EVALUATION OF *Phaseolus vulgaris* EXTRACT IN A RAT MODEL OF CAFETERIA DIET-INDUCED OBESITY: METABOLIC AND BIOCHEMICAL EFFECTS

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การประเมินสารสกัดถั่วขาว (*Phaseolus vulgaris*) ภาวะอ้วนที่เกิดจากอาหารไขมันสูง: ผลกระทบทางเมตาบอลิซึม และชีวเคมี

นายอัฌชา เอื้อวงศ์วัฒนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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คำสำคัญ: โรคอ้วน/อาหารคาเฟทีเรีย/ถั่วขาว/การทดสอบความทนต่อกลูโคส/ค่าเลือด

วิทยานิพนธ์นี้ได้ศึกษาการเพิ่มประสิทธิภาพการผลิตสารสกัดยับยั้งเอนไซม์แอลฟา-อะไมเลสจากถั่วขาว (*Phaseolus vulgaris*) และประเมินประสิทธิภาพ *in vivo* ในการลดภาวะโรคอ้วนและความผิดปกติของระบบเมแทบอลิซึม การออกแบบ Box-Behnken และระเบียบวิธีพื้นผิวตอบสนอง (Response Surface Methodology, RSM) ถูกนำมาใช้เพื่อหาสภาวะที่เหมาะสมในการสกัดเพื่อให้ได้ผลผลิตและฤทธิ์ยับยั้งเอนไซม์แอลฟา-อะไมเลส สภาวะที่เหมาะสมที่สุดสำหรับฤทธิ์จำเพาะ (0.111 units/mg) คือความเข้มข้นของ PBS 0.101 M, เวลาในการสกัด 1 ชั่วโมง และเวลาในการแยก 30 นาที ในขณะที่ผลผลิตสารสกัดสูงสุด (11.89%) ได้จากสภาวะ PBS 0.105 M, เวลาในการสกัด 1 ชั่วโมง และเวลาในการแยก 30.01 นาที ความสัมพันธ์ระหว่างข้อมูลการทดลองและการวิเคราะห์ด้วย RSM ได้ยืนยันประสิทธิภาพของกลยุทธ์การหาสภาวะที่เหมาะสมนี้ ซึ่งปูทางไปสู่การผลิตขนาดใหญ่

นอกจากนี้ การศึกษา *in vivo* ได้สำรวจผลของสารสกัดถั่วขาว (PVE) ในหนู Wistar ที่เป็นโรคอ้วน โดยหนูถูกกระตุ้นให้เกิดโรคอ้วนด้วยอาหารคาเฟทีเรียเป็นเวลา 17 สัปดาห์ จากนั้นได้รับสารสกัด PVE ขนาดต่ำ (200 mg/kg) สารสกัด PVE ขนาดสูง (300 mg/kg) หรือเมทฟอร์มิน (200 mg/kg) ผ่านการป้อนทางปากเป็นเวลา 6 สัปดาห์ ในกลุ่มหนูที่ได้รับอาหารคาเฟทีเรีย PVE ขนาดต่ำสามารถลดระดับน้ำตาลในเลือดภายหลังมื้ออาหารได้อย่างมีนัยสำคัญระหว่างการทดสอบความทนทานต่อน้ำตาลกลูโคส (OGTT) ที่เวลา 60 และ 120 นาที ($p < 0.05$) รวมถึงลดปริมาณอาหารและพลังงานที่ได้รับในช่วงสัปดาห์ที่ 17-20 และ 18-19 ตามลำดับ แม้ว่าพื้นที่ใต้กราฟกลูโคสโดยรวมในการทดสอบ OGTT จะไม่แตกต่างกันอย่างมีนัยสำคัญ ($p > 0.05$) แต่จุดเวลาแต่ละจุดแสดงให้เห็นการเปลี่ยนแปลงในเชิงบวก การศึกษานี้แสดงให้เห็นถึงประสิทธิภาพของการออกแบบ Box-Behnken และ RSM ร่วมกันสำหรับการสกัดสารออกฤทธิ์ทางชีวภาพ และให้การสนับสนุนอย่างมากสำหรับการสำรวจสารสกัดจากถั่วขาวเพิ่มเติมในฐานะส่วนผสมสำคัญในอุตสาหกรรมอาหาร โดยเฉพาะอย่างยิ่งสำหรับการพัฒนาผลิตภัณฑ์ที่ช่วยในการควบคุมน้ำหนักและระดับน้ำตาลในเลือด

สกัดจากถั่วขาวเพิ่มเติมในฐานะส่วนผสมสำคัญในอุตสาหกรรมอาหาร โดยเฉพาะอย่างยิ่งสำหรับการพัฒนาผลิตภัณฑ์ที่ช่วยในการควบคุมน้ำหนักและระดับน้ำตาลในเลือด

สาขาวิชาเทคโนโลยีอาหาร
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม ..... Atcharuyaporn T.

ATCHA UAWONGWATTANA: EVALUATION OF *Phaseolus vulgaris* EXTRACT IN A RAT MODEL OF CAFETERIA DIET-INDUCED OBESITY: METABOLIC AND BIOCHEMICAL EFFECTS.
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Keyword: OBESITY, CAFETERIA DIET, *Phaseolus vulgaris*, GLUCOSE TOLERANCE TEST, BLOOD CHEMISTRY

This thesis investigated the optimization of α-amylase inhibitory extract production from white kidney beans (*Phaseolus vulgaris*) and subsequently evaluated its *in vivo* efficacy in mitigating obesity and metabolic dysfunction. A Box-Behnken design and Response Surface Methodology (RSM) were employed to optimize extraction parameters for yield and α-amylase inhibitory activity. Optimal conditions for specific activity (0.111 units/mg) were determined to be 0.101 M PBS concentration, 1-hour extraction time, and 30 minutes separation time, while the highest extract yield (11.89%) was achieved with 0.105 M PBS, 1-hour extraction, and 30.01 minutes separation. The strong correlation between experimental data and RSM analysis validated the effectiveness of this optimization strategy, paving the way for large-scale production. Concurrently, the *in vivo* study explored the impact of *P. vulgaris* extract (PVE) on obese Wistar rats. Obesity was induced by a cafeteria diet for 17 weeks, followed by a 6-week oral gavage treatment with low-dose PVE (200 mg/kg), high-dose PVE (300 mg/kg), or metformin (200 mg/kg). In the cafeteria diet group, low-dose PVE significantly lowered postprandial glycemia during an oral glucose tolerance test (OGTT) at 60 and 120 minutes ($p < 0.05$), and decreased food and energy intake during weeks 17-20 and 18-19, respectively. While the overall areas under the glucose curves in the OGTT were not significantly different ($p > 0.05$), individual time points showed positive changes. This study demonstrates the effectiveness of a combined Box-Behnken design and RSM approach for optimizing bioactive compound extraction and provides strong support for further exploring *Phaseolus vulgaris* extract as a valuable functional ingredient in the food industry, particularly for developing products that aid

in weight management and glycemic control.

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LIST OF ABBREVIATIONS

°C	=	Degree
αAI	=	α-amylase inhibitor
Akt	=	Engage protein kinase B
ANOVA	=	Analysis of variance
ALT	=	Alanine transaminase
AST	=	Aspartate aminotransferase
BSA	=	Bovine serum albumin
BUN	=	Blood urea nitrogen
CaCl2	=	Calcium chloride
CAF	=	Cafeteria diet
Cr	=	Creatinine
DNS	=	Dinitrosalicylic acid
eWAT	=	Epididymal white adipose tissue
Fig	=	Figure
g	=	Gram
GDM	=	Gestational diabetes mellitus
GLUT4	=	Glucose transporter 4
HCl	=	Hydrochloric acid
HDL	=	High-density lipoprotein
IKK-β	=	I kappa B kinase
IRS	=	Insulin receptor substrate
JNK	=	c-Jun N-terminal kinase
KCl	=	Potassium chloride
kDA	=	Kilodalton
Kg	=	Kilogram
LDL	=	Low-density lipoprotein

LIST OF ABBREVIATIONS (Continued)

m	=	Milligram
M	=	Molar
min	=	Minute
mL	=	Milliliter
mM	=	Millimolar
mTOR	=	Mammalian target of rapamycin
Na_2HPO_4	=	Disodium phosphate
NaCl	=	Sodium chloride
NAFLD	=	Non-alcoholic fatty liver disease
NaH_2PO_4	=	Monosodium phosphate
NaOH	=	Sodium hydroxide
NCDs	=	Noncommunicable chronic disease
NFkb	=	Nuclear factor kappa b
nm	=	Nanometer
PBS	=	Phosphate-buffered saline
pH	=	Potential of Hydrogen ion
PI3K	=	Phosphatidylinositol 3-kinase
PVE	=	*Phaseolus vulgaris* extract
RSM	=	Response Surface Methodology
SD	=	Standard diet
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T1DM	=	Type 1 diabetes mellitus
T2DM	=	Type 2 diabetes mellitus
TC	=	Total cholesterol
TG	=	Triglyceride

CHAPTER I
INTRODUCTION

1.1 Significant of the study

Noncommunicable chronic disease (NCDs), such as hypertension, hyperlipidemia, type 2 diabetes mellitus, cardiovascular disease, metabolic syndrome, high cholesterol levels, and cancer, cause worldwide death. (Ejigu & Tiruneh, 2023). The one factor that increase NCDs risk is Obesity and overweight which cause by life behavior especially eating behavior. The one of reason of overweight and obesity is eating behavior that lead to excessive energy consumption per energy expenditure (Wright and Aronne, 2012, Romieu et al., 2017). behavior.

Type 2 diabetes mellitus is one of noncommunicable chronic disease (NCDs) that body cells have low effective insulin action or insulin resistance that lead to hyperglycemia, also the type 2 diabetes mellitus is found in the majority of peoples who have diabetes mellitus. Type 2 diabetes mellitus usually found in age 30 or upper, obesity (Asian body mass index ≥ 23 kg/m^2), The patient might or might not have any abnormal symptoms, which are usually symptoms not severe but gradually progress, usually appear in parents or sibling. Risk of type 2 diabetes mellitus increased by older age, higher weight, lack of exercise, and more appear in woman who previous history of diabetes mellitus when pregnancy (Clinical Practice Guideline for Diabetes, 2017). The one of reasons that lead to type 2 diabetes mellitus is consuming behavior but it can be prevented by consuming some bioactive compound from some food. The one of those is α-amylase inhibitor (αAI) which found in white bean (*Phaseolus vulgaris*) that can slower starch absorption first reported by Bowman (1945) named as phaseolamin by Marshall and Lauda (1975) So that, there are many works study the effect of αAI in *P. vulgaris* on of type 2 diabetes mellitus, overweight, obesity or other NCDs in animals (Tormo et al., 2004; Fantini et al., 2009; Oliveira et al., 2014; Micheli

et al., 2019; Ezzat et al., 2021) or human subject (Spadafranca et al., 2013; Wang et al., 2020; Jäger et al., 2024)

This study expored the effect of *P. vulgaris* extract on diabetes induced rats. The difference of previous studies and this study are induction of diabetes and this study additionally investigate proteins related in insulin signaling. The induction of diabetes in rats was done using a cafeteria diet (CAF) because it is similar to human food. The preparation *P. vulgaris* extract method with high specific αAI activity and identification of group of bioactive compounds with show αAI activity was investigate too.

1.2 Research objectives

(1) To prepare *P. vulgaris* extract with high specific αAI activity.

(2) To identify group of bioactive compounds with show αAI activity.

(3) To investigate the effect of *Phaseolus vulgaris* extract on insulin resistance and hepatic steatosis in obesity induced Wistar rats.

1.3 Research hypothesis

P. vulgaris extraction condition with high specific α-amylase inhibitory activity could be found. PVE could have alleviation effect in obese male Wistar rats.

1.4 Scope of the study

Perform optimization of *P. vulgaris* extraction condition with a Box-Behnken and Response surface methodology to get high yield and specific α-amylase inhibitory activity. Investigate the effect of PVE on alleviation in male Wistar rats obese induced with CAF.

1.5 Expected results

(1) White bean extract preparation method.

(2) Bioactive compound identification in white bean extract.

(3) Approved in bioactivity of white bean extract in vivo in tern of reduce insulin resistance and hepatic steatosis.

1.6 Reference

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CHAPTER II
LITERATURE REVIEWS

2.1 Diabetes Mellitus (DM)

Diabetes mellitus is a chronic metabolic disorder stemming from either insufficient pancreatic insulin production or the body's impaired insulin utilization, leading to hyperglycemia and progressive damage to various physiological systems, particularly the nerves and blood vessels (WHO, 2021). There are 4 types of DM divided by cause, Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), Gestational diabetes mellitus (GDM), and Specific types of diabetes due to other causes.

T1DM characterized by deficient insulin production requiring daily administration with unknown etiology and prevention (WHO, 2021). T1DM is results oocur by the β-cells in pancreas were destroys by autoimmune (Paschou et al., 2018). T2DM is the most common type, resulting from ineffective insulin use largely due to excess body weight and physical inactivity, with additional influences from race, ethnicity, and age. Undiagnosed or poorly managed diabetes significantly increases the risk of debilitating and irreversible complications, including severe damage to the heart, eyes, kidneys, and nerves, potentially leading to limb amputation, vision loss, and premature mortality. This condition represents a global epidemic, affecting over 420 million people worldwide, approximately 6% of the global population (WHO, 2021). GDM is highly insulin resistance during pregnancy but often disappear after pregnancy (Clinical Practice Guideline for Diabetes, 2017). Mother who used to had GDM increase risk in T2DM. (Jovanovic & Pettitt, 2001) GDM can be used in prediction in incoming T2DM (Farahvar et al., 2018). Specific types of diabetes due to other causes is notable cause DM such as Maturity-Onset Diabetes of the Young (MODY), DM cause by liver

disease, abnormal endocrine, drug, infection, autoimmune, and/or combination (Clinical Practice Guideline for Diabetes, 2017)

2.2 Obesity

Obese individuals develop insulin resistance, which is characterized by impaired insulin action in the liver and reduced glucose uptake in fat and muscle. Increased body mass index (BMI) and abdominal fat distribution linearly increases the risk of T2DM due to alterations in adipose tissue biology that links obesity with insulin resistance and beta cell dysfunction (Klein et al., 2022). Obesity is the most significant single risk factor for the development of fatty liver, Non-alcoholic fatty liver disease (NAFLD) is liver function impairments and tissue damage similar that not related with drinking alcohol, developed by steatosis to advanced fibrosis and cirrhosis (Festi et al., 2004).

2.3 Insulin signaling

Upon insulin binding, the insulin receptor undergoes autophosphorylation, initiating the phosphorylation of insulin receptor substrate (IRS) proteins. These phosphorylated IRS proteins then associate with phosphatidylinositol 3-kinase (PI3K) and engage protein kinase B (Akt), ultimately leading to the RAB10 protein-mediated translocation of glucose transporter 4 (GLUT4)-containing vesicles to the cell membrane, thereby enabling glucose entry into cells (Sano et al., 2003; Velez et al., 2014). Activated Akt additionally promotes glycogen synthesis (Sah et al., 2016) and mediates diverse tissue-specific insulin effects: in the liver, p-Akt upregulates glycogen synthesis, inhibits gluconeogenesis, and stimulates cell growth via mammalian target of rapamycin (mTOR) activation; in skeletal muscle, insulin drives glycogenesis; and in adipose tissue, insulin primarily regulates lipid transport and storage (Velez et al., 2014). Furthermore, in the post-prandial state, high insulin levels increase lipoprotein lipase activity, releasing free fatty acids from circulating triglyceride-containing chylomicrons, which are then captured by adipocytes via acylation-stimulating protein and re-esterified into triglycerides by diacylglycerol transferase (Velez et al., 2014).

2.4 Insulin resistance

Aberrations in the phosphorylation and dephosphorylation of key signaling proteins, from the insulin receptor itself to downstream effectors like Akt, can significantly impair insulin action, culminating in insulin resistance. At a cellular level, such impairments can manifest as down-regulation, deficiencies, or polymorphisms in the tyrosine phosphorylation of the insulin receptor, IRS proteins, or PI3K, as well as compromised Akt activation or functional abnormalities of GLUT4 (Wheatcroft et al., 2003; Sah et al., 2016). Furthermore, proinflammatory cytokines and endoplasmic reticulum stress contribute to insulin resistance through the activation of serine kinases, specifically c-Jun N-terminal kinase (JNK) and I kappa B kinase (IKK-β). These kinases promote the phosphorylation of IRS1 at serine residues (e.g., serine 302 and serine 307), which negatively regulate normal insulin signaling (Ropelle et al., 2010; Sah et al., 2016). Activated IKK-β also phosphorylates the inhibitory protein nuclear factor kappa b (NFkb), which typically sequesters the transcription NFkb. This phosphorylation targets NFkb, for proteasomal degradation, thereby releasing NFkb. Subsequently, NFkb translocate to the nucleus, where it promotes the expression of various target genes whose products contribute to the development of insulin resistance (Sah et al., 2016).

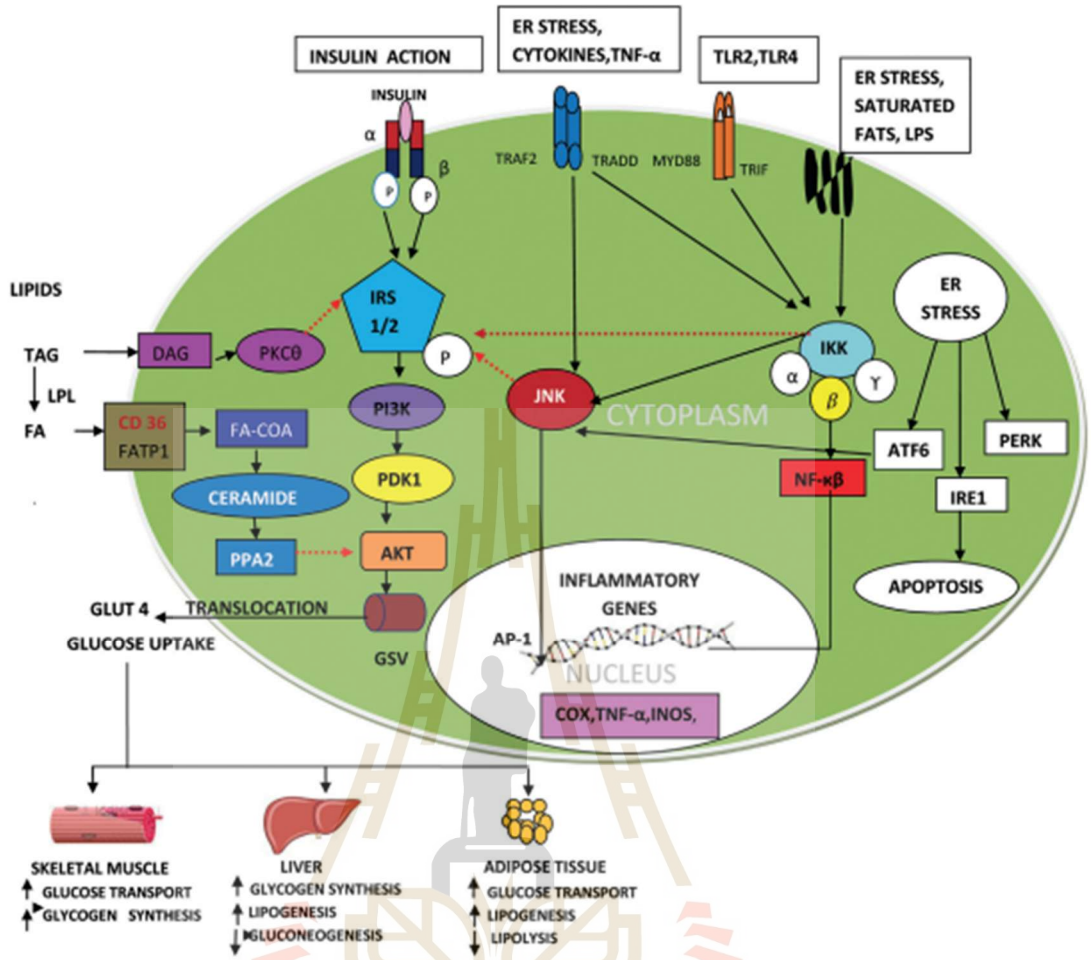

Figure 2.1 Pathophysiology of insulin resistance

(Source: Sah et al., 2016)

2.5 Hepatic steatosis

Accumulation of fat leads to liver steatosis, imbalance in free fatty acid availability, and impairment in the oxidative capacity of mitochondria ultimately causing mitochondrial dysfunction and further accumulation of fats in the cell. (Muoio & Newgard, 2008; Velez et al., 2014) Excessive concentrations of diacylglycerol also interfere with the phosphorylation of IRS proteins and activation of PI3K. This effect is mediated by serine kinases in the protein kinase C family that phosphorylate IRS proteins at serine residues impeding their ability to activate insulin signaling through PI3K. This effect impairs insulin signaling and prevents GLUT4 translocation (Roden, 2004; Velez et al., 2014)

2.6 Cafeteria diet (CAF)

The limitations of the high-fat diet model in accurately reflecting human obesity and its associated metabolic disorders (Yandell, 2015; Bortolin, 2018) have led to the introduction of the CAF model. Both high-fat diet and CAF effectively induce visceral obesity, glucose intolerance, and insulin resistance in mice (Lang et al., 2019). However, for optimal induction of hyperphagia and its metabolic consequences, the CAF model should incorporate a diverse range of palatable high-fat and high-carbohydrate (particularly sugar) food products, encompassing both salty and sweet tastes, as relying solely on high-fat or high-sugar items is less effective (Rodríguez-Correa et al., 2020; Lalanza & Snoeren, 2021).

Lang et al. (2019) conducted a 12-week study on male C57BL/6J mice, initiating dietary interventions at six weeks of age. Mice were assigned to one of four groups: standard chow diet, normal-fat diet, CAF, or high-fat diet. While both CAF and high-fat diet successfully induced visceral obesity, glucose intolerance, and insulin resistance, the CAF diet proved to be more potent in eliciting perivascular adipose tissue dysfunction and vascular dysfunction in the aortas. This suggests that the broader palatability and varied macronutrient composition of the CAF diet may more closely mimic the complex dietary patterns contributing to severe metabolic dysfunction in humans.

2.7 Pancreatic α-amylase

α-amylase (1,4--D-glucan-glucanohydrolase, EC 3.2. 1.1) is digestive enzyme that catalase the α-1,4 glyosidic linkage hydrolysis in starch, maltodextrins and maltooligosaccharides (Tundis et al., 2010). The cofactor of pancreatic α-amylase is calcium ion for structural integrity (Steer & Levitzki, 1973; Vallee et al., 1959) and chloride ion for activation (Levitzki & Steer, 1974), the optimum pH of pancreatic α-amylase is 6.9 (Ishikawa et al., 1990)

2.8 White kidney bean (*Phaseolus vulgaris*)

White kidney bean (*Phaseolus vulgaris*) is a notable source of dietary fiber and resistant starch, with research suggesting a negative correlation between starch intake and colorectal cancer risk, potentially attributed to the protective effects of resistant starch (Soral-Śmietana & Krupa, 2005). Beyond carbohydrates, bean seeds are remarkably rich in protein, constituting 17% to 39% of their dry matter, significantly higher than the 5-15% found in cereals (Bressani, 1993; Krupa & Soral-Śmietana, 2003; Soral-Śmietana et al., 2003). These proteins, primarily storage proteins, are deposited in membrane-bound organelles within cotyledonary parenchyma cells and serve as a crucial reservoir of amino acids, ammonia, and carbon skeletons for the developing seedling upon germination.

A key functional component of *Phaseolus vulgaris* is the α-amylase inhibitor (αAI), which exists in several isoforms: αAI-1, αAI-2 and αAI-3 (or αAI-L). αAI-1 and αAI-2. While αAI-1 and αAI-2 exhibit high amino acid sequence similarity, they differ in their specificity towards α-amylases. Specifically, αAI-1, prevalent in cultivated beans, effectively inhibits porcine pancreatic α-amylase, whereas αAI-2 is found in certain wild bean accessions. The αAI-L isoform, conversely, demonstrates no activity against tested α-amylases and is hypothesized to represent an evolutionary intermediate among plant defense proteins such as phytohemagglutinins and arcelins (Lee et al., 2002; Guzman-Partida et al., 2007; Obiro et al., 2008).

The biosynthesis and maturation of αAI-1, a typical bean lectin, involve several cellular compartments. It is synthesized in the rough endoplasmic reticulum, undergoes modification (signal peptide removal and N-glycosylation) in the Golgi apparatus, and is subsequently transported to protein storage vacuoles for proteolytic processing. Analysis of microsomal fractions via SDS-PAGE indicates that 30-35 kDa fractions are associated with the endoplasmic reticulum, while 14 kDa and 19 kDa fractions are linked to the Golgi body and storage vacuoles, respectively. αAI-1 becomes detectable in the cotyledons and axis of the plant seed approximately 17

days post-pollination, reaching a maximum concentration by 28 days that is maintained until maturity, though its dry weight content may slightly decrease during desiccation (Obiro et al., 2008). Mechanistically, αAI completely occludes the substrate-reducing end of the enzyme cavity and sterically hinders access to the other end. This inhibitor triggers substrate "mimetic" interactions with the enzyme's binding subsites, effectively targeting all catalytically competent components (Payan, 2004; Manatwiyangkool, 2014).

Figure 2.2 White beans (*Phaseolus vulgaris*)

2.9 Effect of white bean extract on vivo

Tormo et al. (2004) investigate the effect of orally administered with 50 mg/kg/days purified α-AI on male Wistar rats (week of age 10) for 3 weeks. They found that Acute effect purified α-AI lower postprandial glycemia. Chronic effect purified α-AI lower body weight, food intake, and glycemia. Fantini et al. (2009) investigate the effect of orally administered with 50, 200, or 500 mg/kg/days *P.valgaris* dry extract on male Wistar rats for 10 days. They found that Acute effect lower postprandial glycemia, and food intake, Chronic effect lower body weight, food intake, and glycemia. Oliveira et al. (2014) investigate the effect of orally administered with commercial phaseolamin on diabetic induced male Wistar rats, the experiment was start when rats had weight rage of 160-210g acclamation for 2 weeks, then rats were induced to diabetic with Streptozotocin, 10 days later rats were orally administered with 100, 500, 1500

mg/kg/days commercial phaseolamin for 20 days. They found that commercial phaseolamin lower glycemia, catalase and superoxide dismutase activity, and tissue damage caused by lipid peroxidation. Song et al. (2016) investigate the effect of orally administered with PVE on diet-induced obesity Male C57BL/6J mice the experiment was start when mice had 4 weeks of age and mice were acclamation for 1 weeks. Mice were induced to obesity with high-fat diet and start orally administered with PVE at the same time of obesity induction. The dose of PVE was 50 mg/kg/days and orally administered for 14 weeks. They found that PVE lower body weight, glycemia, hepatic steatosis, liver cholesterol, liver triglyceride, serum AST, serum ALT, serum glucose, and serum insulin; influence on the gut microbiota. Micheli et al. (2019) investigate the effect of orally administered with PVE on metabolic syndrome induced Male C57BL/6 mice. The experiment was start when mice had 20 gram of body weight and mice were acclamation for 1 weeks. Mice were induced to metabolic syndrome with high-fat diet and start orally administered with PVE at week 11 after the week of metabolic syndrome induction. The dose of PVE was 500 mg/kg/days and orally administered for 8 weeks. They found that PVE lower body weight, glycemia, blood glucose, blood cholesterol, blood triglyceride, blood LDL, plasma insulin, hepatic steatosis and liver lipid peroxidation. Ezzat et al. (2021) investigate the effect of orally administered with *P.vulgaris* fractions on diabetes induced rats the experiment was start when mice had 150-200 gram of body weight and rats were fast for 12 hour then rats were induced to diabetic with Streptozotocin. When Blood glucose level above 300 mg/dl after 48 house the administration with *P.vulgaris* fractions though orogastric tube were conducted for 30 days. The *P.vulgaris* fractions were prepared by *P.vulgaris* pods ethanolic extract and fractions (polar or non-polar). The dose of *P.vulgaris* fractions were 100, or 200 mg/kg/days. They found that the *P.vulgaris* fractions lower blood glucose (polar and non-polar), lower malondialdehyde, nitric oxide, cholesterol, and triglycerides, and increase HDL (non-polar).

In another study, Wang et al. (2020) allocated obese human subjects, balanced by gender, into a control group receiving placebo and an intervention group receiving

PVE. Subjects consumed two PVE capsules (total 2,400 mg/day) before each of three daily meals for a period of 35 days. At the conclusion of the study, the PVE group (n=56; 29 males, 27 females) demonstrated significant weight loss and reduction in subcutaneous fat compared to the placebo group (n=58; 28 males, 30 females), where baseline characteristics (height, weight, age, body fat) were not significantly different between groups ($p < 0.05$).

More recently, Jäger et al. (2024) conducted a randomized trial, assigning subjects to one of three groups: a high-dose PVE group (500 mg/capsule), a low-dose PVE group (350 mg/capsule), or a high-dose placebo group, following a two-week screening period. After 12 weeks of treatment, both PVE dosages significantly reduced body weight, BMI, and fat mass when compared to the placebo group ($p < 0.05$). These collective human studies provide compelling evidence for the beneficial metabolic effects of *Phaseolus vulgaris* extract.

Table 2.1 Reviews of *P. vulgaris* on animals

References	Animals	Induction of metabolic syndrome	Start of experiment (week of age)	Start induction after start of the experiment	Start administration of *P.valgaris* after start of induction	*P.valgaris* administered period	Dose of *P.valgaris* administered (mg/kg/days)	Product for feeding	*P.vulgaris* name	Effect	Main results
Tormo et al. (2004)	Male Wistar rats	-	10	-	-	3w	50	Purified α-AI	White kidney beans	Acute, chronic	[Acute] lower postprandial glycemia; [Chronic] lower body weight, food intake, and glycemia
Fantini et al. (2009)	Male Wistar rats	-	-	-	-	1w 3d	50, 200, 500	Dry extract	Kidney beans	Acute, chronic	[Acute] lower postprandial glycemia, and food intake; [Chronic] lower body weight, food intake, and glycemia
Oliveira et al. (2014)	Male Wistar rats	Streptozotocin	160-210g	2w	1w 3d	2w 6d	100, 500, 1500	Commercial phaseolamin	White beans	Chronic	Lower glycemia, catalase and superoxide dismutase activity, tissue damage caused by lipid peroxidation
Song et al. (2016)	Male C57BL/6J mice	High-fat diet	4	1w	0w	14w	50	Extract	White kidney bean	Chronic	Lower body weight, glycemia, hepatic steatosis, liver cholesterol, liver triglyceride, serum AST, serum ALT, serum glucose, and serum insulin; influence on the gut microbiota
Micheli et al. (2019)	Male C57BL/6 mice	High-fat diet	20g	1w	11w	8w	500	Extract	White kidney beans	Chronic	Lower body weight, glycemia, blood glucose, blood cholesterol, blood triglyceride, blood LDL, plasma insulin, hepatic steatosis and liver lipid peroxidation
Ezzat et al. (2021)	Male Albino Wistar rats	Streptozotocin	150-200g	12h fasted	Blood glucose level above 300 mg/dl after 48 hr of induction	4w 2d	100, 200	Pods ethanolic extract and fractions (polar or non-polar)	Green beans	Chronic	[polar and non-polar] Lower blood glucose [non-polar] Lower malondialdehyde, nitric oxide, cholesterol, and triglycerides; increase HDL

Table 2.2 Reviews of using of *P. vulgaris* human subjects

References	Subjects n (male/female)	Age (years)	BMI (kg/m^2)	*P. vulgaris* administration period	Dose of *P. vulgaris* (mg/days)	Effect	PVE effect
Spadafranca et al. (2013)	Placebo: 12 (6/6) PVE: 12 (6/6)	20 - 26	19.7 – 23.5	7 days of washout period	100	Acute	Lowered postprandial glucose, ghrelin secretion, and eating desire
Wang et al. (2020)	Placebo: 58 (28/30) PVE: 58 (28/27)	18 – 65	≥ 30	35 days	2,400	Chronic	Lowered body weight, BMI, and fat mass
Jäger et al. (2024)	Placebo: 36 (12/24) PVE Low: 18 (6/12) PVE High: 36 (12/24)	18 - 65	25 – 34.9	12 weeks	2100, 3000	Chronic	Lowered body weight, BMI, and fat mass

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CHAPTER III

Unveiling Optimal Extraction Conditions for Potent Alpha-Amylase Inhibitors from White Kidney Beans

3.1 Abstract

This study refines the extraction of alpha-amylase inhibitors, a valuable health-promoting compound, from white kidney beans (*Phaseolus vulgaris*). We employed a data-driven approach to identify the perfect balance between maximizing extract yield and preserving its inhibitory activity.

A Box-Behnken design strategically varied three key factors: solvent concentration, extraction time, and separation time. Response Surface Methodology (RSM) then analyzed the results, revealing a significant correlation between the chosen parameters and the extracted alpha-amylase inhibitory activity. This confirmed the effectiveness of both methods in optimizing the process.

The study analyzes the proximate analysis the composition of white kidney bean, 11.01% moisture, 3.50% ash, 0.06% sand, 0.45% fat, 5.13% crude fiber, and 19.43% protein, identified the ideal extraction conditions as 0.1 M phosphate-buffered saline (PBS), 1 hour extraction, and 30 minutes separation. This optimized approach paves the way for efficient and targeted extraction of alpha-amylase inhibitors from white kidney beans, potentially leading to the development of more potent health supplements and functional food ingredients.

Keywords: White kidney bean *(Phaseolus vulgaris)*, Alpha-amylase, inhibitory activity, Optimization

3.2 Introduction

Archaeological investigations suggest that white kidney beans (*Phaseolus vulgaris*) and other common beans originated in the Americas, specifically the southern United States, Mexico, Central America, and the northern Andes (Geil & Anderson, 1994;

Carai et al., 2009). Introduced to Europe in the 16th century, *P. vulgaris* has since become a vital crop worldwide. Its nutritional value for humans and animals stems from its high content of protein, complex carbohydrates, and dietary fibers (Geil & Anderson, 1994; Carai et al., 2009).

White kidney beans are known for their alpha-amylase inhibitors, first reported by (Bowman, 1945). These inhibitors are concentrated in the embryonic axes and cotyledons, absent in other plant parts (Marshall & Lauda, 1975). They are glycoproteins that specifically inhibit alpha-amylase activity in mammals and insects, leaving plant amylases unaffected (Marshall & Lauda, 1975; Moreno et al., 1990). Studies have shown that *P. vulgaris* extracts can lower body weight and glycemia in animals (Fantini et al., 2009; Song et al., 2016; Micheli et al., 2019).

This study employs a randomized Box-Behnken experimental design to optimize the extraction conditions for alpha-amylase inhibitors from white kidney beans. The research goal is to identify the conditions that yield the highest percentage of extract and the most potent alpha-amylase inhibitory activity.

3.3 Material and Methods

3.3.1 Sample preparation

White kidney bean (*Phaseolus vulgaris*) cultivar Pangda 2 was obtained from Royal Project Foundation, Thailand, was dried and grounded to powder, sieved by mesh size 2 mm, and kept in a vacuum package at 4 °C until used.

3.3.2 Proximate analysis

Dry white kidney beans powder was analyzed for moisture, ash, sand, fat, crude fiber and protein

3.3.2.1 Moisture (AOAC 925.10)

Empty moisture cans were putted in hot air oven at 110°C for 30 min then putted in desiccator for 30 min and weight. White kidney bean powders were placed in moisture cans around 1 gram and putted in hot air oven at 110°C for 24 hour and

then put in desiccator for 30 min and weight. The percentage moisture content was calculated by the formulation:

$$\%\text{Moisture content} = \frac{(B - C - A) \times 100}{B}$$

Where:

A = empty moisture can

B = weight of sample

C = weight of sample + moisture can after oven

3.3.2.2 Ash (AOAC 900.02A)

Empty crucibles were putted in muffle furnace at 550 ℃ for 3 hours then putted in desiccator for 30 min and weight. White kidney bean powders were placed in crucibles around 1 gram and putted in muffle furnace at 550 ℃ for 12-18 hour and then put in desiccator for 30 min and weight. The percentage ash content was calculated by the formulation:

$$\%\text{Ash content} = \frac{(B - C - A) \times 100}{B}$$

Where:

A = empty crucible

B = weight of sample

C = weight of sample + moisture can after furnace

3.3.2.3 Sand (AOAC 900.02D)

Crucibles with ash from ash analysis were added 10% hydrochloric acid and cover with watch glass, boil around 5 min, then filed through ashless filter paper and rinsed with hot water several times. Put filter paper in the same crucible, and put crucibles in muffle furnace at 550 ℃ for 12-18 hours and then put in desiccator for 30 min and weight. The percentage sand content was calculated by the formulation:

$$\%\text{Sand content} = \frac{(B - C - A) \times 100}{B}$$

Where:

A = empty crucible from ash analysis

B = weight of sample from ash analysis

C = weight of sample + moisture can after furnace

3.3.2.4 Crude fat (AOAC 945.16)

Cup and wool were putted in hot air oven at 110°C for 1 hour then putted in desiccator for 30 min, weighted cups then put in cup holder. White kidney bean powders were placed in filter paper around 1 gram wrapped up and putted in thimble with wool cover upper and lower then putted Soxtec extractor raised thimble up placed cups than add petroleum ether 80 ml in each cup. Run the Soxtec extractor around 3 hours. Draw wrapped filter paper with sample inside out of Trimble, putted its and cups in hot air oven at 110°C for 30 min then putted in desiccator for 30 min for cups but until used in crude fiber analysis for wrapped filter paper. Weight cups. The percentage sand content was calculated by the formulation:

$$\%\text{Crud fat content} = \frac{(C - A) \times 100}{B}$$

Where:

A = empty cup

B = weight of sample

C = weight of cup after extraction

3.3.2.5 Crude fiber (AOAC 978.10)

Empty crucibles were putted in muffle furnace at 500 °C for 30 hours then putted in desiccator for 30 min and weight. Weight defatted samples from crude fat analysis in crucibles then load crucibles to hot extractor poured 1.25% sulfuric acid and 1.25% sodium hydroxide to tank in hot extractor then composed each of hot extractor and run around 3 hours. After hot extraction, moved crucibles out washed samples with 95% ethanol 10 ml. Put crucibles in hot air oven at 105 °C for 3 hours then putted in desiccator for 30 min and weight then and putted in muffle furnace at 500 °C for 2 hours then putted in desiccator for 30 min and weight. The percentage crude fiber content was calculated by the formulation:

$$\%\text{Crude fiber content} = \frac{(B - C) \times 100}{A}$$

Where:

A = weight of sample

B = weight of crucible after hot air oven

C = weight of crucible after muffle furnace

3.3.2.6 Crude protein (AOAC 928.08)

0.5 N hydrochloric acid were standardized with titration with sodium tetraborate conducted by weight 5 gram of sodium tetraborate adjusted volume to 250 ml, diluted 0.5 N hydrochloric acid for 10 times, drop methyl red 2 drop and titrate with sodium tetraborate solution, the end point was changing of color from pink to no color.

White kidney bean powders were weight around 1 gram to Kjehdalh flask follow by potassium sulfate and copper sulfate 7 and 0.7 gram respectively, and did blank with same condition without sample. Add 20 ml of sulfuric acid then load in Kjedalh digestion apparatus and digested in 400 °C for 2 hours until become green color. Pour 50 ml of boric acid in Erlenmeyer flask and dropped mixed indicator 4-5 drop then placed under Kjeldahl Distillation system with tube insert in flask, Load Kjehdalh flask in to Kjeldahl Distillation system settled condition, 80 ml of distillated water and 80 ml of 32% sodium hydroxide, and run for 6 min. Brought Erlenmeyer flask to titrate with definite concentration 0.5 N hydrochloric acid, the end point was changing of color from green to pink. The percentage nitrogen content was calculated by the formulation and multiply with protein factor of white kidney bean 6.25.

$$\%N = \frac{\text{Normality of HCl}}{1000} \times \frac{V_s - V_b}{w} \times 14 \times 100$$

Where:

%N = percentage nitrogen

V_s = volume of acid used for sample

V_b = volume of acid used for blank

w = weight of sample

%crude protein = %N x 6.25

3.3.3 White kidney bean extraction

The extraction method was adapted from (Mosca et al., 2008; Manatwiyangkool, 2014). Briefly, ground white kidney beans were suspended in varying PBS (phosphate-buffered saline) solutions (0.05 M, 0.1 M, 0.15 M) at a ratio of 1.65 g beans to 10 ml PBS (10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl). The suspension was stirred for 1, 2, or 3 hours at room temperature. Subsequently, centrifugation was performed at 10,000 rpm for 30, 45, or 60 minutes at 4°C. The supernatant was collected, and the volume was adjusted to 10 ml using the corresponding PBS solution (0.05 M, 0.1 M, 0.15 M). Two milliliters of the adjusted supernatant were then aliquoted into separate tubes (5 tubes total). Finally, the aliquots were freeze-dried and stored at -20°C until further use.

3.3.4 Protein concentration

Method of Bradford (1976) using BSA as a standard. Prepare a series of protein standards using BSA diluted with 0.15 M NaCl to final concentrations of 0 (blank = NaCl only), 0.1, 0.2, 0.3, 0.5, 0.7 and 1 mg BSA/mL. Also prepare serial dilutions of the unknown sample to be measured. Add 100 µL of each of the above to a separate test tube. Add 5.0 mL of Coomassie Blue to each tube and mix by vortex, or inversion. Adjust the spectrophotometer to a wavelength of 595 nm, and blank using the tube which contains 0 BSA. Wait 5 minutes and read each of the standards and each of the samples at 595 nm wavelength. Plot the absorbance of the standards vs. their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.

3.3.5 Specific α-amylase inhibitory activity measurement

Specific α-amylase inhibitory activity was measured using the method described by Bernfeld (1955) with modifications, by measuring the residual α-amylase

activity. The αAI and 40 unit/ml porcine pancreas α-amylase solution in succinate buffer (15 mM, 20 mM $CaCl_2$, and 0.5 M NaCl, pH 5.6) was preincubated at 37 ˚C for 30 min, after that 2% (w/v) soluble starch solution was added and incubated at 37 ˚C for 1 min. The reaction was stopped by addition of 0.8 ml of dinitrosalicylic acid reagent (DNS) solution and boiled for 10 min then cooled and diluted with 6 ml of water then the absorbance was read at 540 nm. Appropriate blank was prepared without α-amylase also the residual activity was measured. A control without enzyme inhibitor addition was done for the specific inhibition activity calculation.

$$\text{Inhibition} = \frac{\text{liberated maltose in control} - \text{liberated maltose in sample}}{\text{sample}}$$

$$\text{Specific inhibitory activity} = \frac{\text{Inhibition}}{\text{mg protein}}$$

3.3.6 Experimental design

Response surface methodology (RSM) was chosen to identify the optimal settings for three critical variables influencing both extract yield and alpha-amylase inhibitory activity. These variables, PBS concentration (denoted as X_1), extraction time (X_2), and separation time (X_3) were investigated using a randomized Box-Behnken design. This specific design ensures all experiments are conducted in a fully randomized order, minimizing bias and strengthening the statistical analysis (Table 3.1).

Table 3.1 Encoded and coded levels of independent variables used in the experimental design.

Symbols	Independent variables	Coded levels		
		-1	0	1
X_1	PBS concentration (M)	0.05	0.1	0.15
X_2	Extraction time (hour)	1	2	3
X_3	Separation time (min)	30	45	60

3.4 Results and Discussion

3.4.1 Proximate analysis results

Proximate analysis results showed in Table 4.4. These results had each attribute, except fat, and crude fiber, near Manastwiyangkool (2014) who reported composition of whit kidney bean, 11.07% moisture, 4.10% ash, 0.01% sand, 1.8%fat, 31.73% crud fiber, and 20.28% protein.

Table. 3.2 Proximate analysis

Nutrient	% Composition
Moisture	11.01 ± 0.05
Ash	3.50 ± 0.12
Sand	0.06 ± 0.01
Fat	0.45 ± 0.04
Crude fiber	5.13 ± 0.17
Protein	19.43 ± 0.07

3.4.2 Optimization of bean extraction conditions

To identify the ideal extraction conditions for maximizing alpha-amylase inhibitory activity in white kidney beans, a response surface methodology (RSM) analysis was conducted on the data presented in Table 3.2 This analysis focused on the influence of three independent variables – PBS concentration, extraction time, and separation time – on the two key responses: percentage yield (Y_1) and alpha-amylase inhibitory activity (Y_2). The RSM analysis revealed a strong correlation between these variables and the desired outcomes. Notably, the analysis identified the following conditions as optimal for achieving the highest alpha-amylase inhibitory activity: PBS concentration: 0.1 M, extraction time at 1 h, and separation time for 30 min. These optimized extraction conditions are expected to yield white kidney bean extracts with both a desirable yield and potent alpha-amylase inhibitory activity, paving the way for further research on their potential health benefits.

Table 3.3 Experimental design and responses of the dependent variables to the extract parameter

	Independent variables				
Exp. No.	PBS concentration (M) (X_1)	Extraction time (hour) (X_2)	Separation time (min) (X_3)	%yield (Y_1)	alpha-amylase inhibitory activity (unit/g) (Y_2)
1	0.1 (0)	1 (-1)	30 (-1)	56.57 ± 0.37	2.58 ± 0.02
2	0.1 (0)	3 (1)	30 (-1)	41.29 ± 0.23	1.88 ± 0.01
3	0.1 (0)	1 (-1)	60 (1)	35.64 ± 0.21	1.62 ± 0.02
4	0.1 (0)	3 (1)	60 (1)	38.88 ± 0.57	1.77 ± 0.02
5	0.05 (-1)	1 (-1)	45 (0)	23.91 ± 0.52	1.09 ± 0.15
6	0.05 (-1)	3 (1)	45 (0)	38.24 ± 0.52	1.74 ± 0.03
7	0.15 (1)	1 (-1)	45 (0)	43.69 ± 0.21	2.00 ± 0.01
8	0.15 (1)	3 (1)	45 (0)	36.18 ± 0.29	1.67 ± 0.01
9	0.05 (-1)	2 (0)	30 (-1)	24.31 ± 0.30	1.11 ± 0.29
10	0.05 (-1)	2 (0)	60 (1)	54.51 ± 0.38	2.49 ± 0.03
11	0.15 (1)	2 (0)	30 (-1)	49.69 ± 0.14	2.26 ± 0.02
12	0.15 (1)	2 (0)	60 (1)	38.39 ± 0.17	1.76 ± 0.03
13	0.1 (0)	2 (0)	45 (0)	37.86 ± 0.57	1.72 ± 0.02
14	0.1 (0)	2 (0)	45 (0)	30.85 ± 0.21	1.41 ± 0.02
15	0.1 (0)	2 (0)	45 (0)	29.91 ± 0.17	1.37 ± 0.01

Significant at $p < 0.05$ expressed by letters

1 = High level, 0 = Medium level, -1 Low level

Table 3.4 ANOVA of independent variables for percentage yield and alpha-amylase inhibitory activity respond

Source	Sum of Squares	df	Mean Square	F-value	p-value		Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	732.42	6	122.07	1.71	0.2357	not significant	Model	1.51	6	0.2523	1.7	0.2388	not significant
A-X1	90.99	1	90.99	1.28	0.2915		A-X1	0.1984	1	0.1984	1.34	0.2812	
B-X2	3.41	1	3.41	0.0477	0.8325		B-X2	0.0066	1	0.0066	0.0445	0.8382	
C-X3	2.46	1	2.46	0.0345	0.8572		C-X3	0.0045	1	0.0045	0.0304	0.866	
AB	119.25	1	119.25	1.67	0.2321		AB	0.2401	1	0.2401	1.62	0.2394	
AC	430.56	1	430.56	6.04	0.0395		AC	0.8836	1	0.8836	5.95	0.0407	
BC	85.75	1	85.75	1.2	0.3048		BC	0.1806	1	0.1806	1.22	0.3023	
Residual	570.7	8	71.34				Residual	1.19	8	0.1486			
Lack of Fit	532.96	6	88.83	4.71	0.1856	not significant	Lack of Fit	1.12	6	0.1859	5.07	0.174	not significant
Pure Error	37.74	2	18.87				Pure Error	0.0734	2	0.0367			
Cor Total	1303.12	14					Cor Total	2.7	14				
		Std.Dev = 8.45							Std.Dev = 0.3855				
		R-Squared = 0.562							R-Squared = 0.5601				
		Mean = 38.66							Mean = 1.76				
		R-Squared = 0.2336							R-Squared = 0.2302				
		C.V. % = 21.85							C.V. % = 21.85				
		Adeq Precision = 4.7653							Adeq Precision = 4.7656				

Significant at $p < 0.05$

Table 3.3 shows the ANOVA of independent variables for percentage yield and alpha-amylase inhibitory activity which is not significantly different at a p-value lower than 0.05 in both percentage yield and alpha-amylase inhibitory.

Figure 3.1 showed response surface plots of the interactive effect of PBS concentration, extraction time, and separation time on percentage yield and alpha-amylase inhibitory activity respectively which had the same trend.

(a)

(b)

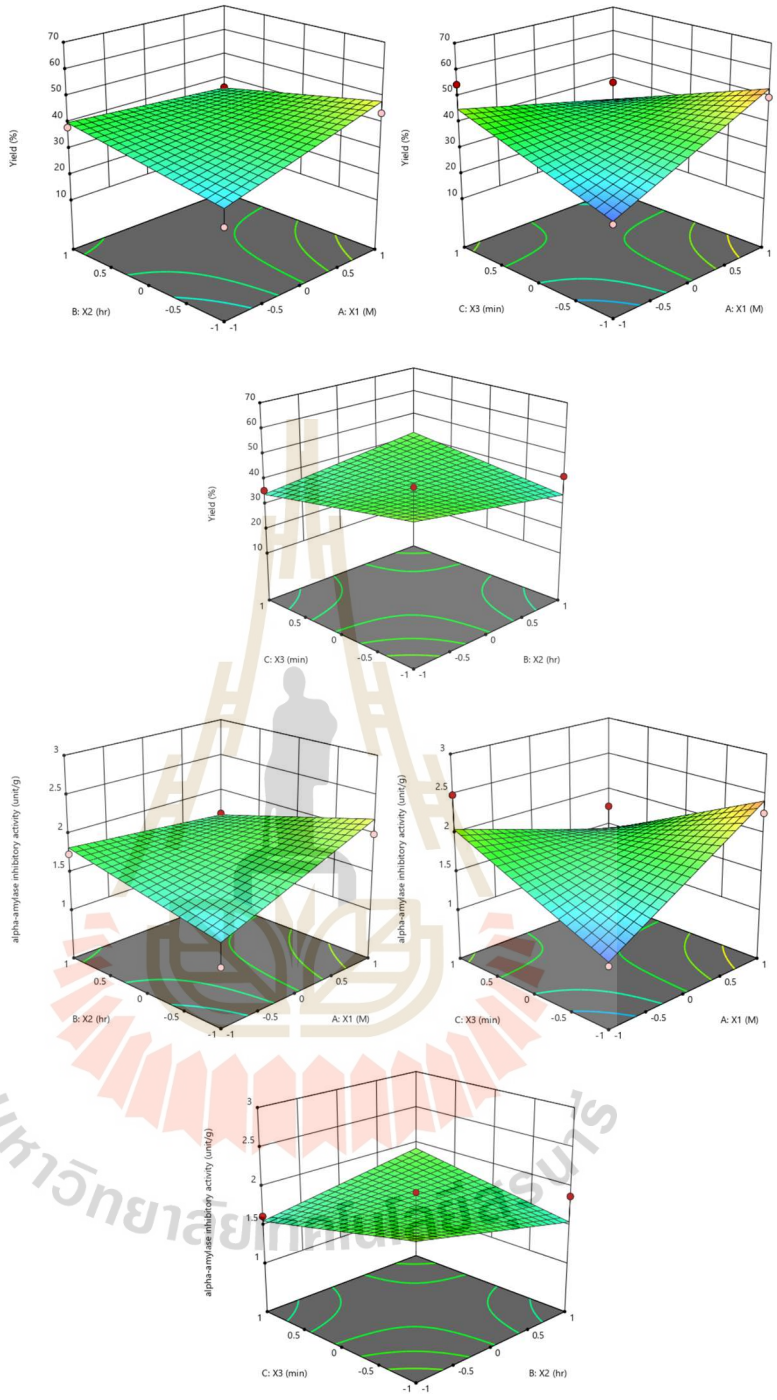

Figure 3.1 Response surface plots indicate the interaction effect of PBS concentration and extraction time, the interaction effect of PBS concentration and separation time, and the interaction effect of extraction time separation time on percentage yield (a) and alpha-amylase inhibitory activity (b).

The regression models are presented in the equation

$Y_1 = 38.66133 + 3.3725\ X_1 - 0.6525\ X_2 - 0.555\ X_3 - 5.46\ X_1\ X_2 - 10.375\ X_1\ X_3 + 4.63\ X_2\ X_3$ (1)

$Y_2 = 1.76467 + 0.1575\ X_1 - 0.02875\ X_2 - 0.02375\ X_3 - 0.245\ X_1\ X_2 - 0.47\ X_1\ X_3 + 0.2125\ X_2\ X_3$ (2)

3.4 Conclusion

This study successfully gained proximate analysis showed the composition of white kidney bean, 11.01% moisture, 3.50% ash, 0.06% sand, 0.45% fat, 5.13% crude fiber, and 19.43% protein, employed a randomized Box-Behnken design to identify the optimal conditions for extracting white kidney bean extract with maximized yield and alpha-amylase inhibitory activity. Under these parameters, the extract achieved a promising yield of 56.57% and a notable inhibitory activity of 2.85 units/g.

However, future research could research deeper into the specific components responsible for the inhibitory activity. Employing high-performance liquid chromatography (HPLC) would enable the quantification of total alpha-amylase inhibitors and active glycoproteins within the white kidney beans. This approach holds the potential to refine extraction conditions even further, potentially leading to even higher yields of extracts boasting even more potent inhibitory activity. By optimizing the extraction process based on specific bioactive components, this research paves the way for the development of more effective functional foods and nutraceuticals derived from white kidney beans.

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CHAPTER IV

Evaluation of *Phaseolus vulgaris* Extract in a Rat Model of Cafeteria Diet-Induced Obesity: Metabolic and Biochemical Effects

4.1 Abstract

Obesity is a global health concern that elevates the risk of non-communicable diseases (NCDs) such as type 2 diabetes, cardiovascular disease, and certain cancers. *Phaseolus vulgaris* (white bean) contains α-amylase inhibitors (αAIs) that can reduce carbohydrate digestion and absorption, potentially mitigating obesity and metabolic syndrome. This study investigated the impact of *P. vulgaris* extract (PVE) on obese rats. Male Wistar rats were fed either a standard diet (SD) or a cafeteria diet (CAF) for 17 weeks to induce obesity. Subsequently, rats in each dietary group were randomly assigned to receive a vehicle, low-dose PVE (200 mg/kg), high-dose PVE (300 mg/kg), or metformin (200 mg/kg) via an oral gavage for 6 weeks. The CAF group exhibited significantly greater weight gain compared to the SD group. In the CAF group, a low dose of PVE lowered postprandial glycemia during an oral glucose tolerance test (OGTT) at 60 and 120 minutes and decreased food and energy intake during weeks 17-20 and 18-19, respectively. In the SD group, a high dose of PVE reduced glycemia at 90 minutes in the OGTT, as well as body weight gain, food intake, and energy intake during week 17. However, the overall areas under the glucose curves in the OGTT were not significantly different across treatment groups ($p > 0.05$), and while individual time points showed changes, the overall glucose exposure (AUC) was not significantly altered. In conclusion, the αAIs present in *P. vulgaris* demonstrate the potential to reduce body weight, weight gain, glycemia, total cholesterol, and triglycerides in vivo, but in the CAF group, neither PVE dose significantly altered TC or TG. This study provides strong support for further exploring *Phaseolus vulgaris* extract as a valuable

functional ingredient in the food industry, particularly for developing products that aid in weight management and glycemic control.

Keywords: Obesity; Cafeteria diet; *Phaseolus vulgaris*; Glucose tolerance test; Blood chemistry

4.2 Introduction

Noncommunicable chronic diseases (NCDs), including hypertension, hyperlipidemia, type 2 diabetes mellitus (T2DM), and metabolic syndrome, are leading causes of global mortality (Ejigu & Tiruneh, 2023). Obesity and overweight are significant modifiable risk factors for NCDs, primarily driven by dietary patterns characterized by an imbalance between energy intake and expenditure (Wright & Aronne, 2012; Romieu et al., 2017). T2DM, a prevalent NCD, is characterized by impaired insulin action and hyperglycemia, often associated with obesity and lifestyle factors (Clinical Practice Guideline for Diabetes, 2017).

Dietary bioactive compounds offer a potential preventative and therapeutic strategy against these conditions, among which α-amylase inhibitors (αAIs), notably found in white kidney beans (*Phaseolus vulgaris*), can retard starch absorption. First identified in 1945 (Bowman, 1945) and later termed phaseolamin (Marshall & Lauda, 1975), αAIs from *P. vulgaris* have been extensively investigated for their effects on T2DM, overweight, and obesity in both animal models (Tormo et al., 2004; Fantini et al., 2009; Oliveira et al., 2014; Micheli et al., 2019; Ezzat et al., 2021) and human subjects (Spadafranca et al., 2013; Jäger et al., 2024). *P. vulgaris* extract (PVE) has been reported to reduce body weight and improve glycemic control in various animal models (Tormo et al., 2004; Fantini et al., 2009; Micheli et al., 2019; Bradford, 1976). In addition, a previous study by Oonsivilai et al. (2024) successfully employed a randomized Box–Behnken design to identify the optimal conditions for obtaining white kidney bean extract with maximized yield and alpha-amylase inhibitory activity. Under these parameters, the extract achieved a promising yield of 56.75% and a notable inhibitory activity of 2.85 units/g.

Collectively, studies on PVE and its active compound, phaseolamin, demonstrate significant potential for managing metabolic disorders in animal models. The core mechanism involves α-amylase inhibition, which delays carbohydrate digestion and absorption, leading to reduced post-prandial glucose levels and contributing to hypoglycemic and anorexigenic effects (Tormo et al., 2004; Fantini et al., 2009; Micheli et al., 2019). This translates into beneficial outcomes such as decreased food intake, reduced body weight, and improved glycemic control (Tormo et al., 2004; Fantini et al., 2009; Micheli et al., 2019). Beyond these direct metabolic impacts, PVE has also shown protective effects against oxidative stress and organ damage associated with diabetes and metabolic syndrome, including the prevention of collagen deposition in the heart and a reduction in hepatic steatosis (Oliveira et al., 2014; Micheli et al., 2019). While the overall evidence supports PVE's efficacy, some inconsistencies regarding dose–response relationships (Oliveira et al., 2014) highlight the need for further research to fully optimize its therapeutic application in various physiological contexts.

Despite the recognized potential of *P. vulgaris* components, the precise relationship between extract dosage and metabolic outcomes remains inconsistent. For instance, some studies have reported that varying doses of phaseolamin uniformly reduced glycemia without demonstrating a clear dose-dependent response, and in certain induced diabetes models, phaseolamin did not significantly alter key metabolic markers (Oliveira et al., 2014). While other research has shown PVE to be comparable to established anti-diabetic drugs like metformin in improving glycemic profiles (Micheli et al., 2019), these contrasting findings underscore a critical need for further investigation into the specific dose–response mechanisms and broader impact of PVE on diverse metabolic parameters.

The cafeteria diet (CAF)-induced obese rat model is a widely used research tool that mimics human obesity by providing rats with ad libitum access to a variety of highly palatable, energy-dense "junk foods" in addition to their standard chow. This encourages voluntary hyperphagia (overeating) due to the appeal and diversity of the

foods, leading to rapid weight gain and increased adiposity. Beyond simple weight gain, the model consistently induces a comprehensive metabolic syndrome phenotype, including hyperinsulinemia, insulin resistance, dyslipidemia, and non-alcoholic fatty liver disease, closely reflecting the complex metabolic dysfunctions seen in humans with diet-induced obesity. This approach leverages the rats' natural preferences to create a robust and translationally relevant model for studying obesity and its comorbidities.

A significant research gap exists in evaluating the therapeutic efficacy of PVE in established diet-induced obesity models, particularly those induced by a prolonged CAF. Much of the existing literature has focused on the preventative or early ameliorative effects of *P. vulgaris* administration, often initiating treatment concurrently with or shortly after obesogenic diet exposure. To address these limitations and provide clinically relevant insights, this study investigated the therapeutic potential of PVE in Wistar rats with well-established obesity induced by a prolonged CAF regimen. This approach facilitates a comprehensive assessment of PVE's capacity to reverse or mitigate the metabolic consequences of chronic diet-induced obesity, specifically focusing on glycemia and key blood biochemical parameters, thereby distinguishing this study from prior research and offering novel insights into PVE as an intervention for existing metabolic complications.

4.3 Materials and Methods

4.3.1 Materials and Sample Preparation

The extraction of αAIs from *P. vulgaris* was optimized based on established protocols (Fantini et al., 2009; Micheli et al., 2019). In brief, 1.65 g of finely ground white kidney bean powder was suspended in 10 mL of 0.1 M phosphate-buffered saline (PBS, pH 7.2 containing 150 mM NaCl), resulting in a 1:6.06 (w/v) ratio. This suspension was continuously stirred at 37 °C for 1 h to facilitate the release of inhibitory compounds. Following incubation, the homogenate was centrifuged at 10,000 rpm (equivalent to approximately 16,000 × g) for 30 minutes at 4 °C using a refrigerated centrifuge (Hettich, universal 16R, USA). The resulting supernatant, containing the crude

PVE, was carefully collected and adjusted to a final volume of 10 mL with the PBS solution. Aliquots of 2 mL were then prepared and immediately freeze-dried using a freezer (GEA, LYOVAC GT2-S, MD) at -80°C under a pressure of 0.010 mBar for 72 h. The freeze-dried PVE aliquots were stored at -20 °C until further biochemical analysis. The protein content of the reconstituted PVE was determined using the Bradford assay with bovine serum albumin (BSA) as a standard (Bradford, 1976). For the Bradford assay, first prepare a BSA solution at a concentration of 1 mg/mL and dilute it to concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL to establish a standard curve. Prepare the Bradford reagent by dissolving 100 mL of Coomassie Brilliant Blue G-250 in 50 mL of 95% ethanol, stirring until the dye is completely dissolved. Next, add 100 mL of 85% phosphoric acid, and adjust the final volume to 1,000 mL with distilled water. Filter the solution using filter paper No. 1 and store it in an amber bottle at 4 °C. For protein measurement, mix 25 µL of the sample or standard solution with 1 mL of the Bradford reagent. Incubate the solution at room temperature for 5 minutes, then measure the absorbance using a spectrophotometer (Genesys 10S UV-VIS, Thermo Fisher Scientific) at a wavelength of 595 nm.

4.3.2. Animals and Experimental design

4.3.2.1 Animal experiment for obesity induction using SD and CAF

Sixty-four male Wistar rats, aged 8 weeks upon arrival, were obtained from the Laboratory Animal Center at Suranaree University of Technology (SUT), Nakhon Ratchasima, Thailand. Upon arrival, rats were housed individually in standard plastic cages under controlled environmental conditions, maintaining a constant temperature (22-24 °C) and a 12-hour light/dark cycle. All experimental procedures adhered strictly to the ethical guidelines for the care and use of laboratory animals established by the Suranaree University of Technology Animal Care and Use Committee, and the study was conducted under the approved protocol (approval code A-12/2020).

Following a two-week acclimation period, the rats were randomly assigned to one of two dietary groups (n = 48; 24 per group): a standard rodent chow diet (SD) group or a cafeteria diet (CAF) group. The CAF, designed to induce obesity, consisted

of a variety of palatable, high-energy foods offered in addition to standard chow for a duration of 17 weeks detail as in supplementary material.

4.3.2.2 Animal experiment during treatment

After the 17-week dietary intervention, both the SD and CAF groups were further randomized into four treatment subgroups (n = 6 per subgroup) for a subsequent 6-week treatment period. The SD group comprised a vehicle control (SDV; distilled water), a low-dose PVE group (SDLP; 200 mg/kg body weight), a high-dose PVE group (SDHP; 300 mg/kg body weight), and a metformin-treated group (SDM; 200 mg/kg body weight). Similarly, the CAF group included a vehicle control (CAFV; distilled water), a low-dose PVE group (CAFLP; 200 mg/kg body weight), a high-dose PVE group (CAFHP; 300 mg/kg body weight), and a metformin-treated group (CAFM; 200 mg/kg body weight). All treatments were administered orally via a gavage once daily throughout the 6-week treatment duration. In addition, the low and high doses of PVE were chosen based on αAI activities determined during the extract preparation process in previous research (Tormo et al., 2004; Fantini et al., 2009; Oliveira et al., 2014; Micheli et al., 2019; Oonsivilai et al., 2024).

All treatments were administered orally via a gavage once daily for 6 weeks. At the end of the 6-week treatment period, all rats underwent an oral glucose tolerance test (OGTT). Following the OGTT, rats were fasted overnight (12 h) before being humanely sacrificed by CO_2 asphyxiation, and subsequently euthanized via cardiac puncture for blood sample collection. Blood samples were processed to obtain serum and plasma for subsequent biochemical analyses.

4.3.3. Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was performed after 6 weeks of oral administration. All rats in the groups were given glucose (2 g/kg) after 12 h fasting. These measurements were taken at 0 (before oral glucose), 30, 60, 90, and 120 min after oral glucose administration at 2 g/kg BW, and the area under the curve (AUC) was

determined by calculation trapezoid area formula and accumulation (Goto et al., 1976; Gómez-Velázquez et al., 2022).

4.3.4. Blood chemistry

Following the experimental period, rat blood collected in anticoagulant-treated tubes was centrifuged at 10,000 rpm at 4 ℃ for 5 minutes. Plasma was then carefully separated, aliquoted, and stored at -20 ℃ pending an analysis performed by a certified laboratory service (RIA Laboratory Co., LTD, Nakhon Ratchasima, Thailand) with the standard methods certified by Laboratory Thailand accreditation by Thailand Medical Technology Council. The blood chemistry profile assessed included the quantification of total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine (Cr).

4.3.5. Statistical analysis

All experiments were performed in biological replicates, and mean values (on a dry basis) with standard deviations are reported. Data were analyzed via an independent-sample T-test with a statistically significant difference at $p < 0.05$. The experimental data were analyzed using an analysis of variance (ANOVA). The software platform SPSS® version 17 (SPSS Inc., Chicago, IL, USA) was used to perform all statistical calculations.

4.4. Results

4.4.1. SDS PAGE

Result of Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed in Figure 4.1 The PVE samples analyzed via SDS-PAGE exhibit a broad distribution of protein molecular weights, ranging from very high molecular weight components (likely above 250 kDa, possibly indicative of aggregates or large protein complexes) down to smaller proteins around 100 kDa, 60-70 kDa, 37 kDa, 25 kDa, and even as low as 20 kDa and possibly 15 kDa. Marshall & Lauda (1975) reported their

phaseolamin molecular weight in range 45,000-50,000 kDa. Pueyo et al. (1993) reported their αAI small polypeptide molecular weight 14,000-18,000 kDa.

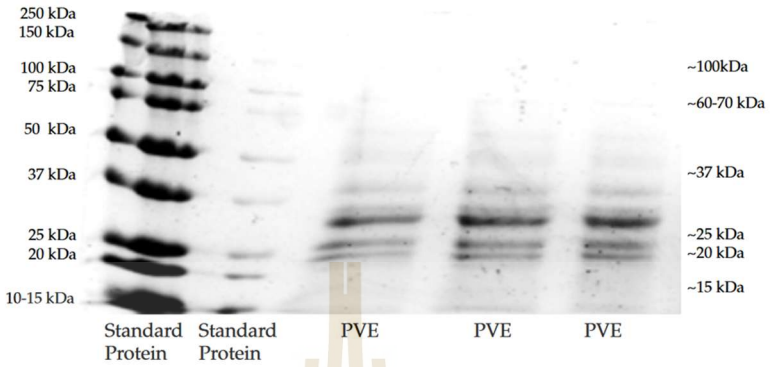

Figure 4.1 SDS PAGE of PVE.

4.4.2. Body weight

Initial body weights did not differ significantly between the standard diet (SD) and CAF groups during the first two weeks ($p > 0.05$; Table 1). Subsequently, from week 3 to week 16 (prior to subgroup allocation), the CAF group exhibited significantly higher body weights compared to the SD group ($p < 0.05$). Following subgrouping at week 17, rats in the CAF group maintained significantly higher body weights than all SD subgroups receiving the same oral administration ($p < 0.05$), except for the metformin-treated subgroups at week 18 ($p > 0.05$). Within the SD group, no significant differences in body weight were observed between the vehicle control and the low PVE, high PVE, and metformin-treated subgroups in the weeks following oral administration ($p > 0.05$), except for the high PVE subgroup at the final week (week 22), which displayed a significantly lower body weight compared to the vehicle control ($p < 0.05$). Similarly, within the CAF group, body weights did not significantly differ between the vehicle control and the low PVE and high PVE-treated subgroups after oral administration ($p > 0.05$), except for the metformin-treated subgroup at week 18 and the final week, which showed significantly lower body weights compared to the vehicle control ($p < 0.05$).

The initial body weights were comparable across all SD and CAF subgroups receiving the same oral administration ($p > 0.05$) (Table 4.1). Furthermore, within both the SD and CAF groups, initial body weights did not significantly differ between the

vehicle control and the subgroups treated with low PVE, high PVE, or metformin ($p > 0.05$). At the conclusion of the study, the CAF group exhibited significantly higher final body weights and total body weight gain compared to the corresponding SD subgroups receiving the same oral administration ($p < 0.05$). Within the SD group, high PVE administration resulted in significantly lower final body weight and body weight gain compared to vehicle control ($p < 0.05$). Low PVE administration in the SD group led to a significant reduction in body weight gain compared to the vehicle control ($p < 0.05$), although final body weight did not differ significantly ($p > 0.05$). Metformin administration in the SD group did not significantly affect final body weight or body weight gain compared to the vehicle control ($p > 0.05$). Conversely, within the CAF group, metformin administration significantly reduced both final body weight and body weight gain compared to the vehicle control ($p < 0.05$). Low PVE administration in the CAF group resulted in significantly lower body weight gain than the vehicle control ($p < 0.05$), but final body weight was not significantly different ($p > 0.05$). High PVE administration in the CAF group did not significantly alter final body weight or body weight gain compared to the vehicle control ($p > 0.05$).

Table 4.1 Initial body weight, final body weight, and body weight gained of male Wistar rats re-ceiving SDV, SDLP, SDHP, SDM, CAFV, CAFLP, CAFHP, CAFM.

Group	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
SDV	319 ± 40	602 ± 64 ^{a}	283 ± 35
SDLP	331 ± 33	578 ± 56 ^{b}	247 ± 25 ^{b}
SDHP	318 ± 12	548 ± 29 ^{b}	230 ± 28 ^{b}
SDM	320 ± 30	597 ± 49 ^{a}	277 ± 25
CAFV	319 ± 32	810 ± 127 ^{*,a}	491 ± 110 ^{*,a}
CAFLP	338 ± 33	722 ± 43 ^{*,ab}	384 ± 45 ^{*,b}
CAFHP	320 ± 39	776 ± 128 ^{*,a}	456 ± 96 ^{*,a}
CAFM	323 ± 14	678 ± 70 ^{*,bc}	356 ± 61 ^{*,b}

Values are expressed as means ± S.D.

* Significant difference between SD and CAF groups with the same treatment ($p < 0.05$).

^{abc} Significant difference between vehicle and treatment groups ($p < 0.05$).

4.4.3. Food intake

Food intake was monitored weekly (Table 4.2). Prior to subgrouping, no significant differences in food intake were observed between the SD and CAF groups during the initial two weeks and at week 6 ($p > 0.05$).

However, from weeks 3 to 5 and weeks 7 to 16, the CAF group consumed significantly less food than the SD group ($p < 0.05$). Following subgrouping, in the vehicle-treated subgroups, the CAF group exhibited comparable food intake to the SD group, except at weeks 17 and 20, where the CAF group showed significantly lower consumption ($p < 0.05$). In the low PVE-treated subgroups, the CAF group consistently displayed significantly lower food intake than the SD group throughout the post-subgrouping period ($p < 0.05$). In the high PVE-treated subgroups, food intake did not significantly differ between the CAF and SD groups, except in weeks 17 and 21, where the CAF group consumed less ($p < 0.05$). Similarly, in the metformin-treated subgroups, the CAF group consistently exhibited significantly lower food intake compared to the SD group after subgrouping ($p < 0.05$).

Within the SD group, food intake did not significantly differ across the orally administered subgroups (vehicle, low PVE, high PVE, and metformin) during the treatment period ($p > 0.05$), with the exception of week 17 for the low PVE, high PVE, and metformin subgroups, and week 18 for the metformin subgroup, where food intake was significantly lower than the vehicle control ($p < 0.05$). Among the CAF group, the low PVE subgroup exhibited significantly lower food intake compared to the vehicle control from weeks 17 to 20 ($p < 0.05$), and the metformin subgroup showed significantly reduced food intake from weeks 17 to 22 ($p < 0.05$). In contrast, the low PVE subgroup from week 21 to 22 and the high PVE subgroup from week 17 to 22 did not show significant differences in food intake compared to the vehicle control within the CAF group ($p > 0.05$). Notably, food intake in the SD group remained relatively stable from week 4 until the end of the experiment, even after subgrouping. Similarly, the CAF group's food intake pattern remained consistent from week 3 until the study's conclusion, despite subgroup allocation.

Table 4.2 Food consumption of male Wistar rats receiving SDV, SDLP, SDHP, SDM, CAFV, CAFLP, CAFHP, CAFM.

	Food consumption (g/week/rat)						
Group	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
SDV	149 ± 9 ^{a}	160 ± 6 ^{ab}	152 ± 3 ^{b}	149 ± 7 ^{ab}	164 ± 6 ^{a}	161 ± 7 ^{ab}	147 ± 11 ^{b}
SDLP	137 ± 16 ^{b}	149 ± 11 ^{b}	150 ± 12 ^{bc}	155 ± 12 ^{ab}	168 ± 7 ^{a}	166 ± 7 ^{a}	150 ± 4 ^{a}
SDHP	144 ± 14 ^{a}	149 ± 6 ^{b}	146 ± 14 ^{c}	143 ± 15 ^{b}	152 ± 18 ^{ab}	155 ± 13 ^{b}	149 ± 20 ^{a}
SDM	149 ± 8 ^{a}	166 ± 4 ^{a}	167 ± 15 ^{a}	159 ± 14 ^{a}	163 ± 13 ^{a}	167 ± 14 ^{a}	152 ± 19 ^{a}
CAFV	129 ± 20 ^{*,a}	136 ± 19 ^{*,a}	151 ± 13 ^{a}	147 ± 15 ^{a}	142 ± 21 ^{*,a}	147 ± 22 ^{a}	157 ± 23 ^{a}
CAFLP	112 ± 5 ^{*,b}	119 ± 8 ^{*,b}	135 ± 7 ^{*,c}	127 ± 11 ^{*,c}	110 ± 15 ^{*,b}	132 ± 5 ^{*,b}	138 ± 6 ^{*,bc}
CAFHP	118 ± 7 ^{*,b}	127 ± 7 ^{*,ab}	147 ± 8 ^{ab}	141 ± 6 ^{b}	140 ± 9 ^{a}	142 ± 8 ^{ab}	149 ± 6 ^{b}
CAFM	101 ± 3 ^{*,c}	117 ± 13 ^{*,b}	137 ± 11 ^{*,bc}	129 ± 12 ^{*,bc}	119 ± 9 ^{*,b}	120 ± 11 ^{*,c}	130 ± 4 ^{*,c}

Values are expressed as means ± S.D.

* Significant difference between SD and CAF groups with the same treatment ($p < 0.05$).

^{abc} Significant difference between vehicle and treatment groups ($p < 0.05$).

4.4.4. Energy intake

Energy intake was calculated based on weekly food consumption (Table 4.3). During the initial 16 weeks (excluding week 4), the CAF group exhibited significantly higher energy intake compared to the SD group ($p < 0.05$). During the oral administration period (weeks 17-22), the vehicle-treated CAF and SD groups did not differ significantly in energy intake ($p > 0.05$). However, for the low PVE, high PVE, and metformin-treated subgroups, energy intake was comparable between the SD and CAF groups at week 17 ($p > 0.05$), but the CAF subgroups showed significantly higher energy intake from weeks 18 to 22 compared to their respective SD counterparts ($p < 0.05$). Within the SD group, energy intake remained consistent across all treatment subgroups (vehicle, low PVE, high PVE, and metformin) after subgrouping ($p > 0.05$), with the exceptions of week 17 where the low and high PVE subgroups had significantly lower energy intake than the vehicle control ($p < 0.05$), and weeks 17 and 18 where the metformin subgroup had significantly higher energy intake than the vehicle control ($p < 0.05$). Within the CAF group, energy intake did not significantly differ across treatment

subgroups after subgrouping ($p > 0.05$), except for the low PVE subgroup at weeks 18 and 19, which showed significantly lower energy intake than the vehicle control ($p < 0.05$). The SD group maintained a stable energy intake trend throughout the experiment, even after subgrouping. In contrast, the CAF group displayed a notably high energy intake in the first week, followed by a consistent trend from week 2 onwards, which was unaffected by subgroup allocation.

Table 4.3 Energy intake of male Wistar rats receiving SDV, SDLP, SDHP, SDM, CAFV, CAFLP, CAFHP, CAFM.

	Energy intake (kcal/week/rat)						
Group	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
SDV	477 ± 30	510 ± 19	489 ± 10 ^{ab}	476 ± 22	525 ± 19	517 ± 23	472 ± 35
SDLP	437 ± 52	478 ± 36	480 ± 40 ^{ab}	496 ± 39	538 ± 24	531 ± 22	479 ± 14
SDHP	459 ± 43	478 ± 18	469 ± 44 ^{b}	458 ± 49	487 ± 58	495 ± 41	476 ± 64
SDM	475 ± 24	530 ± 14	535 ± 49 ^{a}	510 ± 46	523 ± 42	536 ± 44	487 ± 61
CAFV	651 ± 84 *	692 ± 87 ^{*,a}	770 ± 45 ^{*,a}	734 ± 57 ^{*,a}	703 ± 79 ^{*,a}	782 ± 94 *	837 ± 102 *
CAFLP	588 ± 22 *	664 ± 30 ^{*,ab}	701 ± 44 ^{b}	667 ± 51 ^{*,b}	639 ± 37 ^{*,b}	725 ± 22 *	770 ± 40 *
CAFHP	608 ± 38 *	653 ± 40 ^{*,ab}	764 ± 60 ^{*,a}	740 ± 31 ^{*,a}	700 ± 49 ^{*,a}	735 ± 38 *	793 ± 38 *
CAFM	538 ± 17 *	627 ± 65 ^{*,b}	757 ± 52 ^{*,a}	712 ± 43 ^{*,ab}	652 ± 42 ^{*,ab}	729 ± 51 *	779 ± 19 *

Values are expressed as means ± S.D.

* Significant difference between SD and CAF groups with the same treatment ($p < 0.05$).

^{ab} Significant difference between vehicle and treatment groups ($p < 0.05$).

4.4.5. Oral glucose tolerance test (OGTT)

The oral glucose tolerance test (OGTT) results are presented in Table 4.4 In the control group (CAF) receiving the vehicle, fasting blood glucose levels (0 min) and glucose concentrations at 30-, 60-, 90-, and 120-minutes post-glucose administration were significantly elevated compared to the standard diet (SD) group ($p < 0.05$). Following oral administration of both low and high doses of PVE, the CAF group exhibited significantly higher blood glucose levels at 30, 60, 90, and 120 minutes

compared to the SD group receiving the same PVE dose ($p < 0.05$). Similarly, the CAF group treated with metformin showed significantly higher blood glucose concentrations at 60-, 90-, and 120-minutes post-glucose load compared to the SD group administered metformin ($p < 0.05$).

Within the SD group, no significant differences were observed in fasting blood glucose or glucose levels at 30, 60, and 120 minutes following the administration of low PVE, high PVE, or metformin compared to the vehicle control ($p > 0.05$). An exception was noted at the 90-minute time point, where the high-PVE-treated SD group displayed significantly higher blood glucose levels than the vehicle control ($p < 0.05$). Among the CAF groups, no significant differences were found in fasting blood glucose or blood glucose concentrations across all time points following the administration of low PVE, high PVE, or metformin compared to the vehicle control ($p > 0.05$). However, the CAF group receiving low PVE exhibited significantly lower blood glucose levels at 60, 90, and 120 minutes, and the CAF group receiving metformin showed significantly lower blood glucose at 120 minutes compared to the CAF vehicle control ($p < 0.05$).

The area under the curve (AUC) for the OGTT is depicted in Figure 1B. Despite a non-significant trend towards higher AUC values in the CAF groups, no statistically significant differences in AUC were found between the SD and CAF groups across all treatments ($p > 0.05$). Furthermore, within each diet group (SD and CAF), no significant differences in AUC were found between the vehicle control and the groups treated with low PVE, high PVE, or metformin ($p > 0.05$). Notably, the CAF group treated with low PVE (CAFLP) showed a slight decrease in AUC compared to other groups within the same diet.

Table 4.4 Glucose tolerance test (OGTT) of male Wistar rats receiving SDV, SDLP, SDHP, SDM, CAFV, CAFLP, CAFHP, CAFM.

	Blood glucose (mg/dL)				
Group	0 min	30 min	60 min	90 min	120 min
SDV	105 ± 11	145 ± 24	147 ± 19	141 ± 10 [b]	134 ± 7
SDLP	103 ± 5	136 ± 16	145 ± 9	147 ± 9 [a]	135 ± 15
SDHP	106 ± 10	140 ± 9	150 ± 6	151 ± 9 [a]	128 ± 9
SDM	110 ± 8	147 ± 19	154 ± 12	151 ± 21 [a]	137 ± 6
CAFV	120 ± 7 [*]	176 ± 26 [*,a]	185 ± 33 [*,a]	194 ± 37 [*,a]	183 ± 30 [*,a]
CAFLP	110 ± 14	157 ± 17 [*,b]	159 ± 9 [*,b]	161 ± 11 [*,b]	154 ± 11 [*,b]
CAFHP	114 ± 11	181 ± 29 [*,a]	188 ± 33 [*,a]	188 ± 35 [*,a]	170 ± 41 [*,a]
CAFM	119 ± 16	153 ± 27 [b]	175 ± 22 [*,ab]	175 ± 23 [*,ab]	154 ± 21 [*,b]

Values are expressed as means ± S.D.

* Significant difference between SD and CAF groups with the same treatment ($p < 0.05$).

[ab] Significant difference between vehicle and treatment groups ($p < 0.05$).

Table 4.5 Area under curve (AUC) of male Wistar rats receiving SDV, SDLP, SDHP, SDM, CAFV, CAFLP, CAFHP, CAFM.

Group	AUC
SDV	1243 ± 689
SDLP	1075 ± 296
SDHP	1165 ± 328
SDM	1208 ± 209
CAFV	1550 ± 684 [*,a]
CAFLP	1105 ± 303 [b]
CAFHP	1495 ± 514 [*,a]
CAFM	1525 ± 509 [*,a]

Values are expressed as means ± S.D.

* Significant difference between SD and CAF groups with the same treatment ($p < 0.05$).

[ab] Significant difference between vehicle and treatment groups ($p < 0.05$).

4.4.6. Blood chemistry

All groups exhibited consistent but incomparable LDL levels (Table 4.6). When administered orally, there were no statistically significant differences in total

cholesterol (TC), triglycerides (TG), or high-density lipoprotein (HDL) between the SD and CAF groups ($p > 0.05$), with one exception: the CAF group receiving low PVE demonstrated significantly higher TG levels compared to the SD group under the same low PVE administration ($p < 0.05$).

Within the SD group, both low and high PVE administration resulted in significantly lower TC levels compared to the vehicle control ($p < 0.05$), while metformin treatment showed no significant difference from the vehicle. Conversely, within the CAF groups, neither low PVE, high PVE, nor metformin administration significantly altered TC levels compared to the vehicle ($p > 0.05$).

Regarding TG levels in the SD group, only low PVE administration led to a significant reduction compared to the vehicle ($p < 0.05$). High PVE and metformin treatments in the SD group did not significantly differ from the vehicle ($p > 0.05$).

In contrast, no significant differences in TG levels were observed between the low PVE, high PVE, or metformin-treated CAF groups and the vehicle control ($p > 0.05$).

For HDL levels in the SD group, both low and high PVE administration resulted in significantly lower levels compared to the vehicle ($p < 0.05$). Metformin treatment in the SD group did not significantly affect HDL levels compared to the vehicle ($p < 0.05$). Similarly, no significant differences in HDL levels were found between the low PVE, high PVE, or metformin-treated CAF groups and the vehicle control ($p > 0.05$).

No statistically significant differences in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were observed between the SD and CAF groups under the same oral administration ($p > 0.05$), with two exceptions. Firstly, the CAF group administered the vehicle control exhibited significantly lower AST levels compared to the SD group ($p < 0.05$). Secondly, the CAF group treated with metformin showed significantly lower ALT levels compared to the SD group ($p < 0.05$).

More interestingly, within the SD group, both low and high PVE administration resulted in significantly lower AST and ALT levels compared to the vehicle control ($p < 0.05$). In contrast, metformin treatment in the SD group did not significantly alter AST or ALT levels compared to the vehicle ($p > 0.05$). Similarly, in the CAF groups, neither

low PVE, high PVE, nor metformin administration significantly affected AST or ALT levels compared to the vehicle control ($p > 0.05$).

Table 4.6 Blood chemistry

Groups	TC (mg/dL)	TG (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	AST (U/L)	ALT (U/L)	BUN (mg/dL)	Cr (mg/dL)
SDV	81 ± 9	217 ± 51	< 30	58 ± 7	287 ± 91 ^{a}	36 ± 5 ^{a}	15 ± 1 ^{a}	0.5 ± 0.1 ^{ab}
SDLP	64 ± 11	144 ± 39	< 30	49 ± 8	102 ± 31 ^{b}	22 ± 4 ^{b}	12 ± 2 ^{b}	0.4 ± 0.1 ^{b}
SDHP	68 ± 2	200 ± 46	< 30	51 ± 3	169 ± 35 ^{b}	24 ± 5 ^{b}	13 ± 3 ^{ab}	0.5 ± 0.1 ^{a}
SDM	74 ± 17	204 ± 60	< 30	55 ± 10	275 ± 164 ^{a}	32 ± 11 ^{ab}	14 ± 2 ^{ab}	0.5 ± 0.1 ^{ab}
CAFV	88 ± 36	207 ± 83	< 30	62 ± 23	164 ± 50 ^{*,b}	27 ± 15 ^{a}	7 ± 2 *	0.4 ± 0.1 ^{b}
CAFLP	76 ± 22	213 ± 52 *	< 30	53 ± 16	113 ± 49 ^{b}	23 ± 5 ^{ab}	8 ± 2 *	0.5 ± 0.1 ^{*,a}
CAFHP	76 ± 13	176 ± 47	< 30	56 ± 8	124 ± 50 ^{b}	31 ± 9 ^{a}	8 ± 3 *	0.4 ± 0.1 ^{*,b}
CAFM	72 ± 15	186 ± 47	< 30	53 ± 11	198 ± 98 ^{a}	20 ± 7 ^{*,b}	9 ± 1 *	0.5 ± 0.0 ^{a}

Values are expressed as means ± S.D.

* Significant difference between SD and CAF groups with the same treatment ($p < 0.05$).

^{ab} Significant difference between vehicle and treatment groups ($p < 0.05$).

4.4.7. Histology

The CAF groups exhibited a higher steatosis grade than SD groups (Figures 4.2-5). In the SD groups, PVE and metformin lowered steatosis, with metformin reducing steatosis more than PVE, and both doses of PVE showed no differences. In the CAF groups, PVE lowered steatosis, but metformin only slightly reduced steatosis, and a high dose of PVE increased steatosis slightly more than a low dose. Table 8 presents the mean adipocyte area (μm^2) across eight experimental groups, expressed as means

± standard deviation. The data suggests that the "CAF" condition leads to significant adipocyte hypertrophy. Both low and high doses of treatment P, as well as Metformin, effectively reduced adipocyte size in the "CAF" model. Furthermore, the high dose of treatment P demonstrated an ability to reduce adipocyte size even under standard dietary conditions. These findings underscore the potential therapeutic effects of the tested interventions on adipocyte morphology.

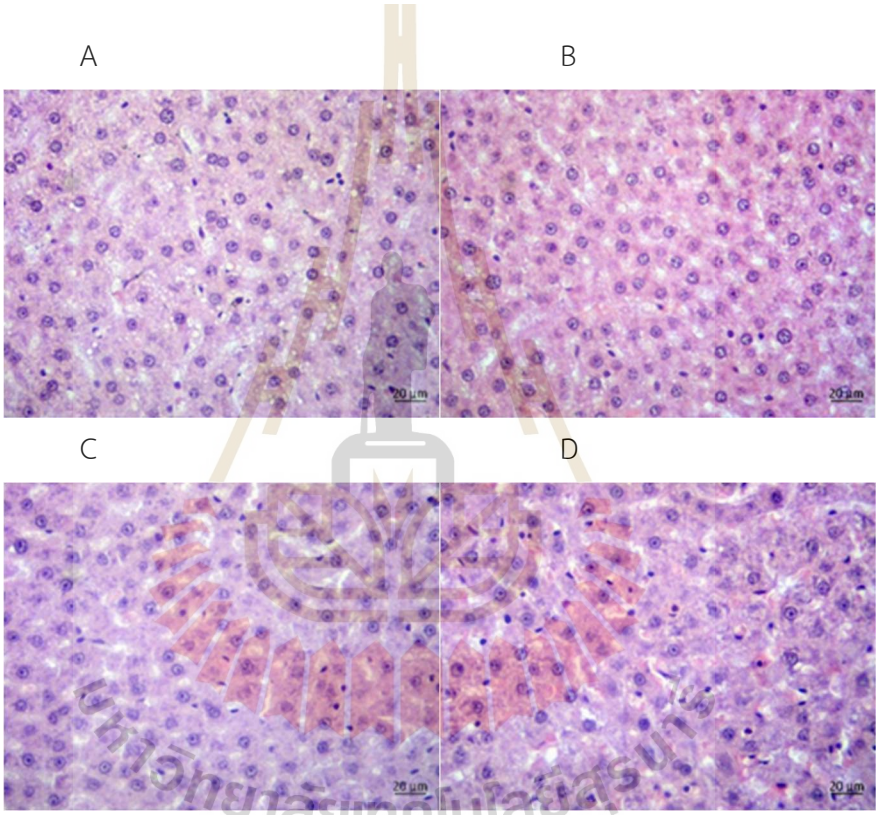

Figure 4.2 Histology of livers of male rats received SDV (A), SDLP (B), SDHP (C), SDM (D).

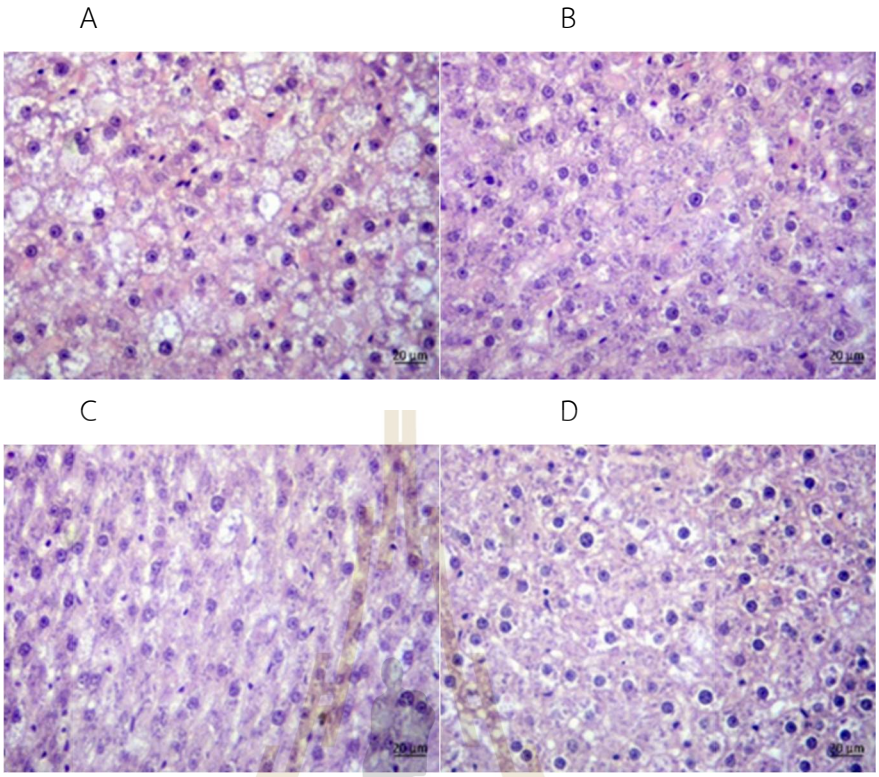

Figure 4.3 Histology of livers of male rats receive CAFV (A), CAFLP (B), CAFHP (C), CAFM (D).

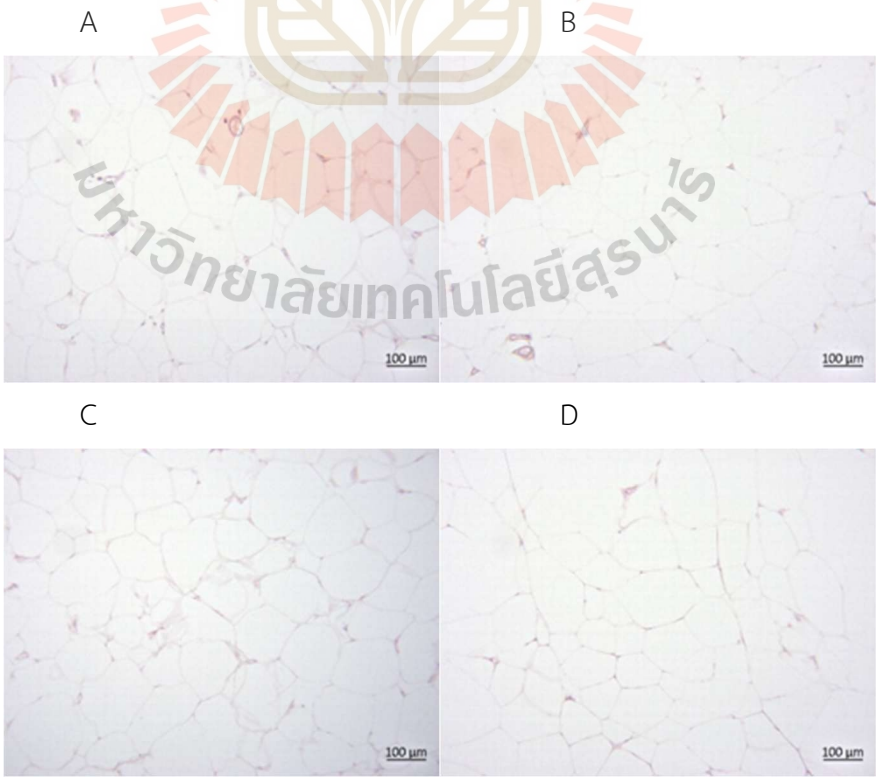

Figure 4.4 eWAT of SDV (A), SDLP (B), SDHP (C), SDM (D).

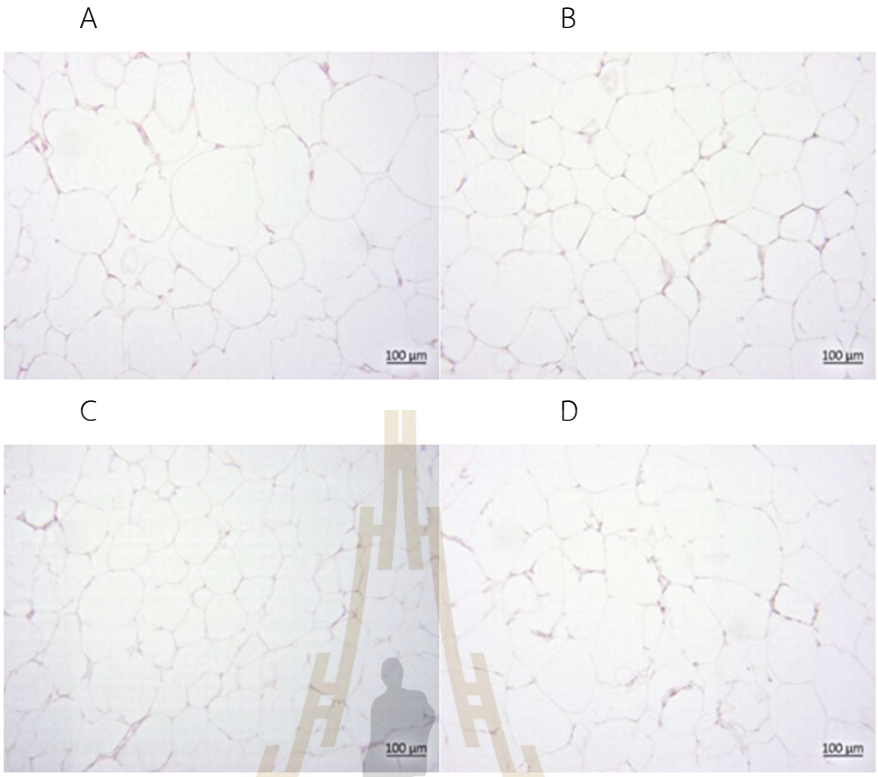

Figure 4.5 eWAT of CAFV (A), CAFLP (B), CAFHP (C), CAFM (D).

Table 4.7 Mean adipocyte area of adipose tissues for male Wistar rats receiving SDV, SDLP, SDHP, SDM, CAFV, CAFLP, CAFHP, and CAFM.

Group	Liver HV/N	Mean adipocyte Area (μm^2)
SDV	0/3	9823 ± 1479 [a]
SDLP	0/3	9574 ± 1553 [ab]
SDHP	0/3	8264 ± 440 [b]
SDM	0/3	11003 ± 3559 [a]
CAFV	1/3	12497 ± 2044 [*,a]
CAFLP	1/3	10032 ± 1305 [ab]
CAFHP	1/3	6159 ± 408 [*,b]
CAFM	1/3	7973 ± 816[b]

The results are expressed as number of rat with pathological finding/total number of rats.

Liver: HV = Hepatic vacuolation/ HD = Hepatic degeneration / N = Normal (very mild lesion)

Values are expressed as means ± S.D.

* Significant difference between SD and CAF groups with the same treatment ($p < 0.05$).
[ab] Significant difference between vehicle and treatment groups ($p < 0.05$).

4.4.8. Tissue index

Tissue index of rat organs was calculated in to percentage of organs weight by final weight and the result show in Table 4.8. SD and CAF had no significantly difference in liver tissue index compare in same orally administration ($p > 0.05$) except administration with vehicle which CAF had significantly higher liver tissue index than SD ($p < 0.05$). Among SD group, metformin had significantly higher liver tissue index than vehicle ($p < 0.05$) while low and high PVE had no significantly difference liver tissue index to vehicle ($p > 0.05$), meanwhile among CAF groups low PVE, high PVE, and metformin had no significantly difference liver tissue index to vehicle ($p > 0.05$).

Orally administration with vehicle, SD and CAF had no significantly difference heart tissue index ($p > 0.05$). While orally administration with low PVE, high PVE, and metformin CAF had significantly lower heart tissue index than SD compared in same orally administration ($p < 0.05$). Among SD groups low and high PVE had significantly lower heart tissue index than vehicle ($p < 0.05$) while metformin had no significantly difference heart tissue index to vehicle ($p > 0.05$). Meanwhile among CAF groups low PVE, high PVE, and metformin had no significantly difference heart tissue index to vehicle ($p > 0.05$).

CAF groups had significantly lower kidney tissue index than SD groups compared in same orally administration ($p < 0.05$). Both of SD groups and CAF groups orally administrated with low PVE, high PVE, and metformin had no significantly difference kidney tissue index to vehicle ($p > 0.05$). In addition, CAF groups had significantly higher VAT tissue index than SD groups compared in same orally administration ($p < 0.05$). Both of SD groups and CAF groups orally administrated with low PVE, high PVE, and metformin had no significantly difference VAT tissue index to vehicle ($p > 0.05$).

Orally administration with vehicle, and low PVE, CAF groups have higher eWAT tissue index than SD groups compared in same orally administration ($p < 0.05$). Orally

administration with high PVE, and metformin, SD, and CAF groups has no significantly difference eWAT tissue index compared in same orally administration ($p > 0.05$).

Among SD groups high PVE and metformin had significantly higher eWAT tissue index than vehicle ($p < 0.05$) while low PVE had no significantly difference eWAT tissue index to vehicle ($p > 0.05$). Meanwhile among CAF groups low PVE, high PVE, and metformin had no significantly difference eWAT tissue index to vehicle ($p > 0.05$). CAF groups had significantly lower soleus muscle tissue index than SD groups compared in same orally administration ($p < 0.05$). Both of SD groups and CAF groups orally administrated with low PVE, high PVE, and metformin had no significantly difference soleus muscle tissue index to vehicle ($p > 0.05$).

Table 4.8 Tissue index of male Wistar rats receiving SDV, SDLP, SDHP, SDM, CAFV, CAFLP, CAFHP, CAFM.

	Tissue index (%Body weight)					
Group	Liver	Heart	Kidney	VAT	eWAT	Soleus
SDV	2.66 ± 0.19	0.23 ± 0.01 [a]	0.56 ± 0.01	9.93 ± 2.07	2.87 ± 0.25 [b]	0.10 ± 0.01 [a]
SDLP	2.71 ± 0.16	0.22 ± 0.01 [b]	0.58 ± 0.01	9.88 ± 1.34	2.86 ± 0.45 [b]	0.10 ± 0.02 [a]
SDHP	2.79 ± 0.19	0.21 ± 0.02 [b]	0.56 ± 0.02	10.84 ± 1.52	3.36 ± 0.42 [a]	0.10 ± 0.01 [a]
SDM	3.09 ± 0.52	0.23 ± 0.02 [a]	0.61 ± 0.02	11.82 ± 2.40	3.42 ± 0.40 [a]	0.09 ± 0.01 [b]
CAFV	3.15 ± 0.48 [*]	0.19 ± 0.06 [b]	0.46 ± 0.06 [*]	16.31 ± 4.88 [*]	3.57 ± 0.64 [*,b]	0.07 ± 0.02 [*,b]
CAFLP	2.79 ± 0.21	0.19 ± 0.01 [*,b]	0.44 ± 0.01 [*]	16.48 ± 1.76 [*]	3.86 ± 0.74 [*,a]	0.07 ± 0.01 [*,b]
CAFHP	2.99 ± 0.62	0.19 ± 0.02 [*,b]	0.45 ± 0.02 [*]	18.86 ± 2.79 [*]	3.97 ± 0.89 [a]	0.07 ± 0.01 [*,b]
CAFM	2.90 ± 0.21	0.21 ± 0.02 [*,a]	0.47 ± 0.02 [*]	17.02 ± 2.51 [*]	4.20 ± 1.01 [a]	0.08 ± 0.01 [*,a]

Values are expressed as means ± S.D.

***** Significant difference between SD and CAF groups with the same treatment ($p < 0.05$).

[ab] Significant difference between vehicle and treatment groups ($p < 0.05$).

4.5 Discussion

This study investigated the impact of PVE on body weight regulation in a CAF-induced overweight rat model. Male Wistar rats (n=48) were initially divided into two groups (n = 24 per group): a control group maintained on a standard diet, and a CAF group designed to induce overweight over a 16-week period. As anticipated, the CAF group exhibited a significantly increased body weight gain and energy intake compared to the control group. Subsequently, both the control and CAF groups were further subdivided into four treatment subgroups (n = 6 per subgroup), receiving daily oral administration of either the vehicle, a low dose of PVE (200 mg/kg), a high dose of PVE (300 mg/kg), or metformin (200 mg/kg) for an additional 6 weeks. The results demonstrated that the administration of a high dose of PVE and metformin significantly attenuated body weight gain in the CAF-induced overweight rats. These findings suggest PVE's potential role in mitigating weight gain associated with a palatable, high-energy diet.

CAF is used in animal models to study obesity and its related metabolic disorders, including diabetes mellitus. This diet typically consists of a variety of highly palatable, energy-dense foods that mimic the "Western diet" commonly consumed by humans. These foods are often high in fat, sugar, and processed ingredients.

The consumption of CAF triggers a cascade of metabolic dysfunctions, initially manifesting as hyperglycemia and compensatory hyperinsulinemia due to elevated blood glucose levels (Sampey et al., 2011; Brandt et al., 2011). This is followed by the development of insulin resistance, characterized by a reduced cellular response to insulin and consequently impaired glucose uptake (Sampey et al., 2011; Brandt et al., 2011). Furthermore, CAF intake leads to dyslipidemia, marked by abnormal lipid profiles including increased triglycerides and cholesterol concentrations. The diet also induces low-grade chronic inflammation in adipose tissue and the liver, a significant factor contributing to the observed insulin resistance (Song et al., 2016). Finally, CAF feeding promotes oxidative stress within adipose tissue, which further exacerbates inflammation and contributes to overall metabolic dysfunction (Castro et al., 2020).

The CAF increases obesity via an increasing body weight associated with energy intake (Sampey et al., 2011). In the study, the CAF groups had higher glycemia than the SD groups because the CAF led to hepatic steatosis and adiposity that involved weight gain, inflammation, insulin resistance, and obesity related with macrophage infiltration (Sampey et al., 2011).

This study investigated the efficacy of PVE in mitigating the detrimental effects of CAF on obese rats. Our findings revealed that PVE administration significantly reduced body weight gain in CAF-fed rats, with lower concentrations exhibiting an effect comparable to that of metformin, a well-established agent in managing obesity and type 2 diabetes. The observed reduction in body weight gain by PVE is likely attributed to decreased food absorption, a mechanism previously reported by Tormo et al. (2004).

Furthermore, PVE treatment demonstrably lowered glycemia in the CAF group, with lower concentrations also significantly reducing the area under the glucose tolerance curve (AUC), indicating improved glucose homeostasis. Beyond glucose regulation, PVE effectively lowered circulating levels of total cholesterol (TC), triglycerides (TG), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), suggesting a protective role against CAF-induced dyslipidemia and hepatic injury. In addition, the SDLP, SDHP, CAFV, CAFLP, and CAFHP groups showed lower AST levels compared to the SDV and SDM groups, with some of these differences being statistically significant ($p< 0.05$). The SDV and SDM groups exhibited the highest AST values and also the largest variability, particularly SDM. The superscript letter "a" and "b" denote statistical significance when compared to their respective control groups, although the specific control for each annotation is not explicitly stated in the table itself.

Histological analyses further supported these findings, revealing that PVE administration attenuated hepatic steatosis and reduced adipocyte size in CAF-fed rats. The beneficial effects of PVE can be primarily attributed to its αAI activity. As evidenced by (Song et al., 2016), the αAIs present in *Phaseolus vulgaris* can suppress

pancreatic α-amylase, leading to diminished starch digestion and absorption in the intestine. This reduced carbohydrate assimilation likely underlies the observed lower body weight gain, improved glycemic control, and the subsequent reduction in hepatic steatosis and inflammation. In conclusion, our data suggest that PVE presents a promising therapeutic strategy for combating obesity and associated metabolic complications arising from the consumption of a high-energy, palatable diet.

The analysis of tissue indices revealed that neither PVE nor metformin administration exerted a significant effect on the relative weights of the heart, kidney, and soleus muscle. Consistently with the induction of obesity, CAF led to a reduction in the tissue indices of these lean tissues (heart, kidney, and soleus), while conversely increasing the tissue indices of visceral adipose tissue (VAT) and epididymal white adipose tissue (eWAT). The lower tissue indices observed in the heart, kidney, and soleus of CAF-fed rats are likely attributable to the lower density of adipocytes compared to lean tissue. The increased adiposity resulting from the CAF leads to fat infiltration and the potential replacement of denser lean tissue with less dense fat cells, thus lowering the overall tissue index. Conversely, the elevated tissue indices of VAT and eWAT in the CAF group are expected, as these represent the primary sites of triacylglycerol (TG) storage in response to an increased energy intake (Cohen & Spiegelman, 2016).

Considering the acute effects of PVE, previous research has indicated its potential to reduce ghrelin levels and diminish the desire to eat, alongside the αAI activity which can slow gastric emptying (Spadafranca et al., 2013). While these acute effects on appetite regulation and gastric motility may have contributed to the observed long-term reductions in body weight gain, they did not translate into significant alterations in the relative weights of the examined lean tissues in this chronic study. This suggests that the primary impact of PVE and metformin on body composition in this model may be mediated through mechanisms affecting overall adiposity rather than directly influencing the relative mass of individual lean organs. Further investigation into the specific mechanisms underlying the differential effects of these treatments on lean versus adipose tissue mass is warranted.

This study, while acknowledging the limitations of CAF-induced obese rat model in fully replicating human obesity's complexity, affirms its utility for preclinical metabolic research. The authors recognize and statistically address intra- and intergroup variability inherent in in vivo studies, maintaining that the observed trends are valid. However, they concede the lack of rigorous extract standardization for probiotics/metformin as a limitation, committing to improved characterization in future work. Despite the modest magnitude of some metabolic effects, the statistically significant improvements in markers like AST, ALT, and BUN suggest a positive physiological impact, warranting further investigation with optimized interventions.

4.6 Conclusions

PVE demonstrates significant potential in mitigating CAF-induced obesity and metabolic dysfunction in rats. Low-dose PVE effectively reduced body weight gain and improved glucose tolerance in obese rats, likely through α-amylase inhibition, leading to reduced carbohydrate absorption and subsequent lower food intakes. Furthermore, PVE exhibited beneficial effects on lipid profiles and may offer protection against hepatic steatosis. These in vivo findings highlight the therapeutic promise of PVE for managing obesity associated with high-energy diets, warranting further investigation into optimal dosage and clinical translation.

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CHAPTER V
CONCLUSION

5.1 Conclusion

This research successfully demonstrated the optimization of α-amylase inhibitory extract production from white kidney beans (*Phaseolus vulgaris*) and subsequently validated its potential in *in vivo* models for weight management and glycemic control. Through a systematic Box-Behnken design and Response Surface Methodology (RSM), optimal extraction parameters were identified, yielding an extract with high specific activity (0.111 units/mg) at 0.101 M PBS, 1-hour extraction, and 30 minutes separation. Slightly adjusted conditions also maximized extract yield (11.89%). This robust optimization strategy provides a clear pathway for efficient and scalable production of this valuable extract.

Furthermore, the *in vivo* study using obese Wistar rats underscored the physiological benefits of the *P. vulgaris* extract (PVE). Low-dose PVE in cafeteria-diet (CAF) fed rats significantly reduced postprandial glycemia and decreased food and energy intake, indicating its role in mitigating obesity-related markers. While the overall glucose exposure (AUC) in the oral glucose tolerance test was not significantly altered, the positive changes observed at individual time points, coupled with reductions in body weight gain, food intake, and energy intake, suggest a promising impact on metabolic health.

In conclusion, the α-amylase inhibitors present in *P. vulgaris* show significant potential to positively impact body weight, weight gain, and glycemia. This comprehensive study provides strong support for further exploring PVE as a valuable functional ingredient in the food industry. Its application could lead to the development of novel products aimed at weight management andglycemic control, offering a natural and effective approach to addressing global health concerns like obesity and type 2 diabetes.

APPENDICES

APPENDIX A

Solution preparation

The solution used in the extraction

- 0.2 M Na_2HPO_4 for 1100 ml (X)

  Weight Na_2HPO_4 for 28.39 g adjust volume with distilled water to 1000 ml. Weight Na_2HPO_4 for 2.84 g adjust volume with distilled water to 100 ml

- 0.2 M NaH_2PO_4 for 500 ml (Y)

  Weight NaH_2PO_4 12 g adjust volume with distilled water to 500 ml

- Stock 0.2 M PBS buffer pH 7.2 (Z) for 500 ml (≥300 ml) (36 Na_2HPO_4: 14 NaH_2PO_4)

  Bring X 1080 ml mix together with Y 420 ml

- 0.05 M PBS pH 7.2 containing 150mM NaCl

  Bring Z for 125 ml and weigh NaCl 4.38 g mix them, adjust volume with distilled water to 500 ml then adjust pH to 7.2

- 0.1M PBS pH 7.2 containing 150mM NaCl

  Bring Z for 250 ml and weigh NaCl 4.38 g mix them, adjust volume with distilled water to 500 ml then adjust pH to 7.2

- 0.15 M PBS pH 7.2 containing 150mM NaCl

  Bring Z for 357 ml and weigh NaCl 4.38 g mix them, adjust volume with distilled water to 500 ml then adjust pH to 7.2

The solution used in pH adjusting

- 1 N HCl 25 ml

  Bring 37% HCl for 9.85 ml adjust the volume with distilled water to 100 ml

- 1 N NaOH 25 ml

  Weight NaOH 4 g, Adjust the volume with distilled water to 100 ml

The solution used in sample dissolving

- Acetate buffer (2 mM buffer pH 4.5, 20 mM $CaCl_2$, 10 mM NaCl) 1000 ml

  Prepare 500 ml of 0.2 M Sodium acetate by weight Sodium Acetate for 13.61 g adjust the volume with distilled water to 500 ml. Prepare 500 ml of 0.2 M acetic acid

by bringing the acetic acid for 6 ml adjust the volume with distilled water to 500 ml. Pour the 0.2 M Sodium Acetate from the previous into the 0.2 M acetic acid to get the pH 4.5 then get the solution for 100 ml, Add the 0.58 g of NaCl, and 2.22 g of $CaCl_2$,and finally adjust the volume with distilled water to 1000 ml.

The solution used in Protein content quantification (Bradford assay)

- 0.15 M NaCl 500 ml

  Weight NaCl 4.38 g, Adjust the volume with distilled water to 100 ml

- 1000 µg/ml of BSA solution 100 ml

  Weight BSA for 0.1 g adjust the volume with the previous 0.15 M NaCl to 100 ml

- Coomassie blue reagent

  Bring 0.05 g of Coomassie blue, 50 ml of methanol, and 100 ml of 85% phosphoric acid, and pour them into distilled water 500 ml. Filtrate the residues off. Finally, add another 300 ml of distilled water. Store in 4 °C.

Solution used in α-amylase inhibition quantification (Bernfeld assay)

- Succinate buffer (15 mM, 20 mM $CaCl_2$, and 0.5 M NaCl, pH 5.6); 500 ml

  Weight 0.88 g of Succinic acid, 1.11 of $CaCl_2$, and 14.61 g of NaCl. Mix them adjust the volume with distilled water to 500 ml and adjust the pH to 5.6

- Porcine pancreatic α-amylase (40 U/ml); 100 ml

  Weight 0.27 g of Porcine pancreatic α-amylase adjust the volume to 100 ml with the previous Succinate buffer.

- 6.7 mmol/L NaCl 250 ml

  Weight NaCl for 0.1 g adjust the volume with distilled water to 250 ml (Z)

- 0.2 M Na_2HPO_4 100 ml (ZX)

  Weight Na_2HPO_4 2.84 g adjust the volume with Z to 100 ml

- 0.2 M NaH_2PO_4 100 ml (ZY)

  Weight NaH_2PO_4 2.4 g adjust the volume with Z to 100 ml

- 20 mmol/L sodium phosphate buffer containing 6.7 mmol/L NaCl, pH 6.9; 100 ml (ZXY)

  Mix 55 ml of ZX and 45 ml of ZY 45 ml adjust the volume with distilled water to 100 ml adjust the pH to 6.9

- Soluble starch 2%(w/v); 100 ml

  Weight Soluble starch for 2 g mixed with ZXY 100 ml

- DNS solution 100 ml

  Weight 1 g of DNS, 30 g of sodium potassium tartrate, and 40 ml of 1 M NaOH 40 ml adjust the volume with distilled water to 100 ml

- Maltose stock solution (10 mg/ml); 25 ml

  Weight maltose for 0.25 g adjust the volume with ZXY to 25 ml

APPENDIX B

Standard curves

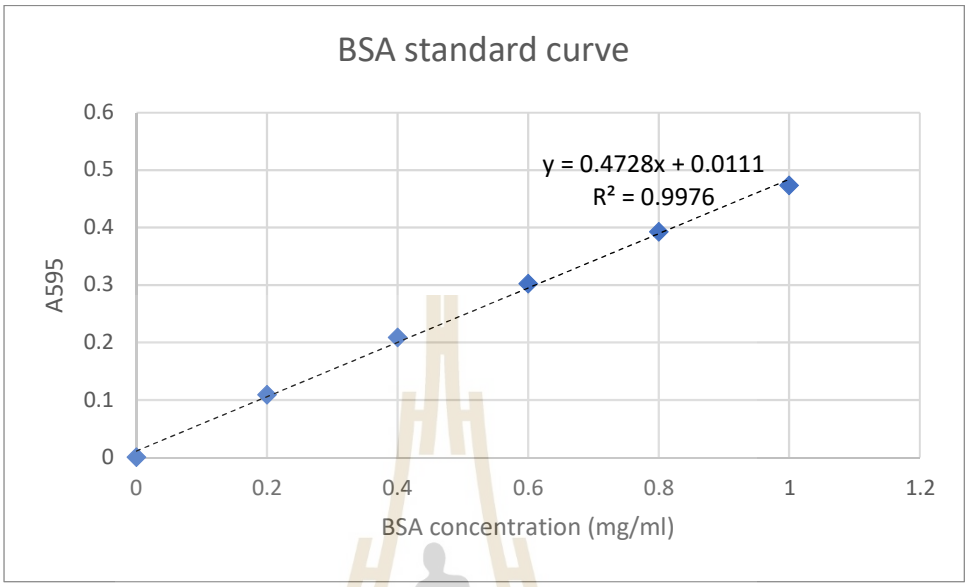

Figure B.1 BSA standard curve

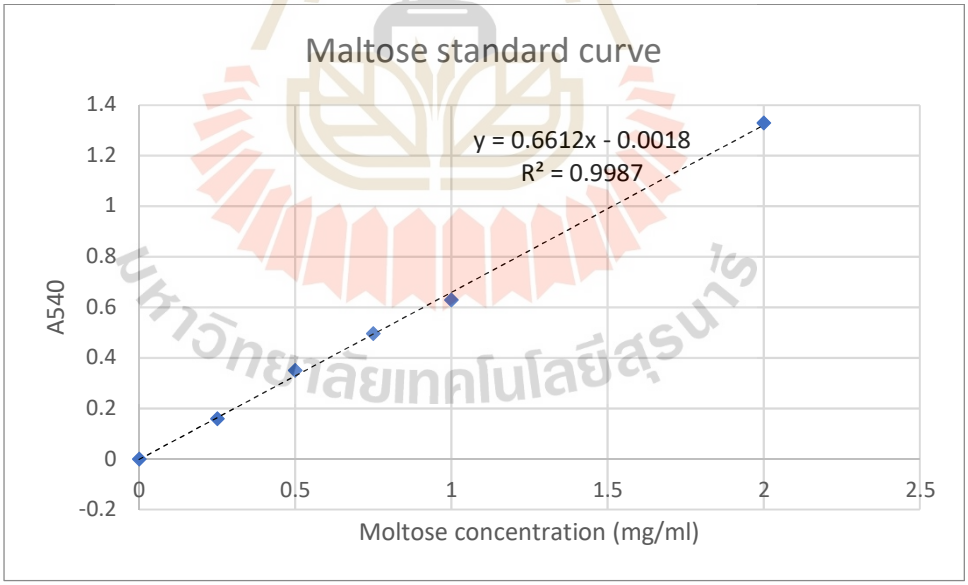

Figure B.2 Maltose standard curve

APPENDIX C

Diet energy and rats water intake

Table C.1 Products energy

Products	Attribute	Energy (kcal/g)
Lay's	salty	5.3
Lay's Stax	salty	5.2
Twistko	salty	4.7
Poo Thai	salty	5.4
Cornae	salty	5.8
Hanami	salty	5
Dino park	salty	5.3
Ellse brand layer vanilla flavoured cake with white cream	sweaty	4.7
Imperial cookies vanilla ring	sweaty	5.2
Sando creamy vanilla	sweaty	5.4
Fun-o sandwich cookies filled with milk cream	sweaty	4.8
Tivoli Twin milk flavoured	sweaty	5
Crispy waters filled with milk cream	sweaty	5
Voiz Cracker Creamy Butter	sweaty	5
Rosy sandwich crackers cheese	salty/sweaty	5.3
Rosy crackers original	salty/sweaty	5
CP Smoked Sausage	salty	2.9

Table C.2 Chow diet energy

chow	(g/g chow)	(kcal/g chow)
protein	0.240	0.960
fat	0.045	0.405
carbohydrate	0.448	1.792
Total energy		3.2

Table C.3 Syrup energy

Syrup	Energy (kcal/ml)
10% syrup	0.4
20% syrup	0.9

Table C.4 Rats water intake

	Water intake (mL/week/rat)						
Groups	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
SDV	191 ± 15 ^{b}	242 ± 21 ^{b}	248 ± 13	212 ± 11 ^{c}	229 ± 2 ^{b}	207 ± 6 ^{b}	242 ± 32
SDLP	202 ± 41 ^{ab}	262 ± 46 ^{b}	272 ± 59	308 ± 27 ^{a}	282 ± 34 ^{a}	270 ± 32 ^{a}	260 ± 38
SDHP	235 ± 41 ^{ab}	305 ± 26 ^{a}	289 ± 33	266 ± 9 ^{b}	291 ± 39 ^{a}	263 ± 29 ^{a}	265 ± 41
SDM	223 ± 22 ^{a}	278 ± 32 ^{ab}	299 ± 54	261 ± 27 ^{b}	264 ± 49 ^{ab}	269 ± 59 ^{a}	260 ± 49
CAFV	292 ± 54 *	305 ± 57 ^{*,b}	257 ± 29 ^{b}	238 ± 38 ^{c}	270 ± 30 ^{*,c}	399 ± 70 ^{*,b}	338 ± 68 ^{*,bc}
CAFLP	300 ± 13 *	407 ± 42 ^{*,a}	259 ± 40 ^{b}	298 ± 22 ^{b}	382 ± 36 ^{*,a}	433 ± 10 ^{*,ab}	385 ± 33 ^{*,ab}
CAFHP	272 ± 59	285 ± 67 ^{b}	266 ± 70 ^{b}	314 ± 36 ^{*,b}	293 ± 38 ^{bc}	331 ± 40 ^{*,c}	311 ± 29 ^{*,c}
CAFM	255 ± 39	326 ± 53 ^{*,b}	372 ± 41 ^{*,a}	358 ± 32 ^{*,a}	342 ± 66 ^{*,ab}	478 ± 50 ^{*,a}	412 ± 24 ^{*,a}

Values are expressed as means ± S.D.

* Significant difference between SD and CAF groups with the same treatment ($p < 0.05$).

^{ab} Significant difference between vehicle and treatment groups ($p < 0.05$).

BIOGRAPHY

Atcha Uawongwattana was born on November 5, 1998, in Chon Buri province, Thailand. He successfully received a Bachelor's degree in science (Food Technology) from Suranaree University of Technology in 2021.