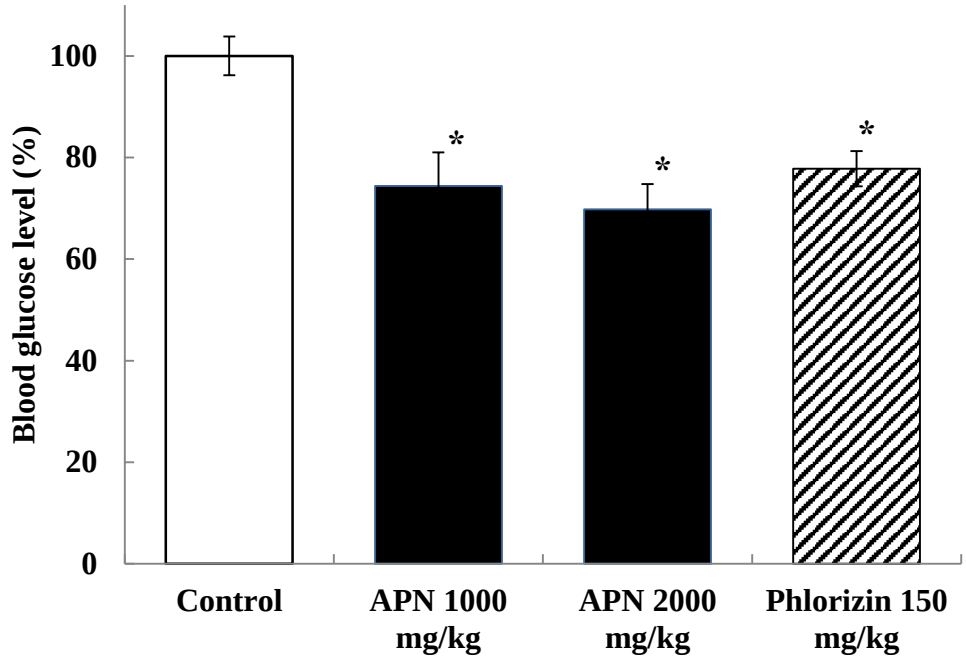


Fig.4-3 Effects of APN crude extracts at rates of 1000 mg/kg of APN 1, 2000 mg/kg of APN 2, 150 mg/kg of phlorizin and vehicle (control) in postprandial elevation in blood glucose level (%) on six-week-old male Slc:ddY mice orally loaded with glucose and measured at 30 min. Data are presented as the mean ± S.E. (n=8). *p<0.05 vs. control.

4.4 Anti-hyperglycemic effects of ethyl acetate and water layers of APN extracts in glucose-loaded mice

Since APN methanol crude extract has potential to decrease blood glucose level from previous results, the extract was separated into two layers on the next part of the procedure. Firstly, the APN dried extract was dissolved in water, and the same volume of ethyl acetate was added. The two layers were mixed and let to stand for a while, collected separately, and concentrated by rotary evaporator. The recovery rate of ethyl acetate and water layer were at 23% and 77%, respectively. Isolation scheme is shown in Fig.4-4.

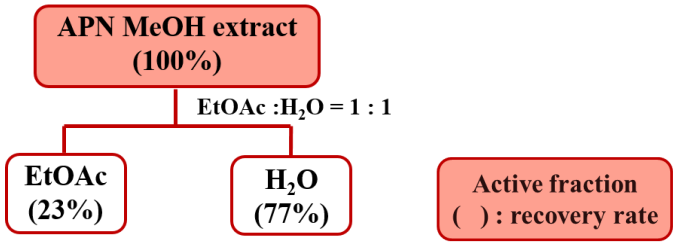


Fig.4-4 Isolation scheme of APN methanol extract.

The effects of ethyl acetate and water layers of APN on the postprandial blood glucose level were examined in glucose-loaded mice, as shown in Fig.4-5. In the control group (glucose administration only), the blood glucose level reached a maximum value of 149 mg/dL at 30 min after the glucose ingestion to the mice, and decreased gradually at 60 min. When ethyl acetate layer (687 mg/kg) and water layer (2304 mg/kg) of APN extract were orally administered simultaneously with glucose, the blood glucose level at 30 min after administration of both two layers were significantly suppressed 25.2% and 17.1%, respectively, when compared with the control group.

Based on the results presented in Fig.4-5, it can be considered that ethyl acetate and water layers have potentials to inhibit postprandial blood glucose level. In addition, the former contains stronger active constituent depending on the amount of dose. After confirming the activity, ethyl acetate layer was divided into two layers from next step to the following steps thereafter to determine possible active substance present in APN.

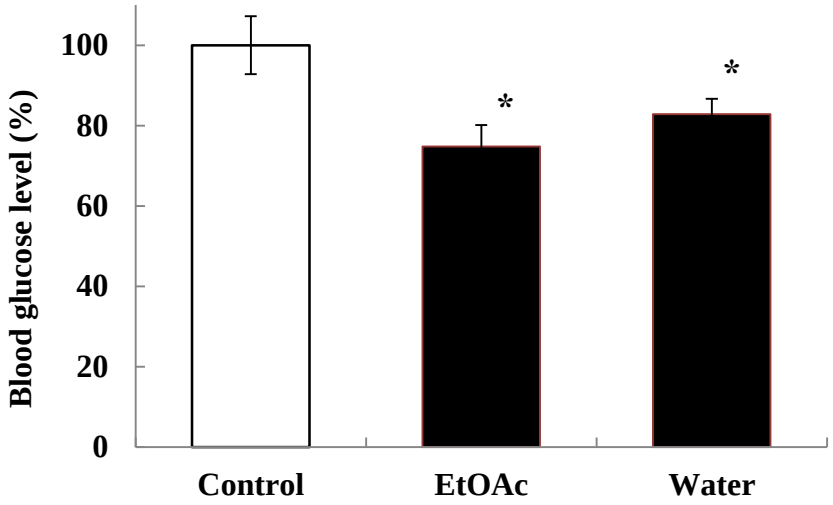


Fig.4-5　Effects of ethyl acetate and water layers of APN at rates of 687 mg/kg of EtOAc layer extract, 2304 mg/kg of water layer extract, and vehicle (control) on postprandial elevation in the blood glucose level (%) on six-week-old male Slc:ddY mice orally loaded with glucose and measured at 30 min. Data are presented as the mean ± S.E. (n=8). *p<0.05 vs. control.

4.5 Anti-hyperglycemic effects of hexane and 90%MeOH layers of APN in glucose-loaded mice

Since ethyl acetate layer of APN was considered to have a potential to decrease blood glucose level from the previous results, the extract was separated two layers from the next part of the extraction procedure. Firstly, dried EtOAc layer extract of APN was dissolved in 90% MeOH, and the same volume of hexane was added. The two layers were mixed and let to stand for a while. The two layers were collected separately and concentrated by rotary evaporator. The recovery rate of two layers are at 4 % and 19 %, respectively. Isolation scheme is shown in Fig.4-6.

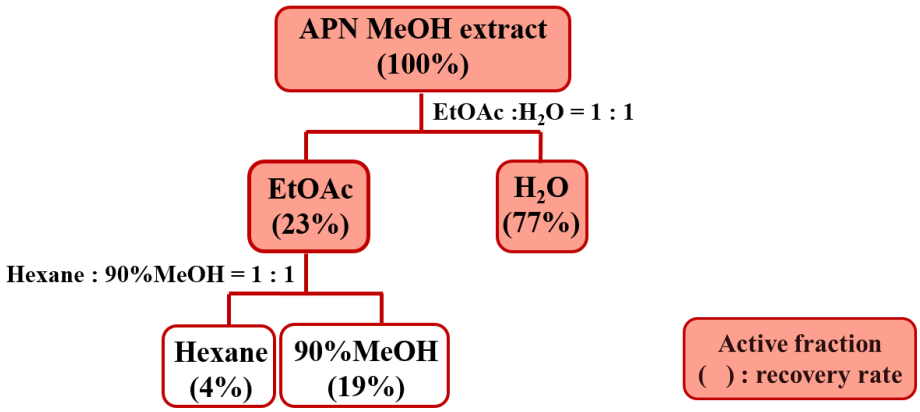


Fig.4-6 Isolation scheme of ethyl acetate layer of APN.

The effects of hexane and 90%MeOH layers of APN on the postprandial blood glucose level were examined in glucose-loaded mice, as shown in Fig.4-7. In the control group (glucose administration only), the blood glucose level reached a maximum value of 160 mg/dL at 30 min after ingestion to the mice, and the level decreased gradually at 60 min. When hexane layer (120 mg/kg) and 90% MeOH layer (560 mg/kg) of APN extract were orally administered simultaneously with glucose, the blood glucose level at 30 min after administration, hexane layer was significantly suppressed 20.3% when compared with the control group.

Based on the results presented in Fig.4-7, can be considered that the hexane layer has a potential to inhibit postprandial blood glucose level. After confirming the activity, hexane layer was divided into eleven layers to further determine which fraction contained the active constituent in APN.

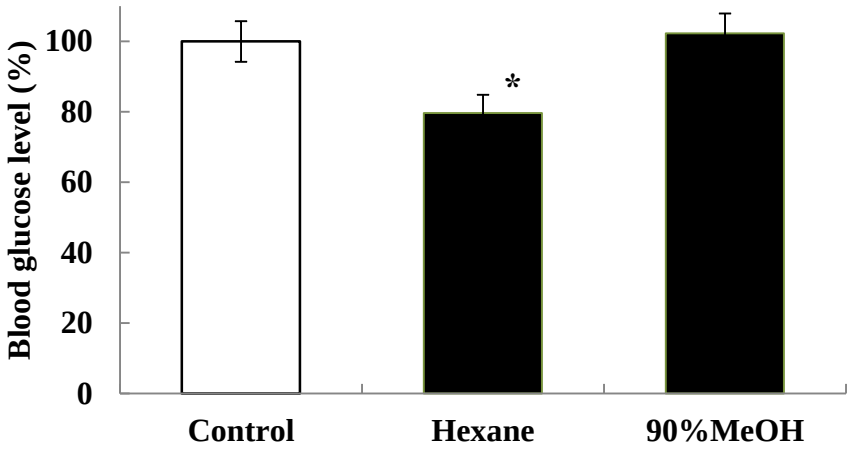


Fig.4-7　Effects of hexane and 90% MeOH layer of APN extracts at rates of 120 mg/kg of hexane layer extract, 560 mg/kg of 90%MeOH layer extract, and vehicle (control) on postprandial elevation in the blood glucose level (%) on six-week-old male Slc:ddY mice orally loaded with glucose and measured at 30 min. Data are presented as the mean ± S.E. (n=8). *p<0.05 vs. control.

4.6.1 Separation of hexane layer of APN by HPLC fractionation

Hexane layer of APN was separated in to eleven fractions by HPLC fractionation. Representative HPLC chromatogram is shown in Fig.4-8. After which, TLC separation was used to confirm the difference in each fraction and divided into four groups for the glucose-loading test (Fig.4-9). R_f value of Fr.2-11 are 0.93, 0.88, 0.80, 0.63, 0.63, 0.55, 0.35, 0.33, 0.25 and 0.00 respectively. The recovery rates of Fr.A1-4, Fr.A5-7, Fr.A8-10, and Fr.A11 are 0.85%, 1.58 %, 0.87% and 0.69 % respectively. Fr.A11 was collected after 60 min washing with EtOAc and MeOH solvent. The isolation scheme is shown in Fig.4-10.

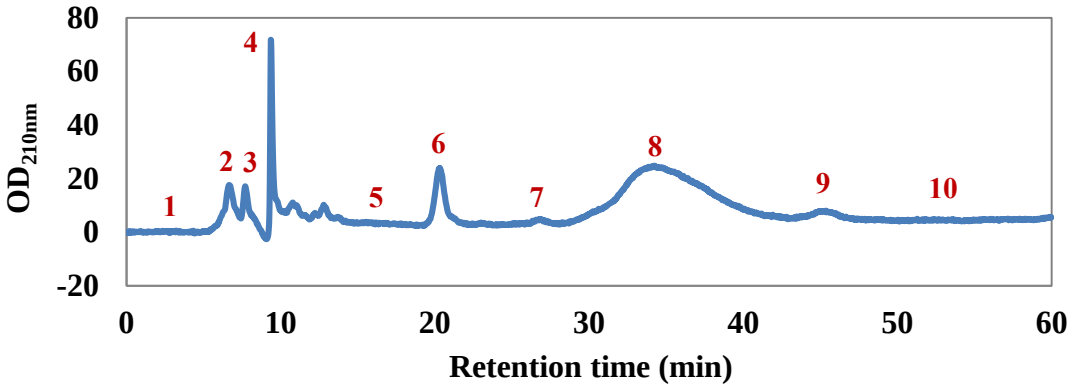


Fig.4-8 Representative HPLC chromatogram of the hexane layer of APN with the following chromatographic conditions: Develosil 30-5 (20 x 250 mm) column, mobile phase: hexane:EtOAc (3:1), λ = 210 nm, 5 mL/min flow rate, 1.5 mL injection volume of 10 mg/mL sample.

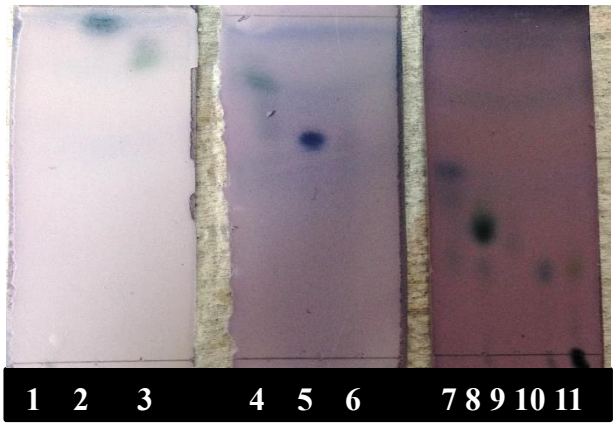


Fig.4-9 TLC chromatograph of each HPLC fractions with the following chromatographic conditions: silica plate, mobile phase: hexane:EtOAc (3:1), *p*-anisaldehyde in ethanol solution stain visualized after heat treatment.

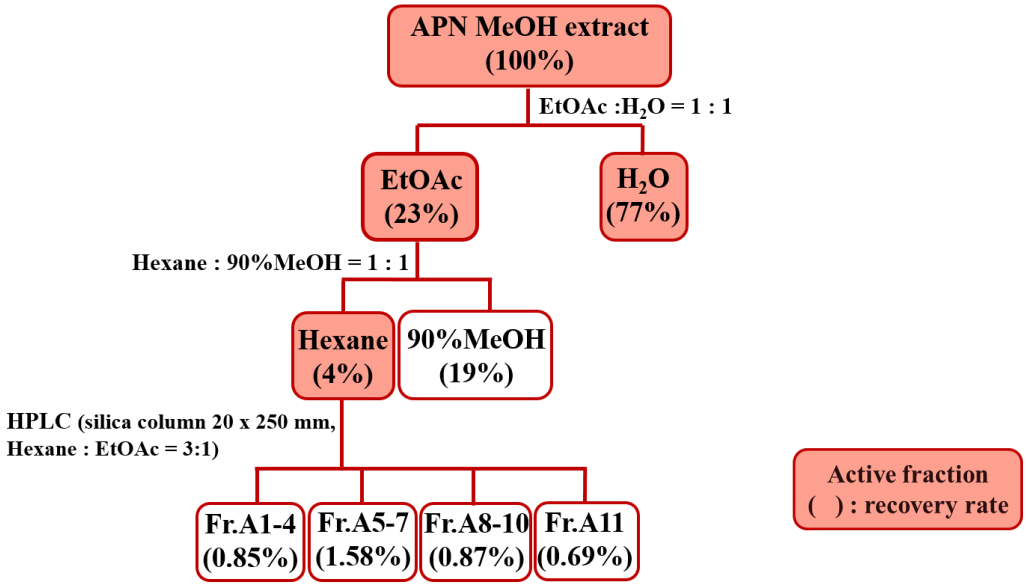


Fig.4-10 Isolation scheme of hexane layer of APN after HPLC fractionation.

4.6.2 Anti-hyperglycemic effects of the different HPLC fractions of the hexane layer of APN in glucose-loaded mice

The effects of each pooled HPLC fraction of the hexane layer of APN on the postprandial blood glucose level were examined in glucose-loaded mice, as shown in Fig.4-11. In the control group (glucose administration only), the blood glucose level reached a maximum value of 127 mg/dL at 30 min after ingestion to the mice, and the level decreased gradually at 60 min. Each group of fraction (Fr.A1-A4: 49.6 mg/kg, Fr.A5-A7: 95.6 mg/kg, Fr.A8-A10: 50.8 mg/kg and Fr.A11: 40.8 mg/kg) of APN was orally administered simultaneously with glucose, the blood glucose level at 30 min after administration was significantly suppressed 10.8% with Fr.A8-A10 when compared with the control group.

Based on the results presented in Fig.4-11, can be considered that Fr.A8-A10 has a potential to inhibit postprandial blood glucose level. After confirming anti-hyperglycemic activity, Fr.A8, Fr.A9 and Fr.A10 were subsequently separately evaluated with glucose-loading test to further confirm which fraction contained the active constituent in the APN.

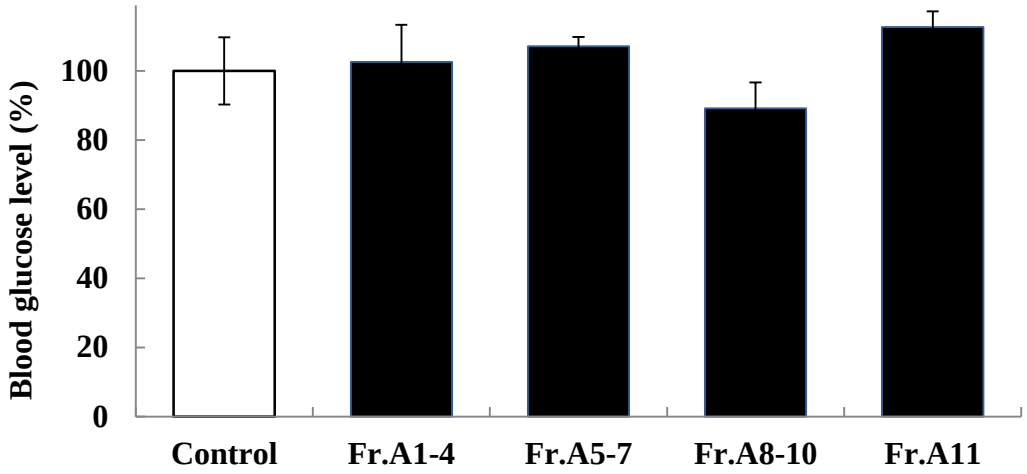


Fig.4-11 Effects of each HPLC fraction of APN extract with rates at 49.6 mg/kg of Fr.(A1-A4), 95.6 mg/kg of Fr.(A5-A7), 50.8 mg/kg of Fr.(A8-A10), 40.8 mg/kg of Fr.(A11), and vehicle (control) on postprandial elevation in the blood glucose level (%) on six-week-old male Slc:ddY mice orally loaded with glucose and measured at 30 min. Data are presented as the mean ± S.E. (n=8).

4.7 HPLC separation of Fr.A8, Fr.A9, and Fr.A10&12 and Fr.A11 in the hexane layer of the APN extracts

The recovery rate of each fraction are 0.79%, 0.05%, 0.80% and 0.06%, respectively. Isolation scheme is shown in Fig.4-12.

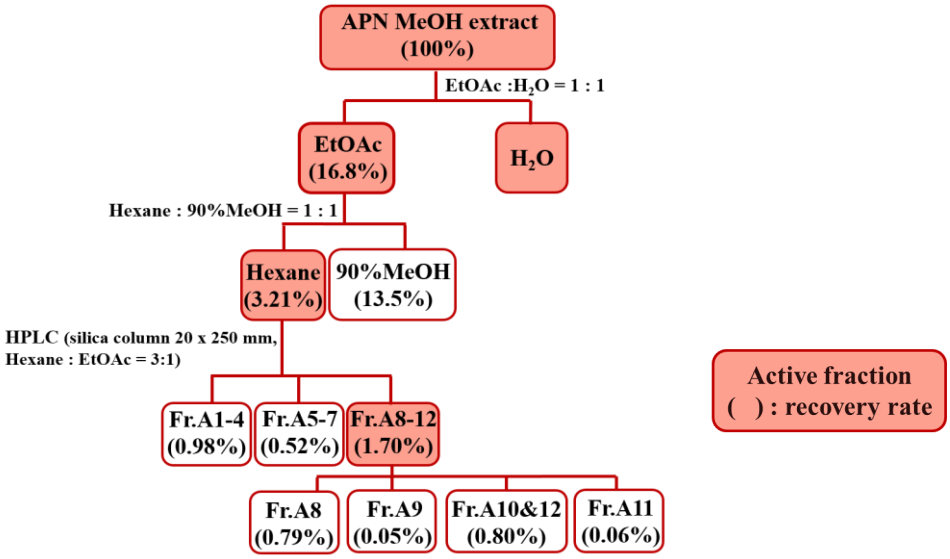


Fig.4-12 Isolation scheme of Fr.A8, Fr.9, Fr.A10&12 and Fr.A11 of APN.

4.8.1 Separation of the water layer of APN by gel column chromatography

Water layer of APN was separated into five fractions by gel chromatography with different ratios of ethanol (EtOH) and water (H_2O) at 50 mL for three times. Firstly, water was added into the column for cleaning. The order of fraction is as follows: 0%EtOH (EtOH: H_2O = 0:1), 25%EtOH (EtOH: H_2O = 1:3), 50%EtOH (EtOH: H_2O = 1:1), 75%EtOH (EtOH: H_2O = 3:1) and 100%EtOH (EtOH: H_2O = 1:0) respectively. The polarity of former is higher than then latter one. After which, TLC was used to confirm the difference in each fraction and were divided into four groups for the glucose-loading test. The recovery rates of Fr.B1, Fr.B2, Fr.B3, and Fr.B4-B5 are at 54.0 %, 12.0 %, 10.6 %, and 0.35 %, respectively.

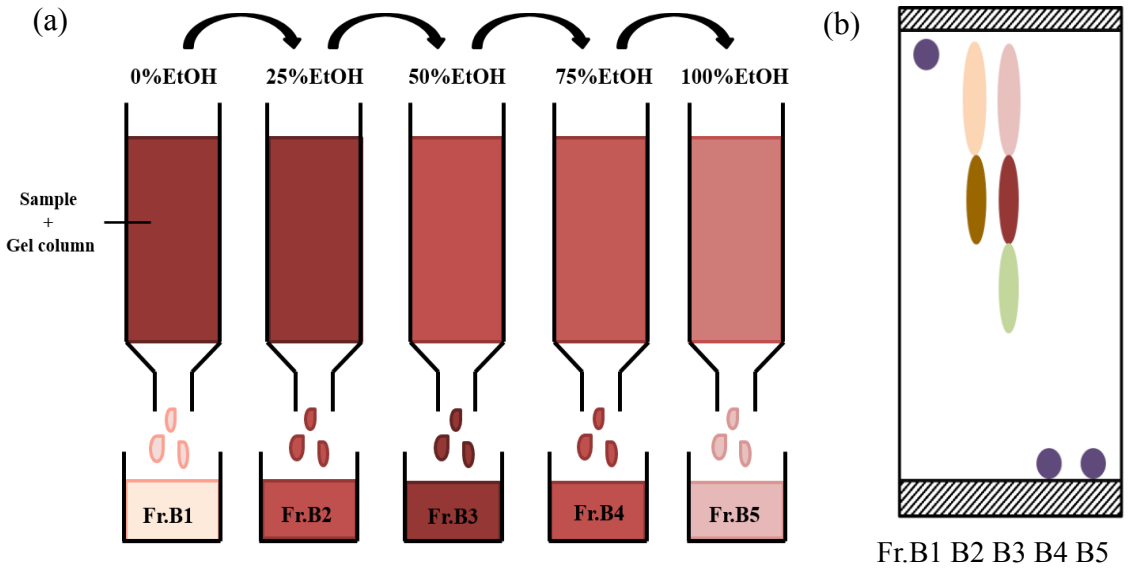


Fig.4-13 Schematic diagram of gel chromatography of the water layer of APN (a) and TLC analysis (b) of Fr.B1-Fr.B5 after HPLC fractionation with the following chromatographic conditions: ODS plate, mobile phase MeOH:H_2O (2:3), *p*-anisaldehyde in ethanol:sulfuric acid = 27:1 solution stain visualized after heat treatment.

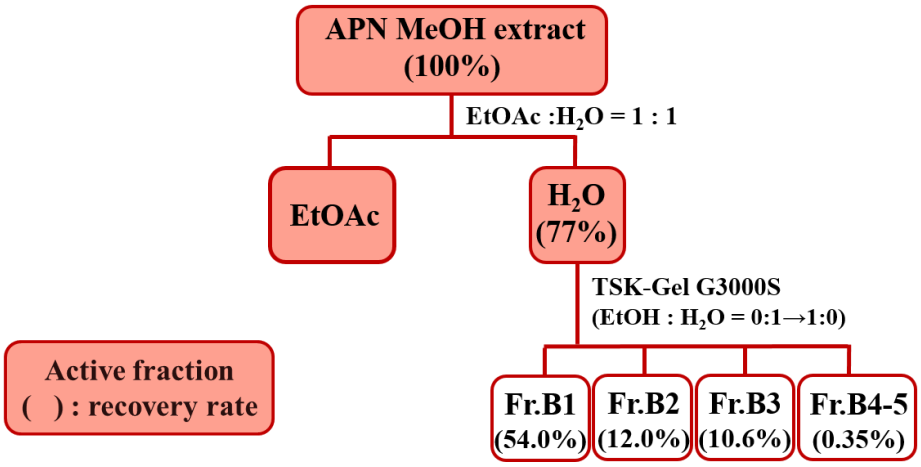


Fig.4-14 Isolation scheme of the water layer of APN after gel chromatography.

4.8.2 Anti-hyperglycemic effects in glucose-loaded mice of the water layer of APN after gel column chromatography

The effects of each group of water layer of APN after gel chromatography on the postprandial blood glucose level were examined in glucose-loaded mice, as shown in Fig.4-16. In the control group (glucose administration only), the blood glucose level reached a maximum value of 161 mg/dL at 30 min after ingestion to the mice, and the level decreased gradually at 60 min. Each fraction (Fr.B1: 1615 mg/kg, Fr.B2: 359 mg/kg, Fr.B3: 318 mg/kg and Fr.B4-B5: 10.6 mg/kg) of APN was orally administered simultaneously with glucose, the blood glucose level at 30 min after administration, was significantly suppressed 11.8% with Fr.B4-B5 when compared with the control group.

Based on the results presented in Fig.4-16, can be considered that Fr.B4-B5 has a potential to inhibit postprandial blood glucose level. After the conformation of the anti-hyperglycemic activity, Fr.B4 and Fr.B5 were divided further into two groups by TLC to conduct the glucose-loading test on the next step to the succeeding steps determine which fraction contained the active constituent in APN.

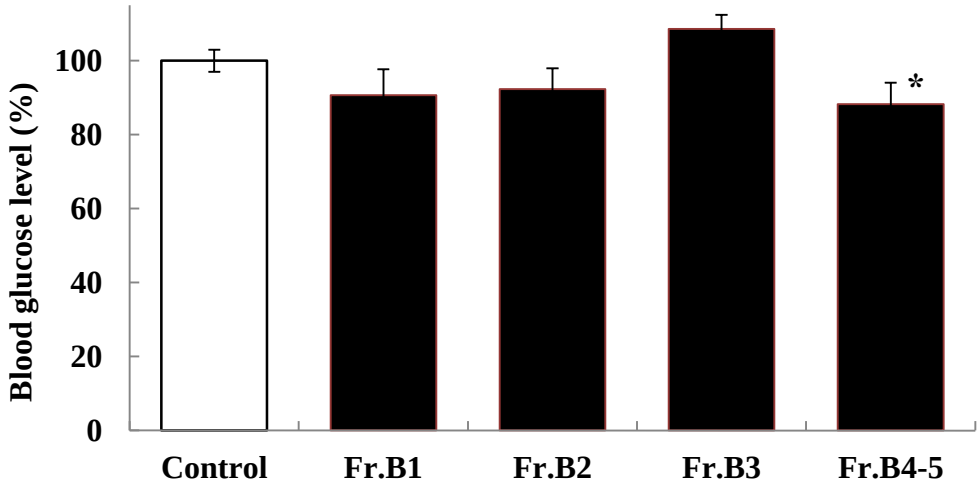


Fig.4-15 Effects of each fraction of water layer of APN extract with rates at 1615 mg/kg of Fr.B1, 359 mg/kg of Fr.B2, 318 mg/kg of Fr.B3, 10.6 mg/kg of Fr.B4-B5, and vehicle (control) on postprandial elevation in the blood glucose level (%) on six-week-old male Slc:ddY mice orally loaded with glucose and measured at 30 min. Data are presented as the mean ± S.E. (n=8). *p<0.05 vs. control.

4.9.1 Separation of Fr.A4 and Fr.A5 from the water layer of APN by gel column chromatography

Fr.B4 and Fr.B5 from the water layer of APN were separated into nine fractions by partition liquid chromatography with mobile phase MeOH:EtOAc (1:1) at 200 mL. Firstly, MeOH was added into the tank to clean the plate. After which, curtained ration solvent was added and the cover was closed to reach saturated equilibrium. PLC schematic diagram is shown in Fig.4-17 (a). The recovery rates of Fr.B4-1 to B4-3, Fr.B5-1 to Fr.B5-6 are 0.05 %, 0.08 %, 0.39 %, 0.12 %, 0.19 %, 0.15 %, 0.39 %, 0.30%, and 0.33 % respectively. The position of each fraction and ^{1}H spectra of two spots were confirmed by TLC analysis, as presented in Fig.4-17 (b). Original Fr.B4 and Fr.B5 were divided into two new groups based on the result of TLC. One group contained Fr.B4-1, Fr.B4-2, Fr.B5-1, Fr.B5-2, and Fr.5-3; and the other group contained Fr.B4-3, Fr.5-5 and Fr.5-6. Fr.B5-4 was excluded for having two spots on the TLC on the following experiment.

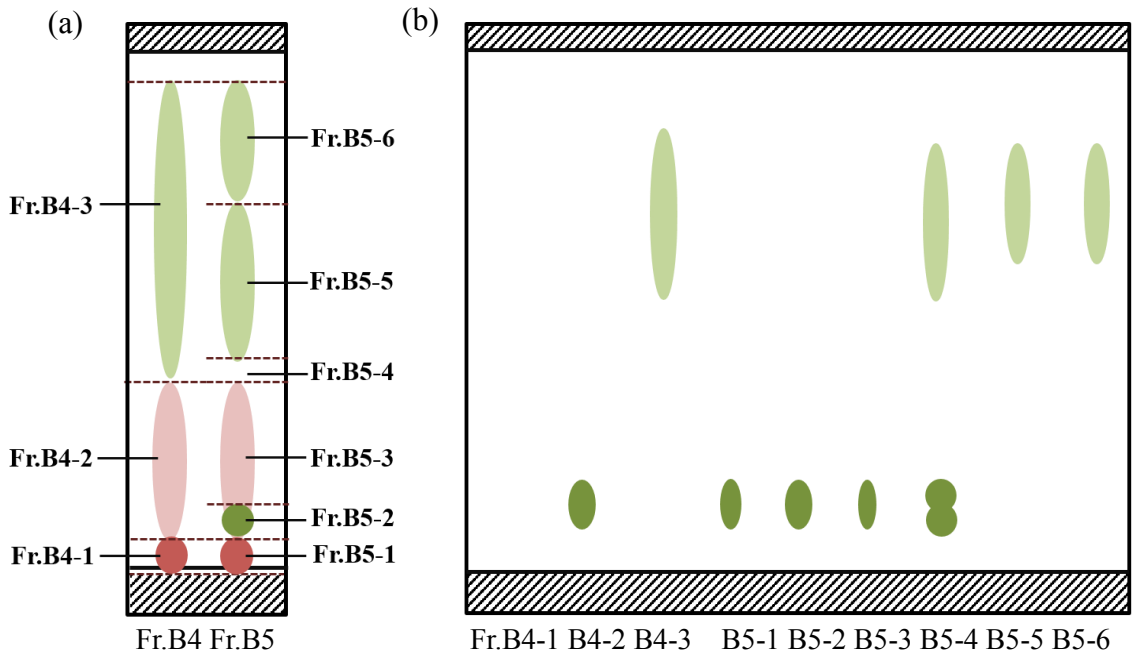


Fig.4-16 PLC plate of silica gel 60 (F_{254} 1 mm x 20 x 20 cm) in Fr.B4-Fr.B5 (a) and TLC plate in Fr.B4 and Fr.B5 were separated into nine fractions (b) after gel chromatography with the following chromatographic conditions: silica plate, mobile phase MeOH:EtOAc (1:1), *p*-anisaldehyde in ethanol:sulfuric acid = 27:1 solution stain visualized after heat treatment.

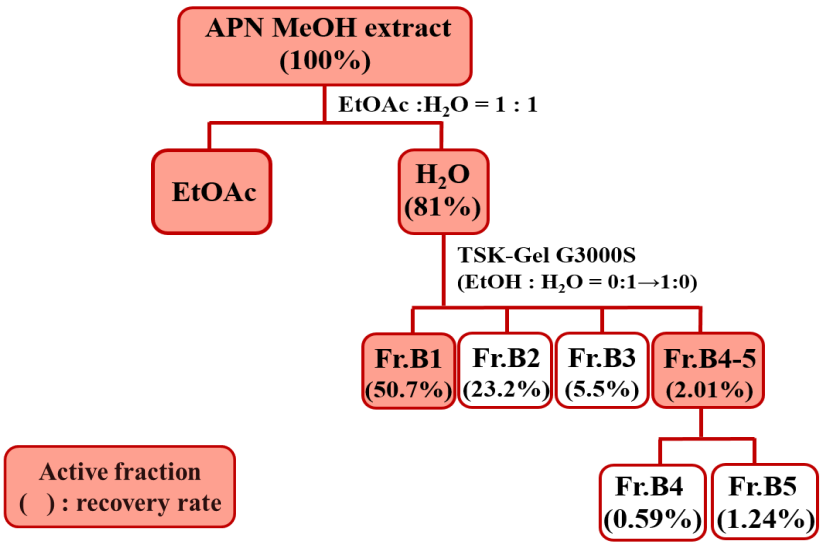


Fig.4-17 Isolation scheme of Fr.B4 and Fr.B5 of water layer of APN after gel chromatography.

4.9.2 Anti-hyperglycemic effects in glucose-loaded mice of Fr.B4 and Fr.B5 from water layer of APN after gel column chromatography

The effects of Fr.B4 and Fr.B5, from water layer of APN after gel chromatography, on the postprandial blood glucose level were examined in glucose-loaded mice, as shown in Fig.4-19. In the control group (glucose administration only), the blood glucose level reached a maximum value of 211 mg/dL at 30 min after ingestion to the mice and the level decreased gradually at 60 min. When each fraction (Fr.B4: 3.33 mg/kg, Fr.B5: 6.33 mg/kg of APN was orally administered simultaneously with glucose, the blood glucose level at 30 min after administration, was significantly suppressed 35.1% with Fr.B5 when compared with the control group.

Based on the results presented on Fig.4-19, it is considered that Fr.B5 has a potential to inhibit postprandial blood glucose level. After confirming the anti-hyperglycemic activity, Fr.B5 has a potential of having an active constituent in the APN. Chemical structure of Fr.B5 was analyzed in the later part of this experiment.

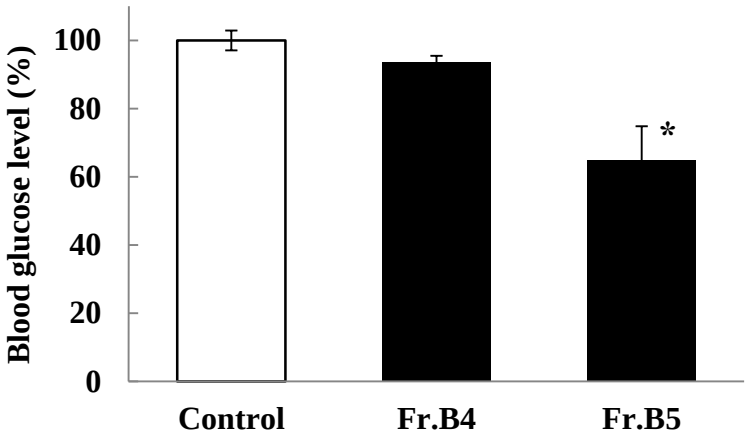


Fig.4-18　Effects of Fr.B4 and Fr.B5 from the water layer water layer of APN extract at rates of 3.33 mg/kg of Fr.B4, 6.33 mg/kg of Fr.B5, and vehicle (control) on postprandial elevation in the blood glucose level (%) on six-week-old male Slc:ddY mice orally loaded with glucose and measured at 30 min. Data are presented as the mean ± S.E. (n=8). *p<0.05 vs. control.

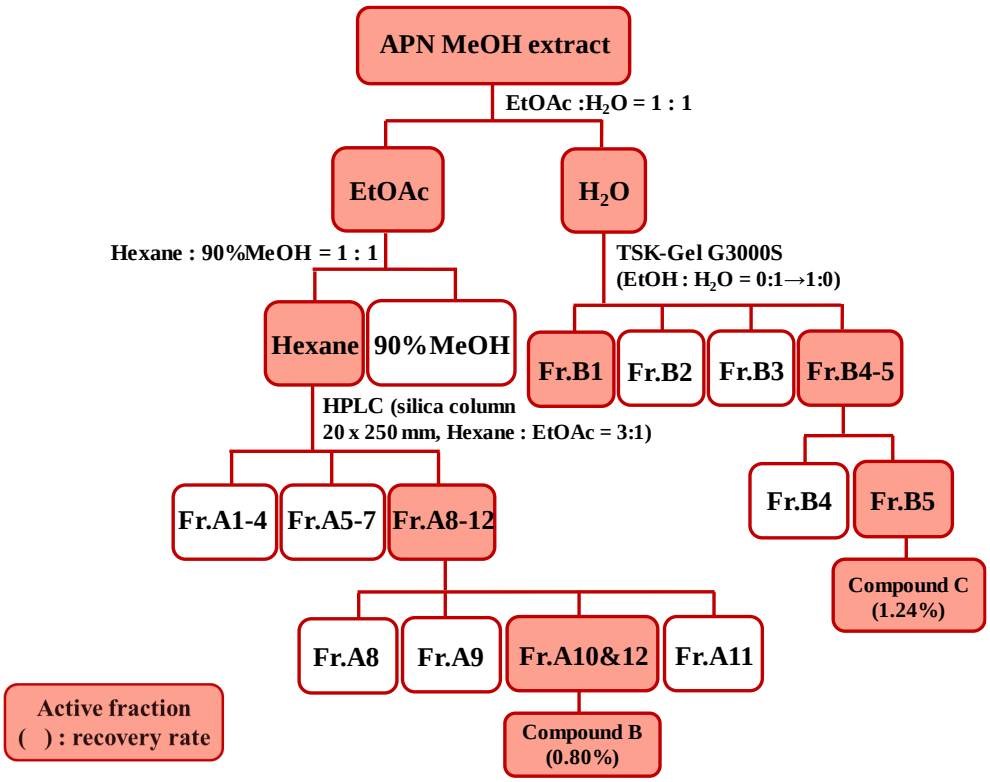


Fig.4-19　Isolation scheme of APN from methanol crude extract to hexane layer of HPLC fractionation and water layer of gel chromatography.

4.10 Structure estimation of compound B in APN

Compound B, Fr.A10&12 of hexane layer in APN was speculated to be an inhibitor of postprandial elevation of blood glucose level, with a single spot was observed on the TLC plate (Fig.4-9). The chemical structure was analyzed using NMR. All the spectral data are shown in the Appendix (^{1}H NMR, ^{13}C NMR, COSY, HMQC and HMBC).

Compound B has a characteristic light green color derived from the hexane-soluble fraction. The molecular weight was measured by electrospray ionization mass spectra (ESIMS) with required molecular peak at [m/z 338.31 $(M+Na)^{+}$].

The chemical structure of compound B is under consideration, other way to certify the integrated structure is needed.

4.11 Structure estimation of compound C in APN

Compound C, Fr.B5 from the water layer in APN was speculated to be an inhibitor of postprandial elevation of blood glucose level (Fig.4-15), with a single spot observed on the TLC plate (Fig.4-16 (a)). The chemical structure was analyzed using NMR, and all the spectral data are shown in the Appendix (^{1}H NMR, ^{13}C NMR, COSY, HMQC and HMBC).

Compound C has a characteristic light green color derived from the methanol-soluble fraction. The molecular weight was measured by electrospray ionization mass spectra (ESIMS) with required molecular peak at [m/z 360.32 $(M+Na)^{+}$].

Based on the evaluation of the low field (δ_H 5.3, 5.4 and 5.7 ppm) ^{1}H NMR spectra, it was observed that ethylene groups are existed. ^{13}C NMR spectra, HMQC, carboxylic acid (δ_C 180.5 ppm) and ethylene groups (δ_C 129.3, 124.9, 117.7, 104 and 100 ppm) were present, also, carbon-oxygen bonds (δ_C 70.3, 69.6, 67.9, 65.8, 64.5, 64.1 and 62.8 ppm) were observed. Aside from the spectra mentioned above, the chemical structure of compound C could be speculated by COSY and HMBC spectra.

The chemical structure of compound C is under consideration, other way to certify the integrated structure is needed.

4.12 Discussion

In this study, the anti-hyperglycemic activity of Japanese maple leaves *Acer palmatum* (APN) was confirmed in glucose-loaded mice. To elucidate the operation of the mechanism the following experiments *in vivo* were performed.

Fig.4-3 shows the effects of APN leaf extract on postprandial elevation of the blood glucose level in glucose-loading test. APN extract (1,000 and 2,000 mg/kg) significantly suppressed the elevation of blood glucose level in glucose-loaded mice when compared with the control group. This results revealed that APN leaf extract is a potential to suppress the postprandial blood glucose level elevation.

The bioassay-guided fractionations (Fig.4-5, Fig.4-7, Fig.4-15 and Fig.4-18) showed that the inhibitory effect on glucose absorption in mice revealed two potential active components derived from the water and hexane layer of the APN extract. The first active component, compound B, which was isolated from Fr.A10&12 of hexane layer of APN after HPLC fractionation, and the other active component, compound C, which was isolated from Fr.B5 of water layer of APN after gel chromatography.

The activity of two active components (compound B and C) was compared with the dose in glucose-loading test which was calculated by the recovery rate. The dose of compound B and C in the glucose-loading test were 48.0 mg/kg and 10.6 mg/kg, respectively. In this study, compound B and C were given approximately 0.80% and 1.24% of the APN leaf extract, respectively.

It is reported that APN leaf has anti-hyperglycemic effect in glucose-loading test. However, there are no studies confirming and charactering the responsible compound(s) in APN leaves that showed the inhibitory effect on postprandial blood glucose level elevation. To date, this is the first report about the anti-hyperglycemic effects of APN leaf extract based on the glucose absorption in the small intestine.

In the prospective study, the pharmacological actions and toxic reaction of the APN extract should be investigated and compared with those of other anti-diabetic drugs present in the market with similar mechanisms of suppression of blood glucose level in the small intestine during a long-term administration in diabetes animal models.

Chapter 5

General Discussion

Chapter 5 General Discussion

The natural materials with anti-hyperglycemic effects was selected as the main topic of this study, aiming to be able to contribute to the control of increasing cases of diabetes worldwide. Hyperglycemic inhibition may be by digestive hydrolytic enzymes or suppression of the postprandial blood glucose level elevation.

As mentioned in chapter 1, in many stages of carbohydrate metabolism, hydrolysis by digestive enzyme and glucose transportation were chosen as the target on the study. These two stages could be evaluated on the residual amount of the starch and blood glucose level elevation after adding each potential natural plants as samples. In other similar studies on OS, tetracyclic triterpenoid structure ($C_{27}H_{46}O_2$) was found to be present [42] and showed hypoglycemic effects or decreased blood glucose level in diabetic-induced rats [49-51]; fresh leaves of OS showed hypoglycemic effects and antioxidant effects after 30 days treated 2 mg/kg/day in albino rabbits. [52] To date, OS research are abundant in anti-diabetic aspect, for instance, anti-hyperglycemic or hypoglycemic effects, however, the mechanism of anti-diabetes is still not yet clearly understood.

In chapter 3, the results were concluded by the α-amylase inhibitory assay, wherein holybasil, *Ocimum sanctum* has an α-amylase inhibitory activity, and the active component compound A in BPK1 extract, is suggested to be a competitive α-amylase inhibitor as the mechanism. This is the first time to elucidate the inhibitory mechanism of α-amylase inhibitory activity in carbohydrate metabolism. Other possibilities of inhibitory mechanism in OS could not be excluded, the further studies and exploration are necessary.

On the other hand, in chapter 4, the results were concluded by the suppression of the blood glucose level elevation using Japanese maple, *Acer palmatum*. No α-amylase inhibitory activity of APN was suggested (Data was not shown). This sample has two active components namely, compound B and compound C which were derived from the APN extract, which are inferred to possibly have the ability to prevent glucose transporters GLUT2 or SGLT1, which transports glucose into the small intestine during blood glucose absorption. Since the property of these two active components are hydrophilic and hydrophobic respectively, it is expected that these two active component may have different functions such as different glucose transporters to suppress blood glucose level elevation in the small intestine. To confirm which glucose transporter would be inhibited, further studies and exploration are necessary.

By comparison with other *Acer* genus plants, it is reported that there have some active components with anti-hyperglycemic effects in α-glucosidase inhibitory activity such as corilagin [25], ginnalin A [26], ginnalin B, ginnalin C [27], and etc. Other than the leaves of the Japanese maple, it is the first time to confirm that *Acer palmatum* leaf extracts have anti-hyperglycemic effects in glucose-loaded mice. Other than natural plants that have been reported to possess suppressive effects on glucose absorption in the small intestine phloridzin [28,53,54] and multiflorin A [30] inhibit glucose transportation through sodium-dependent glucose transporter 1 (SGLT1) on the intestinal epithelia by competitive inhibition.

In this study, the inhibitory effects of potential leaves on hydrolysis of α-amylase and postprandial elevation of the blood glucose level and the properties of their possible active components were elucidated, respectively. Improvement of the hyperglycemic condition is considered as an effective way for preventing diabetes. The findings in this research can lead to the beneficial application of leaves as functional foods which is more preferred by some individuals as compared with synthetic medicine for the prevention of some common diseases like diabetes.

There are many kinds of functional foods for the prevention of diabetes that have shown inhibitory effects on carbohydrate digestive enzymes. On the other hand, there are few functional foods to inhibit the glucose absorption which are available as commercial products. In this study, holybasil *Ocimum sanctum* ethanolic extract was shown to have inhibitory effect on carbohydrate digestive enzymes, and Japanese maple, *Acer palmatum* methanolic extract have inhibitory effects on glucose absorption in the small intestine.

In summary, these two kinds of leaves may prevent diabetes or metabolic syndrome by inhibiting hyperglycemia, provided as functional foods for people who have awareness in the suppression or delay of high blood glucose state in life. Through this study, people could have more chances to prevent disease with their daily meals before the disease progresses and becomes difficult to manage and treat. This study could as well contribute to the enhancement of the quality of life and to help build a healthier society.

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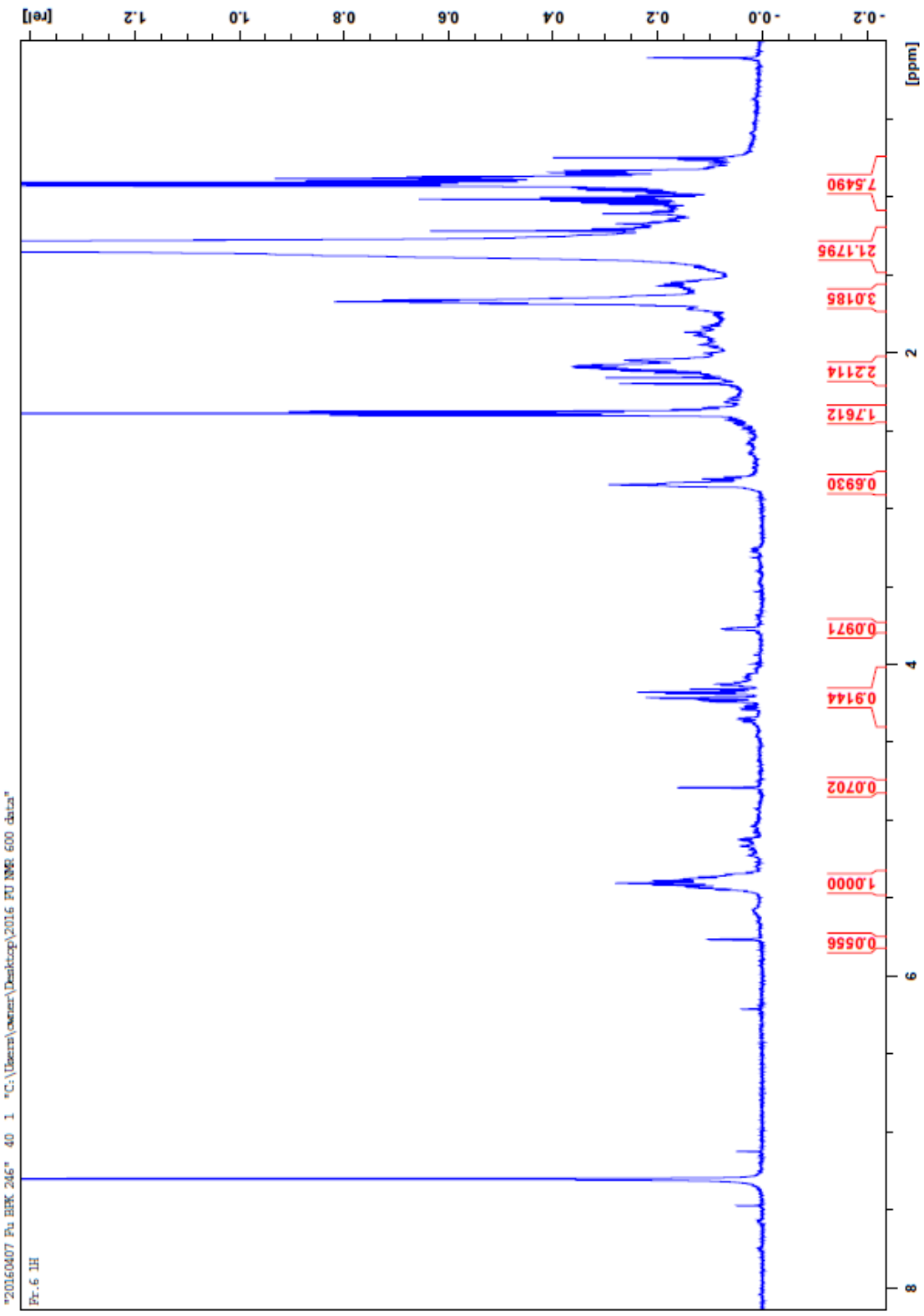
Appendices

NMR chart

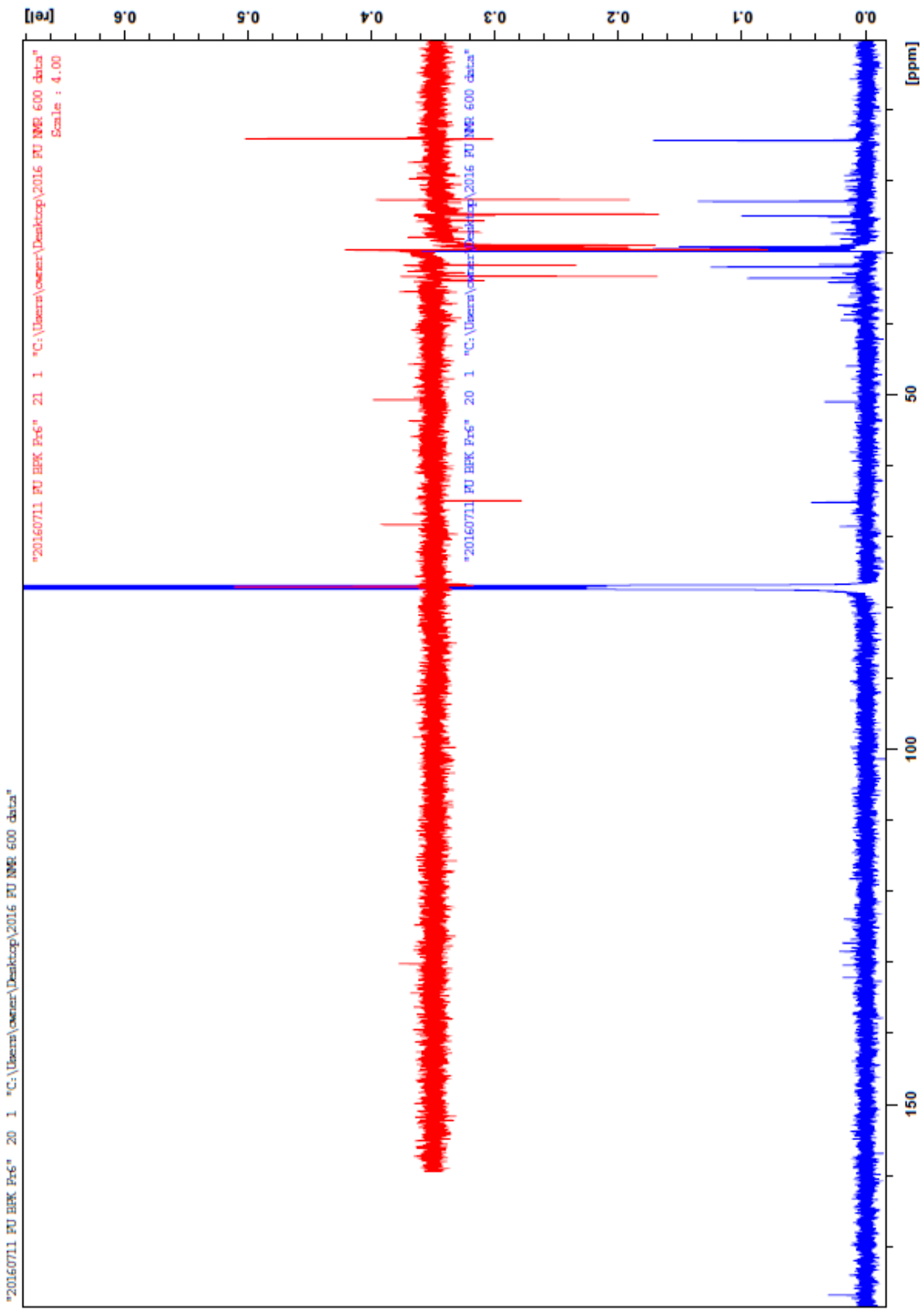
Compound A

Compound B

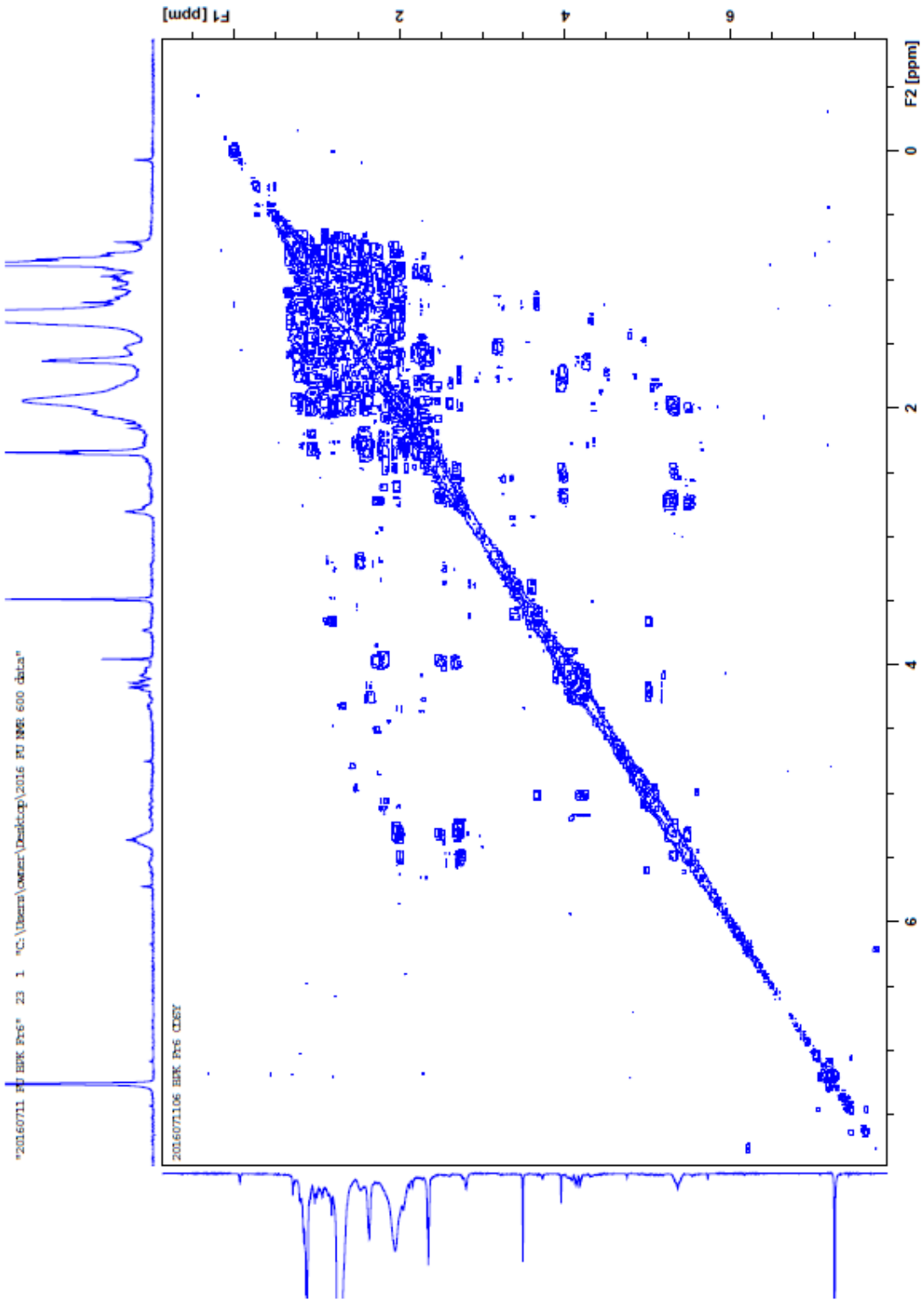
Compound C



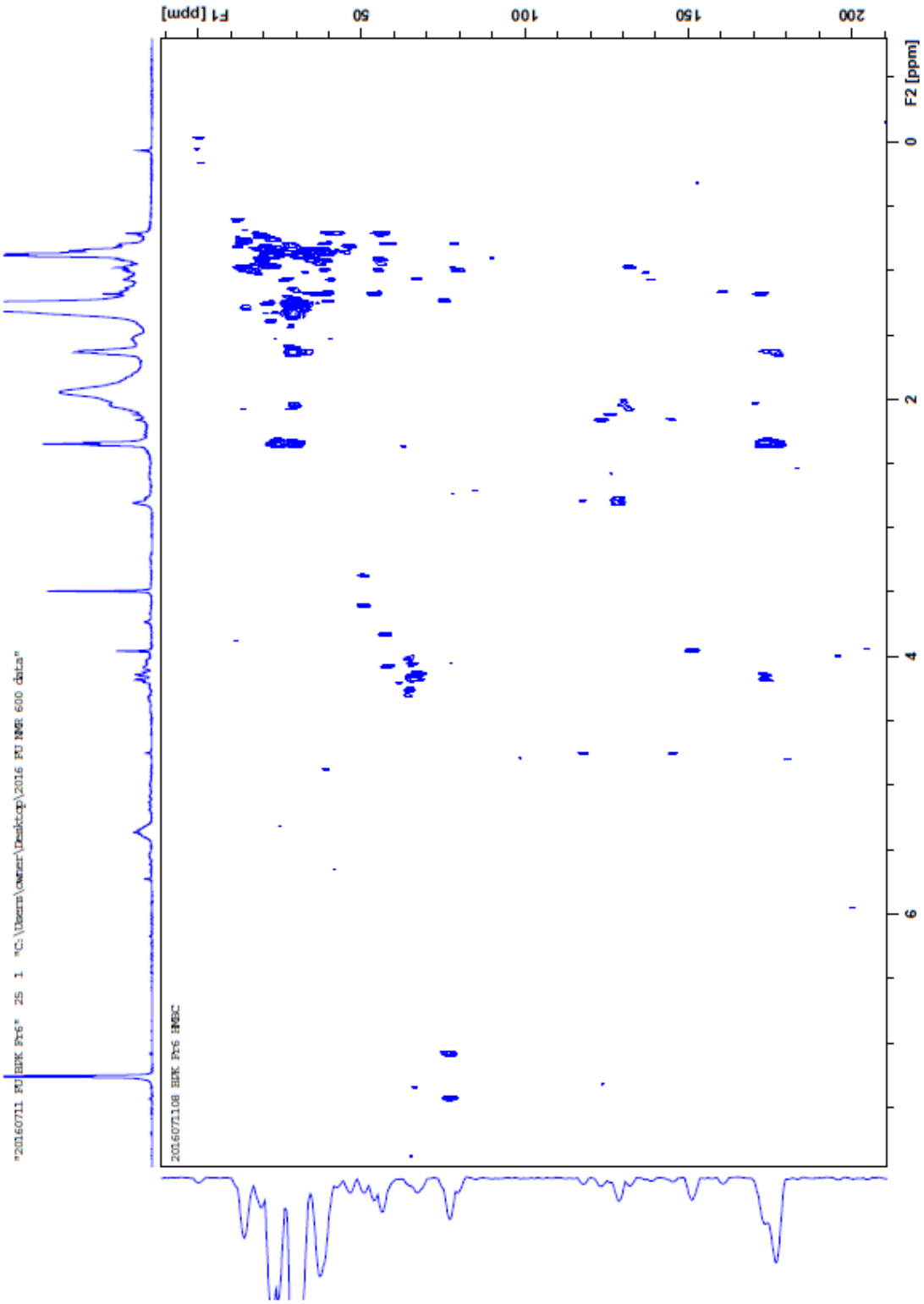
^{1}H NMR spectra of compound A (600 MHz, $CDCl_3$)



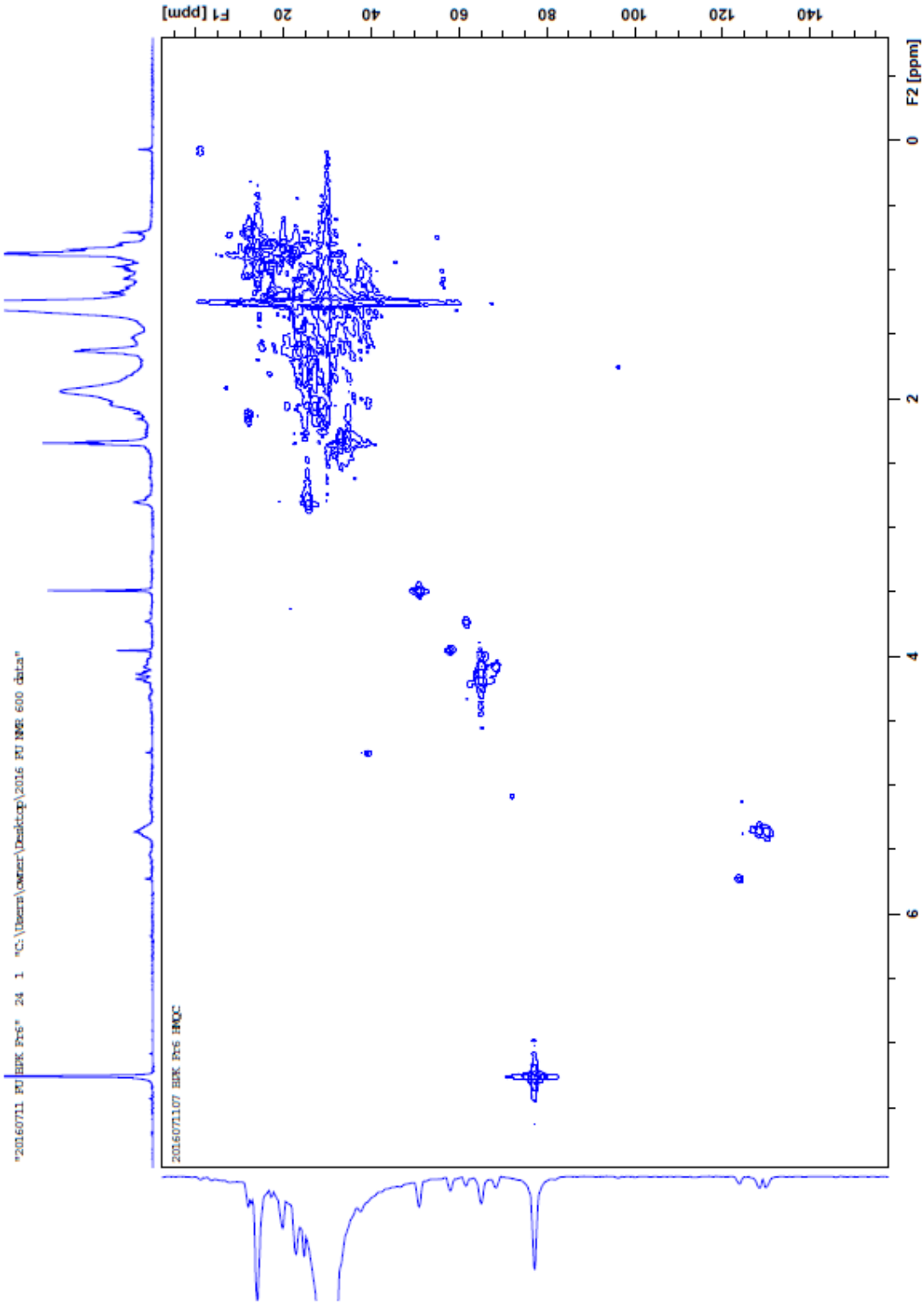
^{13}C NMR and DEPT135 spectra of compound A (600 MHz, $CDCl_3$)



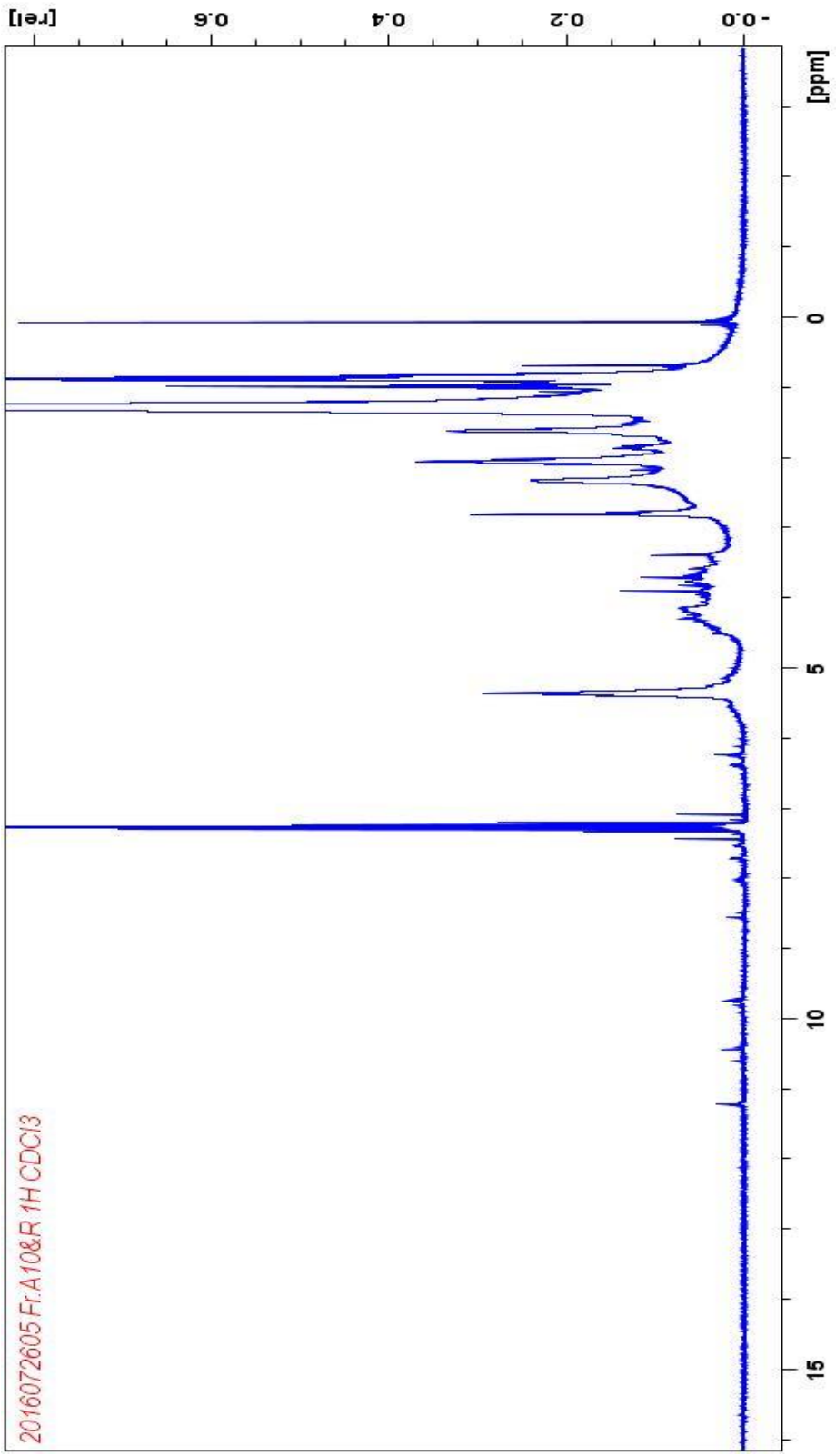
COSY spectra of compound A (600 MHz, $CDCl_3$)



HMQC spectra of compound A (600 MHz, $CDCl_3$)



HMBC spectra of compound A (600 MHz, $CDCl_3$)



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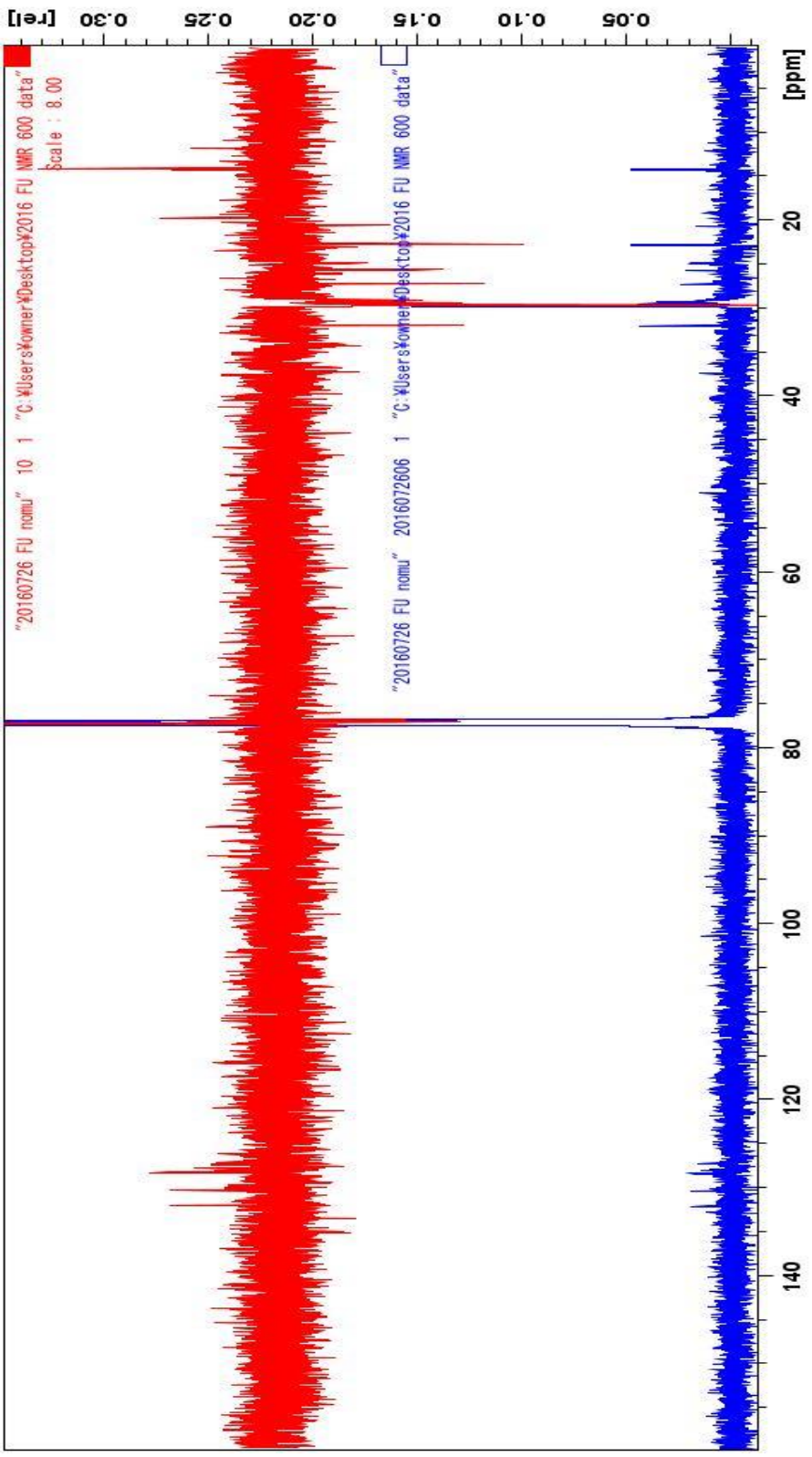
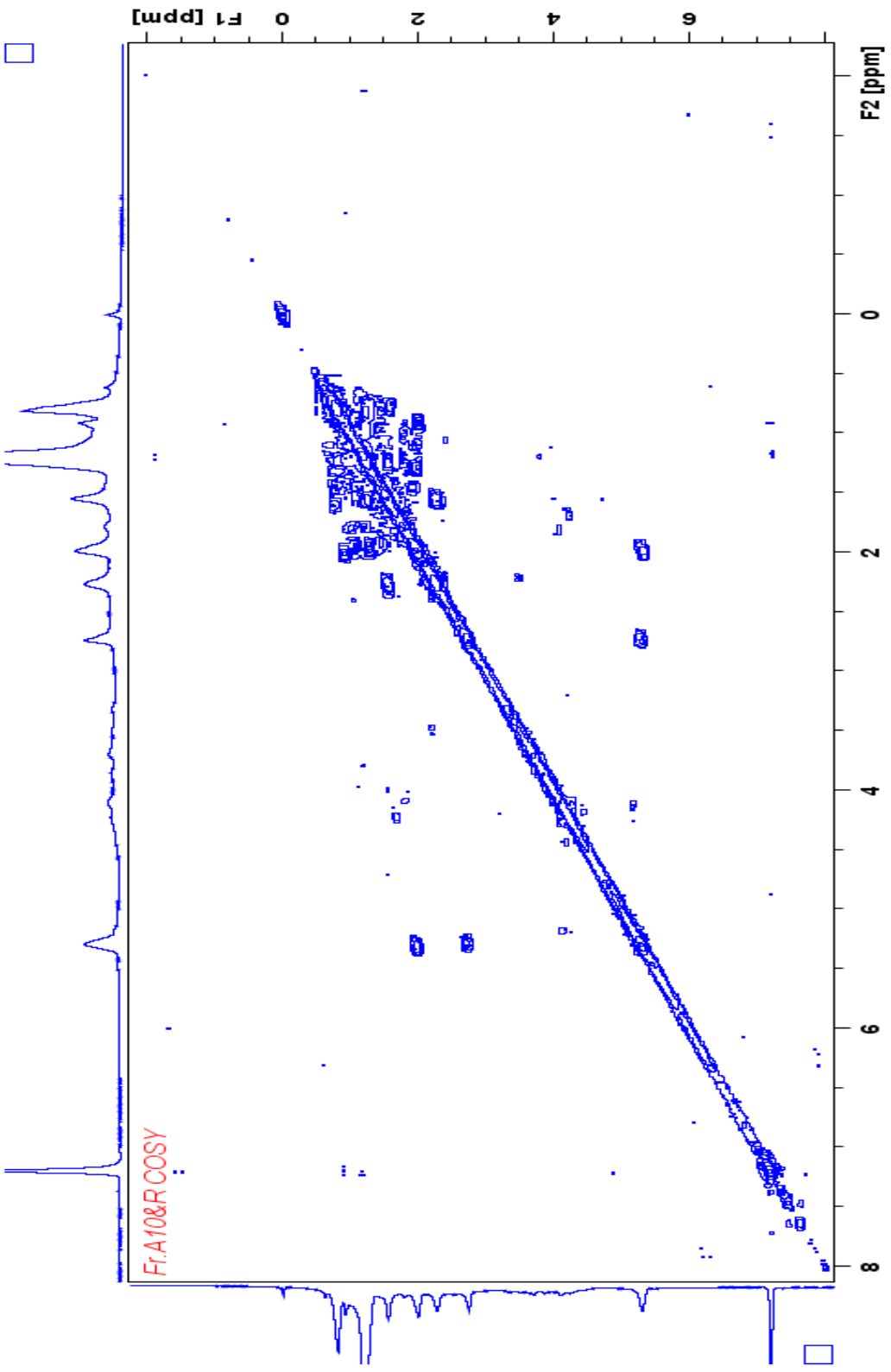
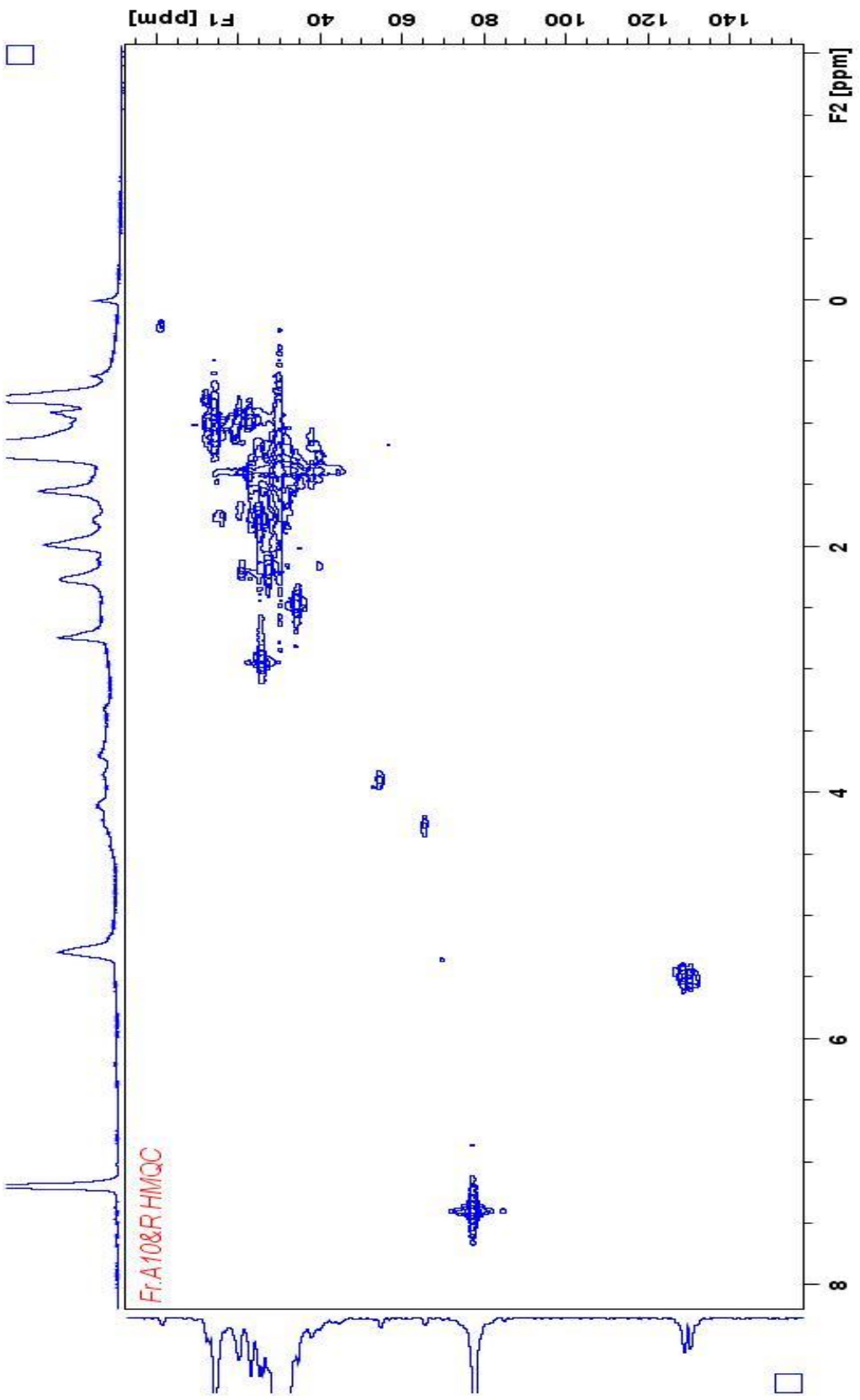


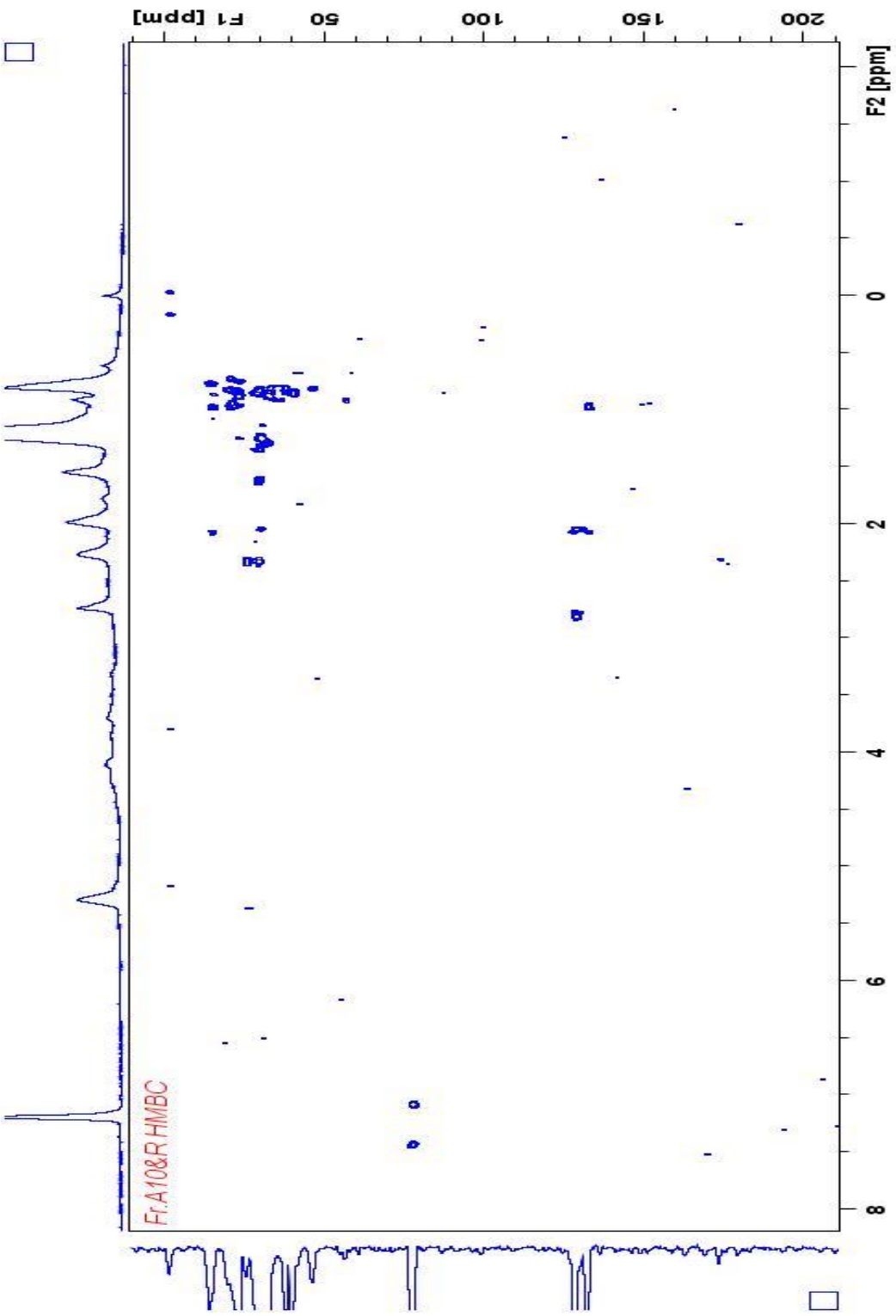
^{13}C NMR and DEPT 135 spectra of compound B (600 MHz, $CDCl_3$)



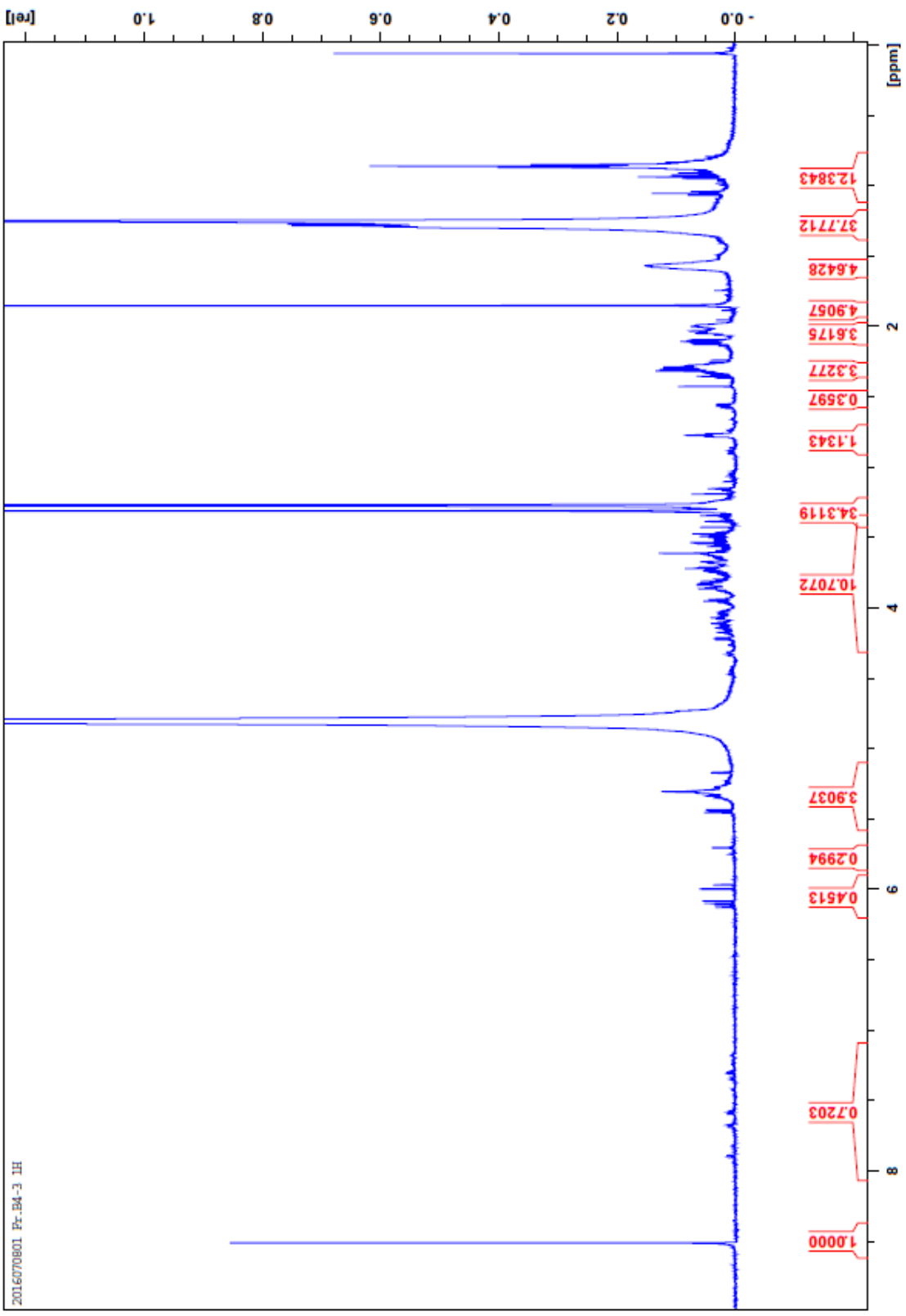
COSY spectra of compound B (600 MHz, $CDCl_3$)



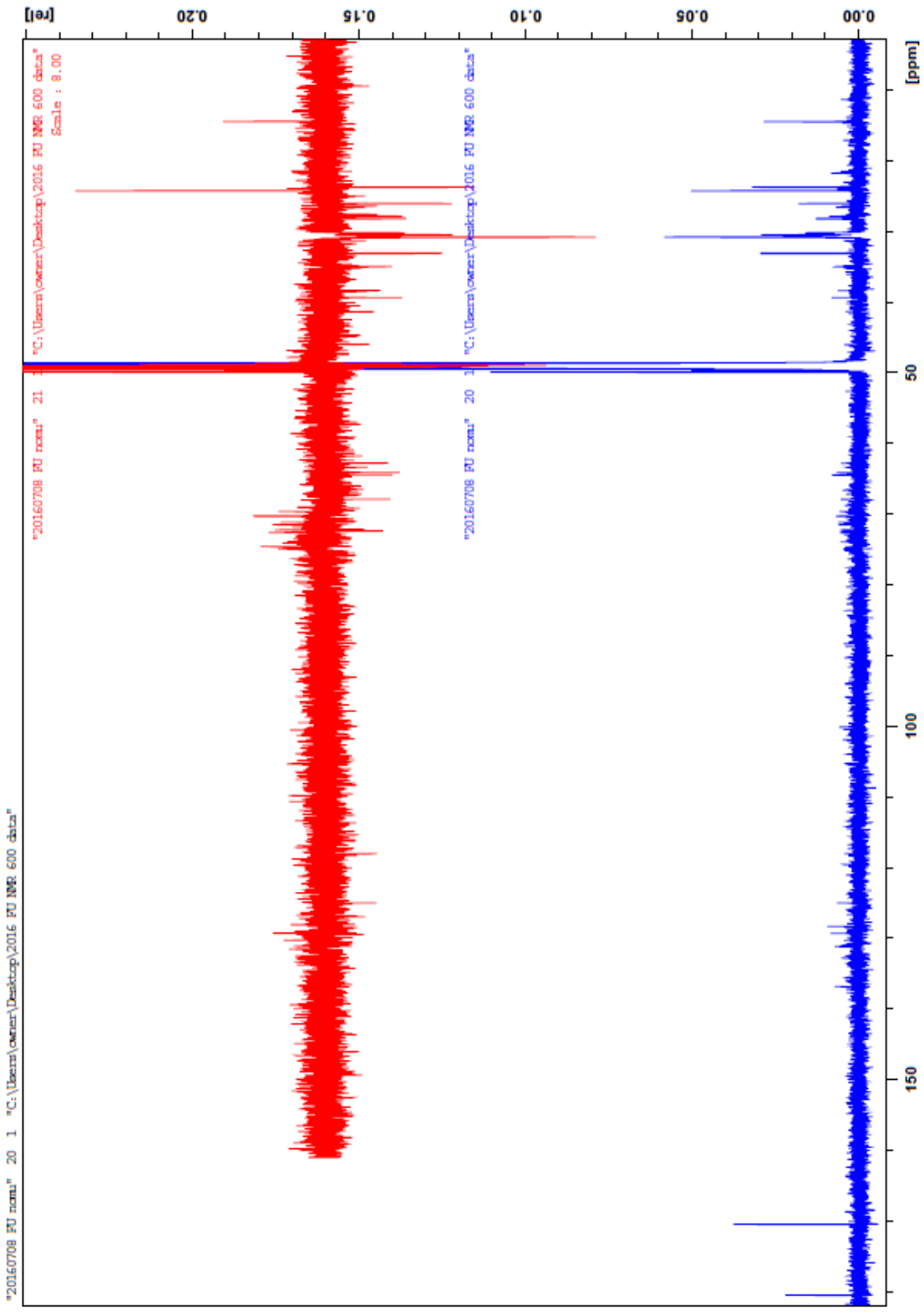
HMQC spectra of compound B (600 MHz, $CDCl_3$)



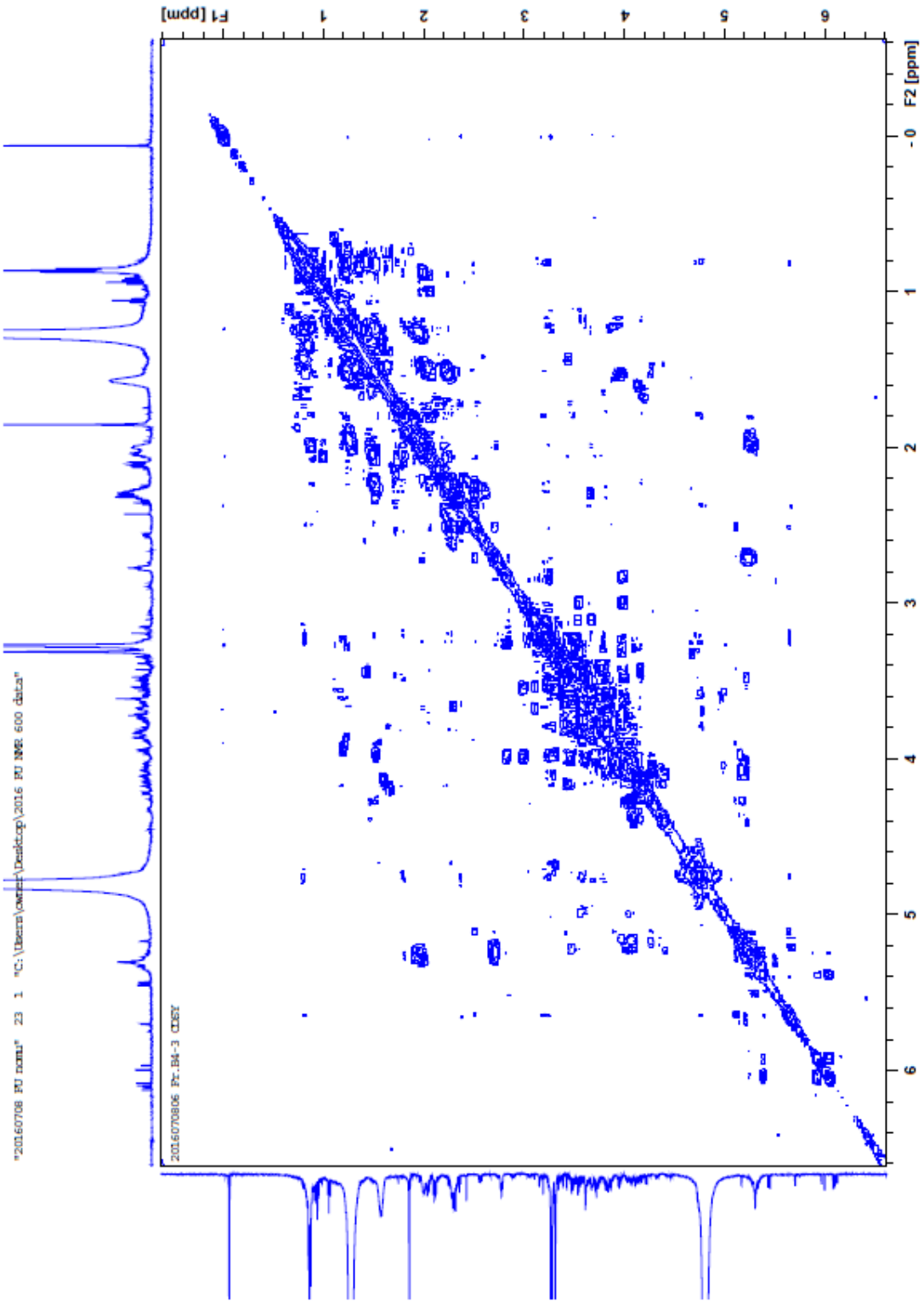
HMBC spectra of compound B (600 MHz, $CDCl_3$)



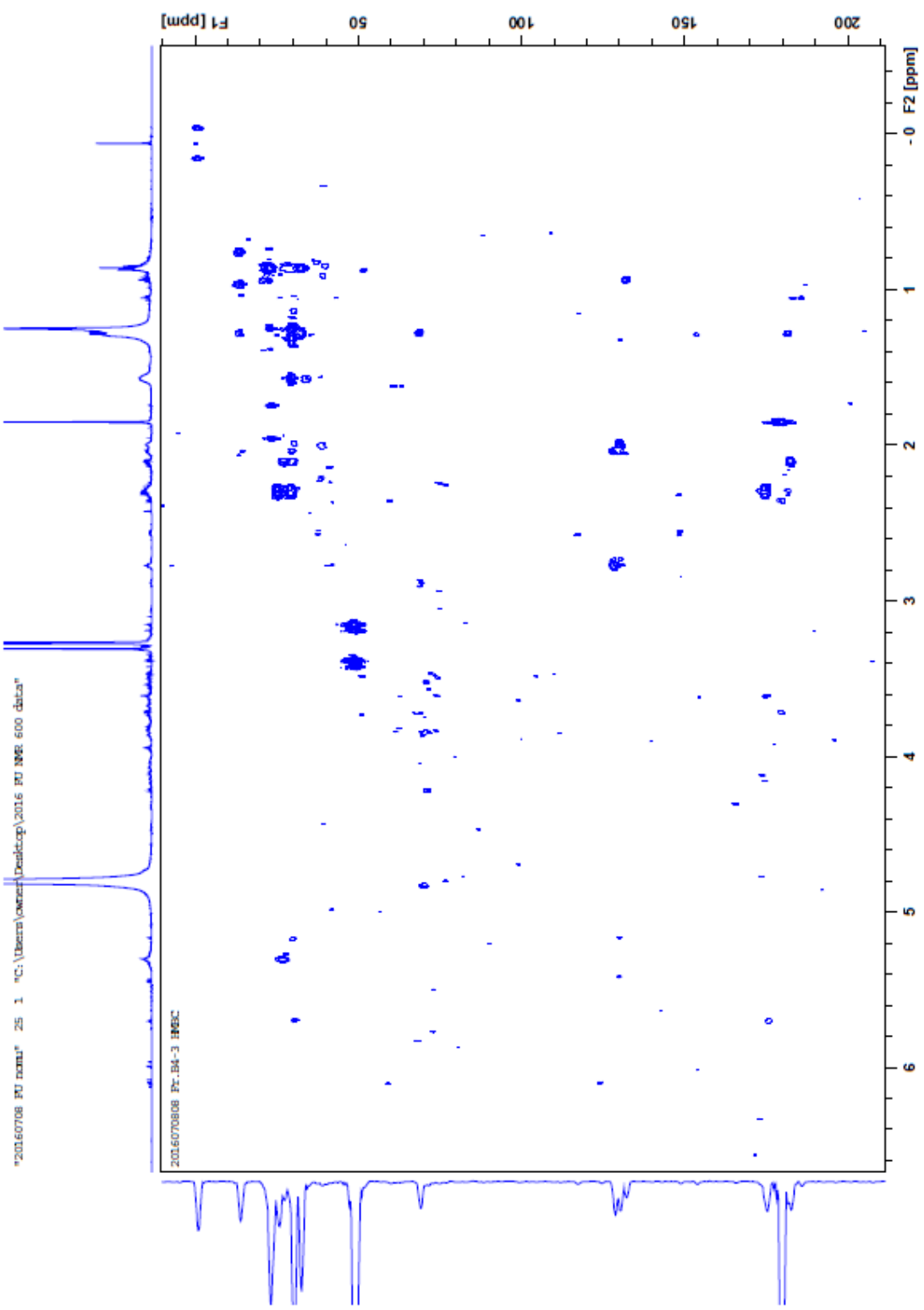
^{1}H NMR spectra of compound C (600 MHz, MeOD)



^{13}C NMR and DEPT 135 spectra of compound C (600 MHz, MeOD)



COSY spectra of compound C (600 MHz, MeOD)



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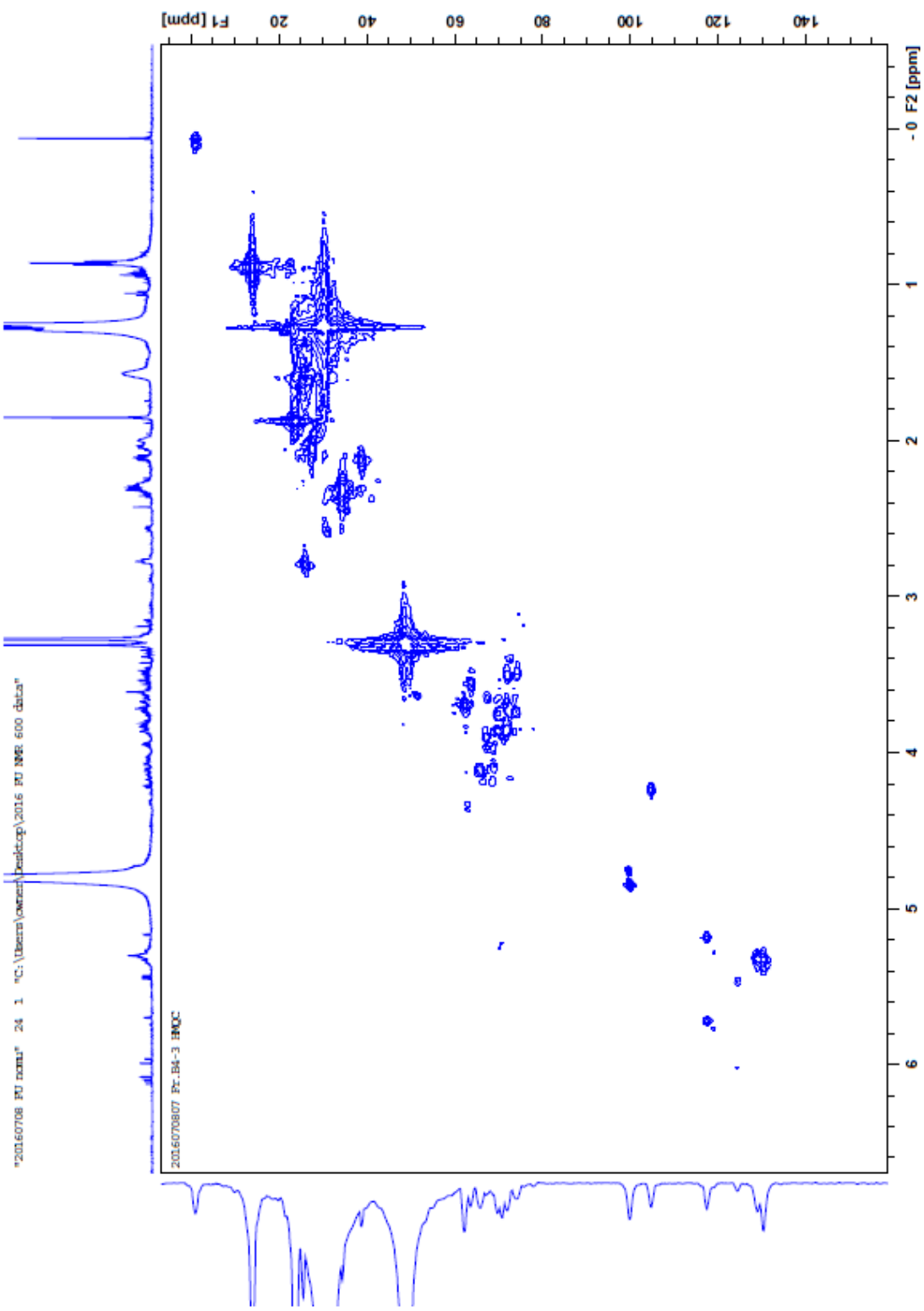

HMBC spectra of compound C (600 MHz, MeOD)