Summary of Doctoral Dissertation

Mechanistic analysis of Thai medicinal plants on anti-obesogenic activity
(タイ薬用植物の抗肥満関連活性に関する作用機構解析)

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1. Introduction

1.1. Obesity

The global incidence of obesity has been steadily increasing over the years. The obese population has experienced a threefold rise since 1975 (WHO, 2000). Moreover, projections for 2025 suggest that the number of obese adults will reach 1 billion. Although there are fluctuations in obesity rates worldwide, the overall prevalence of obesity remains alarmingly high.

Numerous strategies exist for the prevention and management of overweight and obesity (Lin and Li, 2021). The least invasive and safest approaches include self-initiated changes and modifications to lifestyle factors such as dietary habits, sleep patterns, and increased physical activity levels (Lin and Li, 2021). Nevertheless, it is widely recognized that implementing such lifestyle changes can be quite difficult, prompting individuals to explore alternatives with more convenient methods, including the use of medications and surgical interventions.

1.2. Overview of lipid metabolism

1.2.1. Adipocytes

There are three processes focused on the aspect of obesity prevention and treatment. These include adipogenesis, lipogenesis, and lipolysis.

Firstly, adipogenesis is the process by which mature adipocytes develop from fibroblast-like progenitor cells (Ghaben and Scherer, 2019). In the second phase, preadipocytes go through three minor steps: growth arrest, lipid storage, and termination of differentiation. During the growth arrest step, preadipocytes activate adipogenic regulators like peroxisome proliferator-activated receptor γ (PPARγ) and transcription co-activators CCAAT/enhancer-binding protein α/β (C/EBPα and C/EBPβ). This step leads to the differentiation of fibroblast-like cells into adipocytes. In the lipid storage step, cells express marker proteins like adipocyte fatty acid-binding protein (AP2) and insulin-sensitive glucose transporter-4 (GLUT4). Finally, in the termination stage of differentiation, marker proteins including adiponectin, leptin, adipose triglyceride lipase (ATGL), lipoprotein lipase (LPL), and perilipin are expressed, providing evidence that the adipogenesis process has concluded.

Secondly, lipogenesis involves the production of fatty acids and the esterification of these fatty acids into triglycerides (Kersten, 2001). This biological process is regulated by several transcriptional elements, including sterol regulatory element-binding proteins (SREBPs), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS). There are three isoforms of the upstream regulator SREBP: SREBP-2, SREBP-1a, and SREBP-1c, which influence the expression of ACC and FAS. These two enzymes are responsible for fatty acid synthesis (Cristancho and Lazar, 2011).

Lastly, lipolysis is a biochemical pathway responsible for breaking down triglycerides into glycerol and fatty acids, serving as the initial step in utilizing fats as an energy source (Carmen and Víctor, 2006). This process involves the essential participation of key enzymes, including hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), which are pivotal in triglyceride hydrolysis (Carmen and Víctor, 2006).

In addition, there are two signaling pathways, Protein Kinase A (PKA) and Extracellular-signal-regulated kinase (ERK), which are involved in regulating lipolysis (Liu *et al*., 2011). In the PKA pathway, Noradrenaline, released by sympathetic nerves, triggers β-adrenoceptors in adipocytes (Arner and Langin, 2014). These receptors are linked to adenylyl cyclase through stimulatory G proteins (Gs proteins), leading to cAMP-dependent activation of PKA. PKA then phosphorylates HSL and breaks down triglycerides. Additionally, PKA prompts perilipin A to detach from the lipid droplet, allowing lipases to access the droplet and initiate lipolysis. Simultaneously, PKA activates perilipin to release CGI-58, which binds to and activates ATGL.

Furthermore, the Mitogen-activated protein kinase (MAPK) pathway includes various cascades, including the ERK pathway (Bost *et al*., 2005; Shaul and Seger, 2007). This cascade initiates when ligands interact with receptors on the cell membrane. These ligands can include growth factors, hormones, and cellular stress inducers. Once ERK is activated, it stimulates a process similar to the PKA pathway to initiate lipolysis (Greenberg *et al*., 2001).

1.2.2. Hepatocytes

Fatty acids undergo metabolism primarily through a process known as β-oxidation. The key regulatory factor governing fatty acid β-oxidation is the peroxisome proliferator-activated receptor alpha (PPARα) (Pawlak *et al*., 2015). In the initial stages of β-oxidation, fatty acids are transported into the mitochondria, and then these fatty acids are converted into acetyl-CoA. Acetyl-CoA can then follow two pathways: it can either enter the tricarboxylic acid (TCA) cycle to generate energy within the liver or be utilized to form ketone bodies, which are subsequently transported to other tissues.

1.3. Screening of medicinal plants with anti-obesogenic potential

The study involved screening 70 Thai medicinal plants using three in vitro bioassays: pancreatic lipase inhibition (an enzymatic assay), lipolysis enhancement, and reduction of lipid accumulation in cells. By utilizing these assays, the study aimed to evaluate and select the plants with the most effectiveness against obesity. This approach is considered more reliable than a single in vitro assay and more efficient than in vivo screening with numerous samples. Six candidate plants, specifically *Acacia concinna* (Willd.)

DC., *Cymbopogon nardus* (L.) Rendle, *Cyperus rotundus* L., *Caesalpinia sappan* L., *Eurycoma longifolia* Jack, and *Tiliacora triandra* Diels, exhibited dual activity in at least two of the three assays (Ruangaram and Kato, 2020). These plants were subsequently tested at various concentrations to assess their effectiveness and were evaluated for cytotoxicity. The findings suggest that these Thai medicinal plants possess potential anti-obesogenic properties, as summarized in Table 1.

***Table 1. Summary of anti-obesogenic potential of candidate plants during screening process* (Ruangaram and Kato, 2020)**

Medicinal plants	Pancreatic lipase inhibition[1]	Lipolysis enhancement[2]	Lipid accumulation reduction	Toxicity
T. triandra	Low	Weak	Active (CD[3])	Negative
C. nardus	Medium	Strong	NA[3]	Negative
E. longifolia	Low	Strong	Active (CD)	Negative
C. rotundus	Low	Strong	NA	Negative
A. concinna	High	Weak	Active (CD)	Positive
C. sappan	High	NA	Active (constant)	Negative

[1]Low: have less than 70% inhibition at the highest tested concentration; Medium: more than 70% inhibition at highest tested concentration; High: more than 70% inhibition in all tested concentrations.

[2]Weak: negative correlation between concentration of extract and its lipolysis enhancing effect; Strong: positive correlation between concentration of extract and its lipolysis-enhancing effect.

[3]NA: no-activity; CD: concentration dependent

1.4. Objectives

The project's main objective is to assess the anti-obesity potential of medicinal plants. To accomplish this goal, two distinct methods have been employed: (I) a screening study to evaluate the anti-obesity potential of medicinal plant candidates which summarised in Section 1.3 and (II) a mechanistic study aimed at uncovering the underlying mechanisms of these plants. In the context of this thesis, the objectives are to uncover the additional anti-obesogenic potential of the chosen plants (*A. concinna*, *C. nardus*, *C. rotundus*, and *T. triandra*) and to study the mechanistic aspects, providing a deeper understanding of how these plants exert their anti-obesity effects on adipocytes. Furthermore, the potential

impact of these plants on hepatocytes was also explored to aid in the selection of the most promising plant candidates.

2. Materials and methods

2.1. Preparation of plant extraction

Thai medicinal plants (as shown in Table 2) were finely ground, and the resulting particles were soaked in a 50% (v/v) aqueous methanol solution for 3 days. The suspension was subsequently filtered to collect the filtrates, which were then evaporated under vacuum conditions at 30°C to yield the dried samples. Afterwards, the samples were re-dissolved with 10% aq. DMSO.

Table 2. List of selected Thai medicinal plants

Scientific name	Voucher specimen (authentic crude drug)		Part used
	Collector number	TTM-c*	
Acacia concinna (Willd.) DC.	WR063	10000621	Pods
Cymbopogon nardus (L.) Rendle	WR037	10000595	Leaves
Cyperus rotundus L.	WR056	10000614	Rhizomes
Tiliacora triandra Diels,	WR036	10000594	Roots

* Thai traditional medicine crude drug

2.2. Cell culture

2.2.1. 3T3-L1 adipocytes

The murine 3T3-L1 preadipocytes (cell number JCRB9014) were sourced from the Japanese Collection of Research Bioresources Cell Bank in Osaka, Japan. These preadipocytes were cultured in multi-well tissue culture plates, with 96-well plates used for adipogenesis and lipolysis assays and 6-well plates for gene expression analysis. The cells were nurtured in a growth medium consisting of D-MEM (high glucose) with L-Glutamine and Phenol Red, supplemented with 10% (v/v) fetal bovine serum, 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 50 µg/mL of gentamicin, at 37˚C in a 10% carbon dioxide atmosphere.

Upon achieving 70-80% confluence, the cells were exposed to a differentiation medium (comprising growth medium with 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX), 0.25 µM of dexamethasone, and 10 µg/mL of insulin) to initiate the differentiation process, designated as Day 0. On Day 2, the medium was switched to an insulin medium (growth medium supplemented with 5 µg/mL of

insulin). Subsequent steps varied depending on the specific experiment and are detailed in the following section.

2.2.2. NCTC 1469 hepatocytes

NCTC 1469 hepatocytes (cell number JCRB9075) were sourced from the same place as adipocytes. These cells were cultured in DMEM (high glucose) supplemented with L-Glutamine and Phenol Red, along with 10% (v/v) horse serum (HS), allowing them to proliferate until Day 3. Subsequently, plant extracts were diluted in a 10% HS/DMEM medium and introduced to the cells. The cells were then incubated at 37˚C in a 10% carbon dioxide atmosphere for 24 hours before being harvested for further analysis.

2.3. Adipogenesis assay

After pre-adipocytes were differentiated, on Day 2, the cells were treated with or without the plant extracts. Then on Day 4, the lipid content was measured using AdipoRed^{TM} reagent (LONZA, Japan).

2.4. AMPK activation assay

The procedure was the same as adipogenesis assay except for that there was an addition of an AMPK inhibitor (Compound C, 20 μM) one hour before introducing the plant extracts. Additionally, an AMPK activator (AICAR, 1 mM) was employed as a positive control.

2.5. Lipolysis assay

3T3-L1 cells underwent a differentiation process, and on Day 2 and 4, the medium was altered to insulin medium to promote differentiation. Following this, the medium reverted to growth medium on Day 6 and was incubated for 2 days. On Day 8, the cells were subjected to serum starvation using D-MEM without L-Glutamine and Phenol Red for 24 hours. Subsequently, on Day 9, specific inhibitors for each pathway were introduced one hour prior to the plant extracts or activators, and the cells were incubated for another 24 hours. Finally, glycerol levels in the medium were assessed using free glycerol reagent (Sigma-Aldrich, St. Louis, MO, USA) as an index of lipolysis.

2.6. RNA extraction and quantitative real-time PCR

Total RNA was isolated utilising the ReliaPrep^{TM} RNA Cell Mini System kit and the obtained total RNA was subsequently converted into complementary DNA through the utilisation of the ReverTra Ace™ qPCR RT Master Mix kit, following the manufacturer's instructions. Gene expression levels were assessed using the Takara Thermal Cycle Dice Real-Time system (Takara Bio Inc., Shiga, Japan) with KAPA SYBR FAST Universal reagent (Kapa Biosystems, MA, USA). The reference gene used is *YWHAZ*.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). Statistically significant differences were detected by Student's t-test or one-way ANOVA with Tukey's test. Differences were considered significant at $p < 0.05$.

3. Results

The outcomes of the effect of plant extracts from *A. concinna*, *C. nardus*, *C. rotundus*, and *T. triandra* on gene expression and protein activation associated to adipogenesis, lipogenesis, lipolysis, fatty acid β-oxidation are summarised as follows.

A. concinna, in *in vitro* studies, exhibited anti-obesity properties by inhibiting adipogenesis and lipogenesis. This effect was achieved through the downregulation of genes associated with these processes and the activation of AMPK. Furthermore, it promoted lipolysis by downregulating *Plin1* while activating PKA and ERK. In hepatocytes, *A. concinna* treatment reduced the expression of lipid metabolism genes, indicating its potential to prevent lipogenesis and reduce liver lipid accumulation.

C. nardus did not exhibit the ability to reduce lipid accumulation in adipocytes during both adipogenesis and in mature adipocytes. However, it demonstrated anti-obesogenic potential as a lipolysis enhancer by activating PKA and downregulating *Plin1*. Additionally, *C. nardus* downregulated lipogenesis-related genes in hepatocytes, suggesting its potential to inhibit lipogenesis and reduce lipid accumulation in the liver.

C. rotundus extract employed in this study does not possess the capacity to inhibit lipid accumulation during either adipogenesis or lipogenesis. However, when applied to hepatocytes, *C. rotundus* demonstrated anti-lipogenic properties by downregulating genes associated with lipogenesis, potentially reducing hepatic lipid accumulation. This plant's potential against lipid metabolism is quite similar to *C. nardus*.

T. triandra exhibited anti-adipogenic properties, despite an observed upregulation in *Cebpa*. It influences lipid metabolism by inhibiting lipogenesis through the AMPK pathway and enhancing lipolysis via the PKA pathway in adipocytes, thereby reducing lipid accumulation. However, its impact on gene expression in hepatocytes indicated limited effectiveness against liver lipid synthesis.

4. Discussion

4.1. Differential effects of plant extracts on gene expression in 3T3-L1 adipocytes and NCTC 1469 hepatocytes

In this study, the same genes associated with lipid metabolism in both adipocytes and hepatocytes were examined. The results indicated that medicinal plants had a stronger impact on the tested genes in adipocytes compared to hepatocytes. Moreover, the study showed that the same plant had varying effects on the same gene in different cell types. For example, *A. concinna* downregulated several genes related to adipogenesis and lipid metabolism in adipocytes but had a minimal impact on these genes in hepatocytes. This difference may be attributed to the distinct expression of glucose transporter (GLUT) isoforms in each cell type (Mazibuko-Mbeje *et al*., 2018).

Furthermore, in a complex living system, plant extracts may exhibit diverse activities in different tissues. For instance, an in vivo study demonstrated that *Melissa officinalis* has tissue-specific effects on lipid metabolism in both the liver and adipose tissue (Brochot *et al*., 2019). *M. officinalis* affected the *Acaca* gene in adipose tissue but not the liver, while it targeted *Pck1* (phosphoenolpyruvate carboxykinase 1), a glucose/lipid metabolism-related gene, in the liver but not adipose tissue.

On top of that the complex interactions between tissues and organs in the body further complicate the effects of these molecules. For example, adipokines released from adipocytes can impact fatty acid oxidation and suppress lipid accumulation in the liver, while fibroblast growth factor-21 (FGF21) from the liver can influence glucose uptake and energy expenditure in adipose tissue (Duwaerts and Maher, 2019). This makes *in vivo* studies essential for a comprehensive understanding of the impacts of plant extracts on adipocytes and hepatocytes within the entire organism.

4.2. Correlation between mechanistic study and anti-obesogenic activity

The mechanistic study conducted in this research revealed that changes in gene expression do not consistently reflect the anti-obesity activity of the tested plants. Protein activation showed a more direct correlation with anti-obesity effects. This can be seen by the result of *C. nardus* and *C. rotundus* exhibiting downregulation of lipogenesis-related genes, but they cannot reduce the lipid accumulation in adipocytes. The findings highlight the limitation of using a gene expression analysis for a sole criterion when choosing the most suitable plant for anti-obesity purposes.

Concurrently, *A. concinna* and *T. triandra* were found to be the most effective plants against both adipogenesis and lipogenesis, with AMPK activation identified as the key factor responsible for reducing lipid accumulation. Besides, this study also confirms the lipolysis-stimulating activity of all the tested plants

via PKA or/and ERK pathways, reinforcing the importance of assessing protein activation to elucidate the underlying mechanisms of their anti-obesity potential.

Meanwhile, the understanding of the impact of these plants on hepatic lipid metabolism remains limited. Further research is needed to establish a concrete effect on liver lipid accumulation, potentially involving various regulators and signalling proteins such as CFLAR/JNK, AMPK, or ERK, as well as genes associated with lipogenesis, fatty acid oxidation, and fatty acid transport (Choi *et al*., 2017; Liu *et al*., 2019).

5. Conclusion

The objective of this research is to assess the ability of specific medicinal plants to combat obesity and to uncover the mechanisms responsible for their effectiveness both in adipocytes and hepatocytes, with the goal of finding the most promising plant candidates.

In summary, by introducing the extracts to adipocytes, *A. concinna* emerges as the most promising anti-obesity plant, closely followed by *T. triandra*, both of which possess anti-adipogenic and anti-lipogenic properties. Although *C. nardus* and *C. rotundus* also showed potential as candidates, their effectiveness is not as robust as the former two.

Additionally, the study also explored the gene expression changes in hepatocytes. However, the concrete results were not concluded. At this stage, gene expression analysis can only propose preliminary results and suggest their potential for liver metabolism. Thus, future investigations should be conducted to explore and prove the effectiveness of these plants against obesity.

6. References

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